UNIVERSITAT DE BARCELONA

Variety improvement in rice *(Oryza sativa* L.): proteomic, hormonal and *in vitro* studies

Camilo Alonso López Cristoffanini

Rice (*Oryza sativa* L.) is without doubt one of the major crops worldwide, as its consumption is continuously increasing, especially in less developed countries where it is the most important staple. This cereal has been domesticated for a long time, fruit of which several species and varieties are now available. Moreover, it was key during the green revolution where its production more than doubled due to the development of dwarf varieties, and has a deep cultural background in all the regions it is grown. Hence, it is of uttermost importance for researchers and breeders to broaden and expand the knowledge we have on this cereal on all the study and research areas, especially now that we are living in the 21st century, which is marked by climate change. This natural phenomena is one of the most menacing as it will reduce the quantity and quality of arable land due to salinization of soil as well as water scarcity which is the single most important factor that determines global crop yields. In this sense, this thesis addressed three important topics on rice that will help researchers and breeders for the improvement of rice varieties in the forthcoming future:

(i) Analysis of a salinity tolerant rice cultivar subjected to high salt concentration through a combined approach of shotgun proteomics and physiological characterization for the identification of new key proteins involved in the tolerance to this stress.

(ii) Phytohormones analysis through a phytohormone profiling method developed during this thesis for the characterization of the phytohormone levels in three rice varieties with contrasting heights that will allow have more information for developing new dwarf mutant varieties.

(iii) Improvement and enhancement of anther culture protocols in rice for obtaining higher rates of stabilized green double haploid plants using different cold-pretreatments, hormones and antimitotics in the growing media for their subsequent commercialization.

Barcelona, March 2019

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DOCTORAL THESIS

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Thesis presented by Camilo Alonso López Cristoffanini to obtain the Doctor degree from the *Universitat de Barcelona*. This thesis is framed within the Doctoral Program *Ecologia, Ciències Ambientals i Fisiologia Vegetal* corresponding to the period between 2014-2019 of the *Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals*.

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Pre-doctoral student	Thesis supervisor	Thesis supervisor
Camilo Alonso López Cristoffanini	Marta López-Carbonell	Salvador Nogués Mestres

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NEURICE
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Guarda fuerza que esto recién empieza, guarda fuerza que esto recién empieza…

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SUMMARY

Rice is one of the most important foods in the world, as it is a staple in several regions. In fact, it is the cereal which production is mainly destined to direct human consumption (80%) followed by wheat and maize with 70 and 15%, respectively (Trade and Markets Division 2018). Moreover, rice consumption per capita is increasing due to the concomitant population growth (Mohanty 2013; FAO 2018a).

Rice belongs to the Poaceae family and genus *Oryza*. It has been present in the human agriculture for long time where it underwent several domestication processes. These events have led nowadays to the cultivation of two of its species: *Oryza sativa* and *Oryza glaberrima*. Nevertheless, *O. sativa* stands out as it is cultivated worldwide whereas *O. glaberrima* only in West Africa (McLean et al. 2013; López-Cristoffanini et al. 2016). Moreover, the domestication of *O. sativa* led to the emergence of two subspecies: *O. sativa spp. japonica* and *O. sativa spp. indica* (Garris et al. 2005; Kovach et al. 2007). In Europe, contrarily to the rest of the world, the production of *O. sativa ssp. japonica* is superior to that of the *indica* spp, as it has been traditionally used for cooking paellas and risottos in Spain and Italy respectively (Franquet Bernis and Borràs Pàmies 2004).

This cereal is a semiaquatic annual grass that, according to Counce et al. (2000), has three distinct morphological phases: seedling, vegetative and reproductive. The whole process, from dry seed to a fully mature rice grain passing through the three morphological stages, lasts for three to six months depending greatly on the variety (genetic background) and the environment (McLean et al. 2013).

During the mid-1960s, the green revolution happened, which allowed researchers, breeders and farmers to double the rice production with only a 20% of crop area increase. This incredible feat was due to a more efficient use of fertilizers, pesticides, water irrigation and the introduction of the so-called high-yielding varieties [HYVs, Zeng at al. (2014)]. These varieties display dwarf and semi-dwarf traits, which allows to allocate more efforts on grain production, thereby explaining the yield increment (Hedden 2003; Wang et al. 2017). It is nowadays known that these HYVs were fruit of mutations in the *GA20ox-2* gene, which led to the *sd1* phenotype which are semi-dwarf plants (Ashikari et al. 2002; Monna et al. 2002; Sasaki et al. 2002; Spielmeyer et al. 2002; Hedden 2003). Moreover, there are nowadays four known mutations in the *GA20ox-2* gene that cause the dwarfism phenotype (Ashikari et al. 2002; Sasaki et al. 2002).

The rice HYVs allowed for a substantial growth of the human population; however, in the forthcoming years these varieties will not be able to sustain by their own a continuous growth since yields are not increasing, leading to a rice stock shortage (FAO 2018a). Moreover, all the progress reached during the green revolution are now optimized at their best and there is a global consciousness to reduce the environmental impact of fertilizers and pesticides (Thrall et al. 2010; Zeng et al. 2014).

In addition to this, the 21st century is being marked by climate change, one if not the gravest danger, that agriculture and consequently humanity is facing, which is mainly driven by a CO_2 increase. Climate change is salinizing the fields and reducing water availability due to the higher abnormalities in temperature and the augmentation of the sea level (Dunwell 2010; Mickelbart et al. 2015; Shrivastava and Kumar 2015). It has been reported that if climate change continues and nothing is done to preven this, food shortages will definitely occur (Kumar Dubey et al. 2017). Among all the effects that the climate change imposes on land

and agriculture, salinity is one of the most severe stresses, which can be influenced by water scarcity and soil salinization. For rice this represents a great problem, as it is the most salt-sensitive cereal worldwide, being most sensitive at the seedling and reproductive stages which could lead to yield losses (Heenan et al. 1988; Lutts et al. 1995; Sahi et al. 2006; Munns and Tester 2008; Kumar et al. 2013; Negrão et al. 2013; Abdollah Hosseini et al. 2015; Reddy et al. 2017). Nevertheless, rice as well as other plants has mechanisms for reducing the damage imposed by the salinity stress such as reduced salt intake through roots, efficient ion compartmentation and non-specific responses to this stress such as antioxidant metabolism activation and protein protection (Abdollah Hosseini et al. 2015; Das et al. 2015; Lakra et al. 2017; Reddy et al. 2017). Three main mechanisms have been proposed to be considered for plants in regard to salinity tolerance: (i) tissue tolerance, (ii) osmotic tolerance or exclusion and (iii) ion exclusion (Roy et al. 2014; Reddy et al. 2017).

Due to the nutritional, cultural and agricultural importance that rice has on the world, this cereal has been profusely studied in terms of salinity tolerance. Despite this, the majority of the quantitative trait locus (QTLs) that are known to be related to salt stress tolerance only explain 10-30% of the tolerance (Lin et al. 2004; Singh et al. 2007; Kumar et al. 2015). However, a benchmark discovery occurred during the beginning of the 2000s, when a major QTL for salinity tolerance was discovered. This QTL, which has been named *Saltol* and is located on chromosome 1, explains *ca.* 60% of the salinity tolerance (Bonilla et al. 2002; Lin et al. 2004). This QTL was studied in-depth and there is substantial evidence to affirm that the high salinity tolerance could be explained by a gene within the *Saltol* region that encodes for an HKT-type transporter, named *OsHKT1;5* (Ren et al. 2005; Platten et al. 2006, 2013; Thomson et al. 2010). In addition, it has been used in some programs involved in variety improvement for salinity tolerance (Babu et al. 2017).

As mentioned previously, salinity is one of the major stresses faced by plants, especially for rice (Parihar et al. 2015). Phytohormones are small molecules acting at very low concentrations and producing several responses (Davies 2010; Kudo et al. 2013). They have been studied regarding abiotic stress including salinity, and also in the scope of variety improvement programs (Wani et al. 2016). Among all phytohormones, abscisic acid (ABA) has long been a well-known player involved in regulating plant's water status (Fahad et al. 2015). In fact, this hormone is involved in closing stomata in guard cells as well as involved in decreasing Na^+ accumulation in leaves. Regarding other phytohormones involved in salinity, there is also some evidence suggesting that salicylic acid (SA) and gibberellins (GAs) could also be involved in the salinity stress tolerance (Peleg and Blumwald 2011; Khan et al. 2012).

Regarding phytohormones, GAs and their biosynthesis have been widely studied due to their crucial role during the green revolution and the availability of HYVs. These hormones are a large group, in fact more than 136 have been identified, although only 4 of them are actually active in plants: GA_1, GA_3, GA_4 and GA_7 (Hedden and Phillips 2000; Yamaguchi 2008; Binenbaum et al. 2018). GAs biosynthesis starts in the plastids and when it arrives to the cytosol there are two possible pathways they can undergo: the GA_{12} or the GA_{53}-pathway, in which their only difference is that the latter has an extra hydroxyl (OH^-) group. Interestingly, independent of the pathway, it is the same enzyme, GA20-oxidase, that transforms those two in the subsequent molecules. This specific enzyme is the one behind the HYVs phenotype, producing height reduction which is explained by a disruption in the GAs production pattern as they show accumulation of GA_{53} (a precursor of the bioactive GAs) and low content of the bioactive ones (Ashikari et al. 2002; Sasaki et al. 2002; Spielmeyer et al. 2002). Nevertheless,

it has been shown that external applications of GA_3 make plants recover their normal height (Hedden 2003).

Large efforts have been put into understanding, or trying to understand, several aspects of rice, due to its importance for humankind. But, in 2005, a milestone for rice research occurred: the publication of its full genome sequence (IRGSP 2005). This allowed researchers worldwide to expand their studies, works and insights in relation to this cereal. In addition, the concomitant advances in several techniques and methodologies led researchers to the omics and high-throughput era, which consists in the analysis of large-scale data. This includes studies at the genomic, transcriptomic, proteomic, metabolomic and phenomics level, that is from DNA to whole organisms (Sumner et al. 2003; Oikawa et al. 2008; Langridge and Fleury 2011; Van Emon 2016; Itoh et al. 2018; Wing et al. 2018). This new and rapidly generated information has served to two ends: (i) increasing our knowledge on fundamental sciences and (ii) using all these resources and findings towards variety improvement programs in order to develop new cultivars with higher yields, higher abiotic and biotic tolerance among others (Langridge and Fleury 2011). The latter is not surprising as humans have been, since rice domestication, selecting traits which were advantageous for the plants in order to please humankind needs such as higher yields, higher tolerances or less problems during harvest (Gepts 2002).

This knowledge and resources combined to the techniques for variety improvement will allow to obtain new rice varieties during for the forthcoming years. To this extent, in this thesis three studies related with rice variety improvement are depicted: (i) understanding salinity response of a *Saltol*-harbouring variety at the proteomic level through a shotgun proteomics approach, (ii) developing a fast and broad method for analyzing gibberellins in relation to dwarfism, and

(iii) improvement of anther culture protocols for obtaining double haploid plants in Mediterranean *japonica* varieties. A summary of each chapter is shown below.

CHAPTER 1 - Proteome profiling in shoots and roots of the FL478 genotype of rice (*Oryza sativa* L. *ssp. indica*) by shotgun proteomics during early salinity stress

As it has been explained, salinity is one of the most menacing stresses for rice plants, and due to climate change it is going to worsen in the following years. Therefore, it is very important to gather all information possible in regard to salinity tolerance with a view towards rice variety improvement. In this context, the main aim of this chapter was to characterize the proteome of FL478, a salinity tolerant variety harboring the *Saltol* region, and to perform a physiological characterization of early stages of salinity tolerance [6, 24 and 48h subjected to 0 (mock treatment) and 100 mM NaCl] in shoots and roots. The salt stress imposes both an ionic and an osmotic stress, which triggers several adaptive responses at the molecular, cellular, metabolic, and physiological levels for coping with it (Kumar et al. 2013; Gupta and Huang 2014; Das et al. 2015). It is known that the seedling stage is heavily affected by salt stress (Reddy et al. 2017). The *OsHKT1;5* gene contained in the *Saltol* region is thought to be the one determining the greater tolerance to salinity in varieties that harbor this region, as evidence suggest that is involved in the Na^+ unloading from the xylem (Ren et al. 2005; Platten et al. 2006, 2013; Thomson et al. 2010).

Shotgun proteomics was the technique chosen for characterizing the proteome, as it has three main advantages: (i) direct analysis of peptides, which are easily fractionable, (ii) allows to detect hydrophobic and low abundant proteins, and (iii) peptide quantification of different samples simultaneously, (Neilson et al. 2007; Chandramouli and Qian 2009; Gupta et al.

2015). For the physiological characterization we analyzed growth (length and weight), as well as water and Na^+/K^+ content, being the latter a good marker for salinity tolerance (Frouin et al. 2018).

As a general overview, it was observed that roots showed faster and more coordinated proteomic responses that shoots. In fact, significant protein abundances was observed only after 6h of treatment and maintained during the whole treatment. In contrast, shoots only showed a significant protein accumulation at 48h which seems non-controlled. This can be easily explained by the fact that roots are the first tissue to be in contact with salinity (Yan et al. 2005). Moreover, transcription- and translation-related proteins were highly increased, which indicates that there is an important activation in this tissue for the synthesis of proteins involved in the salinity tolerance responses. In addition, roots compared with shoots, showed a higher accumulation of stress-related proteins in response to salinity treatment, like peroxidase and *Salt* which are both contained in the *Saltol* region. Moreover, several enzymes involved in the antioxidant responses and protein protection were also up-regulated during the salinity treatment, especially in roots. As also evidenced by Frouin et al. (2018), we observed activation of several proteins related to Ca^{2+} sensing which is crucial for ion homeostasis. Regarding the HKT-type transporter *OsHKT1;5* it was not detected it in our study, not even using a shotgun proteomics approach. Nevertheless, several authors studying salinity tolerance in *Saltol* varieties have not yet seen its presence as a protein (Singh and Jwa 2013; Abdollah Hosseini et al. 2015; Lakra et al. 2017).

At the physiological level, it was observed that the relative chlorophyll content (SPAD) increased at 6 and 24h, but at 48h it was significantly reduced. Moreover, the fresh weight of both shoots and roots displayed a reduction which was significant at the 48h of treatment. Contrarily, only shoot reduced its length as roots displayed higher length throughout the

treatment, probably in an attempt to find less salinized mediums. The water content was only reduced in shoots as roots, maintaining the same levels in all the time points of the treatment. Regarding Na^+/K^+ ratio, it was evident that roots have a protective role in salinity stress tolerance as they showed a 5-fold of shoots ratio, in order to avoid Na^+ to reach the leaves, which is in agreement with other works (Khan and Panda 2008; Lakra et al. 2017).

Our findings indicate that roots display more efficient and adaptive responses to salinity stress than roots, which can be explained by a better adaptation to the growing medium (Deinlein et al. 2014; Roy et al. 2014). In our study, more than 2000 proteins were found to be involved in the salinity response, which could be also used and looked when improving varieties. Finally, it is also important to analyze roots when studying salinity tolerance as it is the first tissue to sense the stress. Altogether, our results suggest towards variety improvement, that roots should be majorly considered due to their crucial role in the tolerance to this stress.

CHAPTER 2 - Phytohormone profiling method for rice: effects of GA20ox mutation on the gibberellin content of Mediterranean japonica rice varieties

Gibberellins were crucial phytohormones during the green revolution when it was discovered that a mutation in one of its biosynthesis genes explained the dwarfism traits of the HYVs (Hedden 2003). Despite this knowledge, there is still a lot of research involved in obtaining semidwarf high-yielding varieties and the study of the GAs biosynthesis disruption caused by the mutations (Wang et al. 2017). In the context that this study is situated, we reported for the first time a protocol that allows to identify in one analysis 13 gibberellins as well as ABA,

JA and IAA in rice samples. Finally, this protocol was used to characterize the GAs profile of three rice Mediterranean *japonica* varieties with contrasting heights.

Our protocol is simple and broad, as it only uses one SPE (Solid Phase Extraction) cartridge for sample clean-up and phytohormone concentration and allows detection of 16 phytohormones. Phytohormones were detected and quantified by HPLC-MS/MS using a QqQ instrument with limits of detection (LOD) and limits of quantification (LOQ) for the 13 GAs that varied between 0.1-0.7 and 0.3-2.3 $pg \cdot g^{-1}$ (f.w.) respectively, of rice samples. Moreover, a good reproducibility of the method was obtained; in fact, using a 40 $ng \cdot mL^{-1}$ standard the relative standard deviation (RSD) was less than 1% for all the phytohormones. Therefore, our method allows to quantify hormones present at very low levels with high reproducibility.

The protocol was applied to analyze the GA contents of three rice Mediterranean *japonica* varieties with contrasting heights. First, we sequenced the GA20ox-2 exon 2 of these varieties, which showed that one of them (*dwarf*-Bomba) had a point mutation that results in a single amino acid substitution (leucine to a phenylalanine). This mutation corresponds to the Calrose mutation also found in other *japonica* varieties such as Jikkoku (Japan) and Calrose76 (USA) (Hedden, 2003; Sasaki et al., 2002). The other two, Bomba and NRVC980385, were wild-type for this gene. In addition, Bomba showed significant higher height than both, NRVC980385 and *dwarf*-Bomba, and NRVC980385 was higher during the first half of the development (week 1 to 11). From week 13^{th} and forth, *dwarf*-Bomba surpassed NRVC980385's height.

Regarding the study of GAs content in several rice varieties we found that, on one hand, GA_1 was absent in 7-day old coleoptiles, indicating that this phytohormone is not needed for coleoptile elongation as it has been detected in 4-day old coleoptiles (Liu et al. 2018). On the

other hand, GA_{19} (precursor of GA_3 and GA_4) seems to be crucial to the complete and normal development of rice plants as its levels were very high in all varieties and all analyzed stages. In accordance with this, GA_4 is the bioactive gibberellin that in general shows the higher levels through the rice plant development in all varieties, which is in agreement with Binenbaum et al. (2018). Finally, the height increment displayed by *dwarf*-Bomba at the second part of its development is in correlation with a significantly elevated content of the 4 bioactive GAs in the internode between flag leaf and previous leaf and the flag leaf node. Therefore, although the mutated variety displayed a semi-dwarfism trait, it did not show drastically lower gibberellin levels in comparison with the other two varieties. In fact, the tallest variety, Bomba, has similar GA contents than NRVC980385 which is almost half its height. Regarding JA, ABA and IAA they were detected in all tissues at least in one variety; and although this does not provide much information it gives insights in phytohormones in different tissues for further studies. Finally, our results suggest that the GA20ox-2 mutation is not the only factor determining height in rice plants.

CHAPTER 3 - An improved anther culture procedure for obtaining new commercial Mediterranean temperate japonica rice (*Oryza sativa*) genotypes & Antimitotic and hormone effects on green double haploid plant production

In the last chapter of this thesis two studies regarding anther culture protocol in rice were performed. Anther culture is an *in-vitro* technique first developed by Niizeki and Oono (1968). It allows to produce double haploid (DH) plants, therefore it has great interest for breeding programs where it has been extensively used (Germanà 2011; Mishra and Rao 2016). The technique is a two-step process that involves the development of a *calli* from the anthers and

the subsequent plant regeneration from the *calli*. This allows to obtain stabilized lines without having to submit the varieties through a pedigree process. Since it has been profusely used in several varieties it has been greatly improved, however, one of the main limiting factors is the genotype (Germanà 2011; Mishra and Rao 2016). Therefore, even though it has been optimized, there is still some need and space for enhancing its efficiency in other varieties. Hence, in both studies we reported optimization of anther culture in both, the first and second steps. Even more, some of the varieties obtained through anther culture were tested in field and their agronomical and production traits were evaluated.

In the first study, an improvement through the application of a cold-pretreatment at 5ºC during a different duration (7 to 12 day) was tested on 4 different rice lines that were product of crosses between different Mediterranean rice *japonica* accessions. This cold-pretreatment has been proven to give satisfactory results for other *japonica* rice varieties (Chen et al. 1986; Trejo-Tapia et al. 2002a, b). Results showed that 9 days was the optimal cold-pretreatment duration and proved to be more efficient for *callus* induction than the original method proposed by Serrat et al. (2014). This increment in *calli* induction rate also translated into a greater percentage of green plants that regenerated from these *calli*, however it did not improve the frequency of green double haploid plants compared to NRVC980385 in the study by Serrat et al. (2014). Therefore, improvements can still be made for increasing the quantity of green double haploid plants obtained through the anther culture procedure.

The aim of our second study was also to improve the anther culture efficiency on two *japonica* rice genotypes, NRVC980385 and H28. In this work a broader set of tests were performed: (i) effect of different growth regulators in the *callus* induction medium, (ii) effect of colchicine in the *callus* induction medium, and (iii) a post anther culture procedure to transform green haploid plants in green double haploid plants. In addition, in this study we performed a cold-

pretreatment at 10ºC during 9 days which improved the *callius* induction and green double haploid plantlets frequency reported by Serrat et al. (2014) in a 51- and 33-fold increase respectively. This is backed-up by several authors that claim that the cold-pretreatment has a stimulatory effect on androgenic responses [microspore or pollen embryogenesis; Tian et al. (2015), Herath et al. (2007), Touraev et al. (2009)]. Regarding the different hormones used in our tested mediums, the one that provide better overall results was the colchicine-free D2 treatment (2 $mg \cdot L^{-1}$ of 2,4-D and 1 $mg \cdot L^{-1}$ of kinetin), which is in agreement with other authors (Chen et al. 2002; Herath et al. 2009). Colchicine, an antimitotic agent, has been reported to increase DH recovery from anther-derived *callus* (Alemanno and Guiderdoni 1994; Chen et al. 2002; Zapata-Arias 2003). Our study presented contradictory results, as *calli* induction was diminished and incremented in NRVC980385 and H28 respectively. This trend was also observed for the regeneration of green plantlets, where colchicine had positive effects only in H28. Nevertheless, independent of the variety and even in colchicine-supplemented media, we observed in general a higher rate of albinism which is contrast with the literature (Barnabás et al. 1991; Kumari et al. 2009; Ferrie et al. 2014). The only exception was H28 treated with 150 $mg \cdot L^{-1}$ of colchicine during 24h.

As reported by the first and the second study, even though anther protocols were optimized according to the literature, the results is not always the expected which indicates the crucial role that genotype has on this procedure. Nevertheless, our post anther culture treatment assayed in the second study, which must be improved, could ameliorate the situation as those green regenerated plantlets could develop into double haploids if the correct treatment is applied. Lastly, of all the field assayed lines of the first study, one of them (F_2-40.D266) showed promising results as its yield was higher than NRVC980385 which is an elite variety

cultivated in the region of *Delta del Ebro* where the assay took place (Català et al. 2009). In addition, its tolerance to fungi and insects was similar to NRVC980385.

Finally, all the works presented in this thesis greatly contribute to the rice research knowledge towards variety improvement as new information and protocols improvement have been presented in the three chapters. New information regarding proteins involved in tolerance responses to salinity in both shoot and root have been presented, as well as new insights into gibberellin contents in wild-type and mutated varieties. In addition, improvements of anther culture protocols have been developed which allows to fasten the development of new varieties. Due to the importance that this cereal has on the human population, all the work carried out in this thesis is of great usefulness. Nowadays, breeders' programs are focused on improving several traits of rice varieties which is facilitated by the availability of several techniques. Therefore, the results and findings of this thesis greatly enhance the knowledge we have on rice.

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ABBREVIATIONS

2,4-D	:	2,4-dichlorophenoxyacetic acid
2-DE	:	Two-Dimensional Electrophoresis
4N	:	4th node
4N5	:	Internode between 4th and 5th node
5N	:	5th node
50H	:	Panicle and florets
A5L	:	Apical part of the 5th leaf
AB	:	Ammonium Bicarbonate
ABA	:	Abscisic Acid
ACN	:	Acetonitrile
AFL	:	Apical part of the flag leaf
ANOVA	:	Analysis of Variance
APX	:	Ascorbate Peroxidase
AUX	:	Auxin
B	:	Bomba (rice variety)
B5L	:	Basal part of the 5th leaf
BC	:	BackCross
BEH	:	Ethylene Bridged Hybrid
BFL	:	Basal part of the flag leaf
BS	:	Brassinosteroids
CAT	:	Catalase
CE	:	Collision Energy
CK	:	Cytokinin
CI	:	*Callus* Induction
COL	:	Coleoptile
ConInt	:	Confidence Intervals
CPratio	:	*Callus* Production ratio
CRISPR	:	Clustered Regularly-Interspaced Short Palindromic Repeats
*d*B	:	*dwarf*-Bomba (rice variety)
DH	:	Double haploid
DMSO	:	DiMethyl SulfOxide
DNA	:	Deoxyribo Nucleic Acid

DP	:	Donor parent
DeP	:	Declustering Potential
EBI	:	The European Bioinformatics Institute
EC	:	Electrical Conductivity
ECe	:	Electrical Conductivity on extract
ESI	:	ElectroSpray Ionization
ET	:	Ethylene
FA	:	Formic Acid
FAO	:	Food and Agriculture Organization
FAOSTAT	:	Food and Agriculture Organization Corporate Statistical Database
FCM	:	Flow Cytometry
FDR	:	False Discovery Rate
FN	:	Flag leaf node
G/A	:	Green/Albino plantets ratio
GAs	:	Gibberellins
GBS	:	Genotyping By Sequencing
GO	:	Gene Onthology
GR	:	Green plantlets
GRDH	:	Green Double Haploid plantlets
gs	:	stomatal conductance
GWAS	:	Genome-Wide Association Study
H_2O_2	:	hydrogen peroxide
HAc	:	Acetic acid
HCD	:	High Energy Collision Dissociation
HCl	:	Hydrochloric Acid
HKT	:	High-affinity Potassium Transporters
HPLC	:	High-performance Liquid Chromatography
HRMS	:	High Resolution Mass Spectrometry
HSD	:	Tukey's Honest Significant Difference
HYV	:	High Yielding Variety
IAA	:	Indole-3-Acetic Acid
IPCC	:	Intergovernmental Panel on Climate Change
IRGSP	:	The International Rice Genome Sequencing Project
IRRI	:	International Rice Research Institute
iTRAQ	:	Isobaric Tag for Relative and Absolute Quantitation
JA	:	Jasmonates / Jasmonic Acid
KASP	:	Kompetitive Allele-Specific PCR
KEGG	:	Kyoto Encyclopedia of Genes and Genomes

KO	:	KEGG Orthology
KOH	:	Potassium Hydroxide
LC-MS	:	Liquid Chromatography-Mass Spectrometry
LOD	:	Limit Of Detection
LOQ	:	Limit Of Quantification
LOWESS	:	Locally Weighted Scatterplot Smoothing
LTQ	:	Linear Trap Quadropole
MABC	:	Marker-Assisted BackCross
MeOH	:	Methanol
MRM	:	Multiple Reaction Monitoring
MS	:	Mass Spectrometry
MS/MS	:	Mass Spectrometry in Tandem Mode
N	:	NRVC980385 (rice variety)
NaCl	:	Sodium Chloride
Na_2HPO_4	:	Disodium phosphate
NAA	:	Naphtalen Acetic Acid
NCBI	:	the National Center for Biotechnology Information
NEURICE	:	New EUropean commercial RICE
P1	:	Parental cultivar 1
P2	:	Parental cultivar 2
PAS	:	Pool of Analyzed Samples
PCR	:	Polymerase Chain Reaction
PDX	:	Peroxidase
pFW	:	plantlet Fresh Weight
pL	:	plantlet Length
pN	:	Node previous to the flag leaf node
pNF	:	Internode between flag leaf and previous leaf
QqQ	:	Triple quadrupole mass spectrometer
qPCR	:	Quantitative PCR
QTL	:	Quantitative Trait Locus
RCP	:	Representative Concentration Pathways
rFW	:	root Fresh Weight
RIL	:	Recombinant Imbred Line
rK^+	:	root K^+ content
rL	:	root Length
rNa^+	:	root Na^+ content
RP	:	Recurrent Parent
RPI	:	Rice Production Index

RSD	:	Relative Standard Deviation
RT^{o}	:	Room Temperature
RT	:	Retention time
RuBisCO	:	Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase
rWC	:	root Water Content
SA	:	Salicylic Acid
SE	:	Standard Error
sFW	:	shoot Fresh Weight
sK^{+}	:	shoot K^{+} content
S/N	:	Signal to Noise
SL	:	Strigolactones
SDN	:	Site-Directed Nucleases
SDS	:	Sodium dodecyl sulphate
SGR	:	Super Green Rice
SILAC	:	Stable Isotope Labelling by Amino acids in Cell culture
SKC	:	Shoot K^{+} concentration
sL	:	shoot Length
SLR	:	Sea Level Rise
sNa^{+}	:	shoot Na^{+} content
SNP	:	Single Nucleotide Polymorphism
SOD	:	SuperOxide Dismutase
SPE	:	Solid Phase Extraction
SSR	:	Simple Sequence Repeat
STRING	:	Search Tool for the Retrieval of Interacting Genes/Proteins
sWC	:	shoot Water Content
TALEN	:	Transcriptional Activator-Like Effector Nuclease
TCEP	:	Tris-(2-Carboxyethyl) phosphine
WC	:	Water Content
ZFN	:	Zinc Finger Nuclease

1

GENERAL INTRODUCTION

1.1. Rice features and characteristics

1.1.1. Rice origin, morphology and life cycle

Rice is a member of the Poaceae family and belongs to the genus *Oryza* (Chang et al. 1965; McLean et al. 2013). It is a plant that has been present in the world for very long time, and the archaeological evidence suggest that the birthplace for humans cultivations is the Yangtze River Valley of China (Kovach et al. 2007). Two domestication processes occurred, one that led to *Oryza sativa* and another one that led to *Oryza glaberrima* (Figure 1.1).

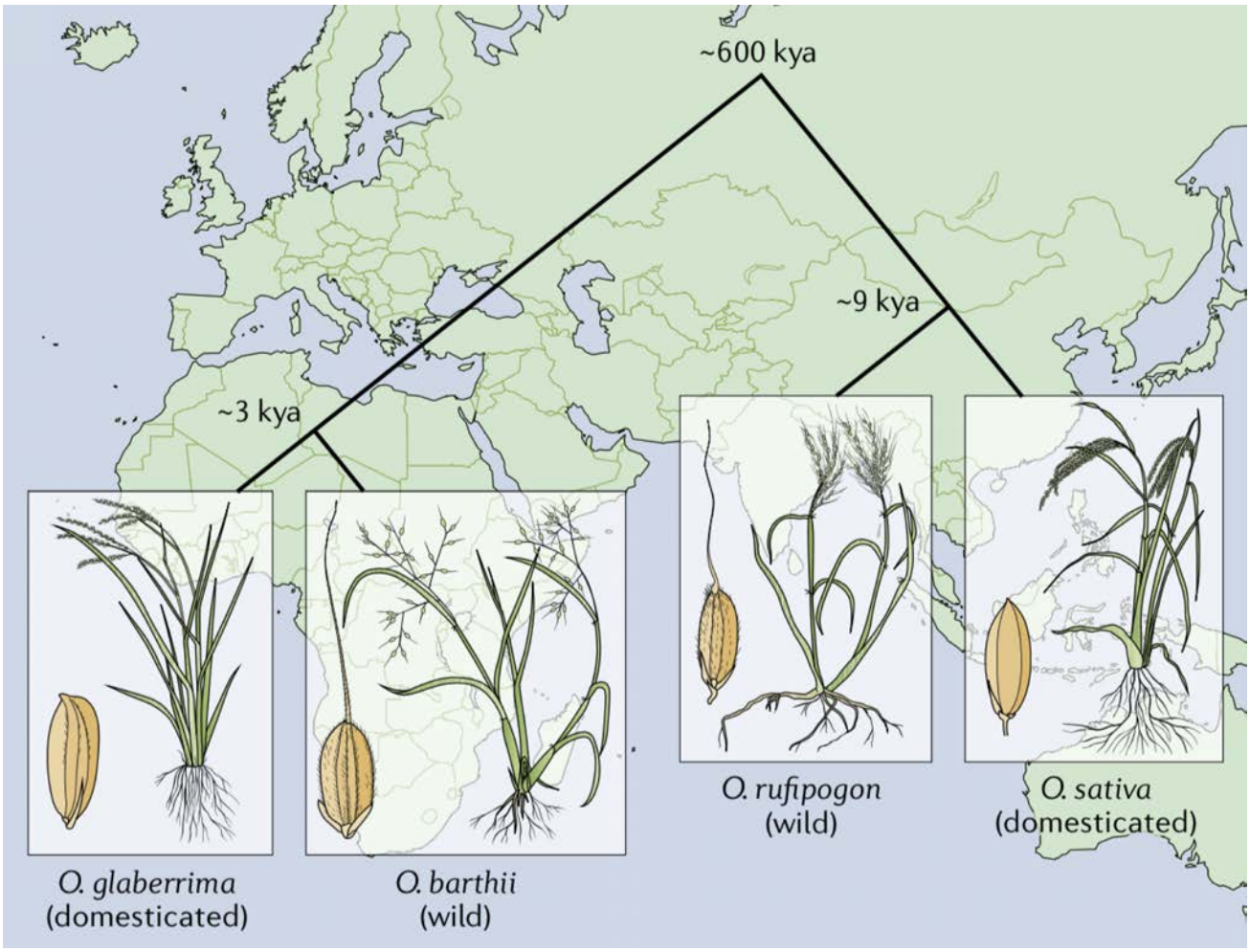


Figure 1.1. Domestication of *Oryza sativa* (Asian rice) and *Oryza glaberrima* (African rice) occurred independently *ca.* 6,000 years apart. kya: thousand years ago. Retrieved from Wing et al. (2018).

The first domestication occurred between 9,000 and 10,000 years ago and constitutes a milestone in human history and corresponds to the “Asian rice” or *O. sativa* (Kovach et al. 2007; Sang and Ge 2007; McLean et al. 2013). The second domestication process 6,000 years later occurred in Africa, where *Oryza glaberrima* was domesticated from the wild *Oryza barthii* (Wing et al. 2018). Regarding *Oryza sativa* progenitor, genomic data has posed some controversy as it suggests that it has been originated from *Oryza rufipogon* but some *Oryza nivara* genes have also been detected (Sang and Ge 2007; Wing et al. 2018). The two domesticated species, *O. sativa* and *O. glaberrima* are cultivated worldwide and in West

Africa respectively (McLean et al. 2013; López-Cristoffanini et al. 2016). Independently of the species, rice plants underwent a continuous selection for desirable features or traits that led to their establishment (Sweeney and McCouch 2007). This domestication process involved a series of profound genetic changes that transformed those wild species into more amenable ones in terms of cultivation and consumption by humans (Kovach et al. 2007). Some of these genetic changes have been characterized, such as the QTLs (quantitative trait locus) for grain shattering resistance (*sh3*, *sh4* and *sh8*), the *GS3* gene for grain size and shape and QTLs for panicle length (Xiong et al. 1999; Cai and Morishima 2002; Li et al. 2006). Moreover, the domestication process within *O. sativa* caused the emergence of two subspecies: *O. sativa spp. japonica* and *O. sativa spp. indica*, which includes 5 subpopulations that are recognized and can be seen in Figure 1.2 (Garris et al. 2005; Kovach et al. 2007).

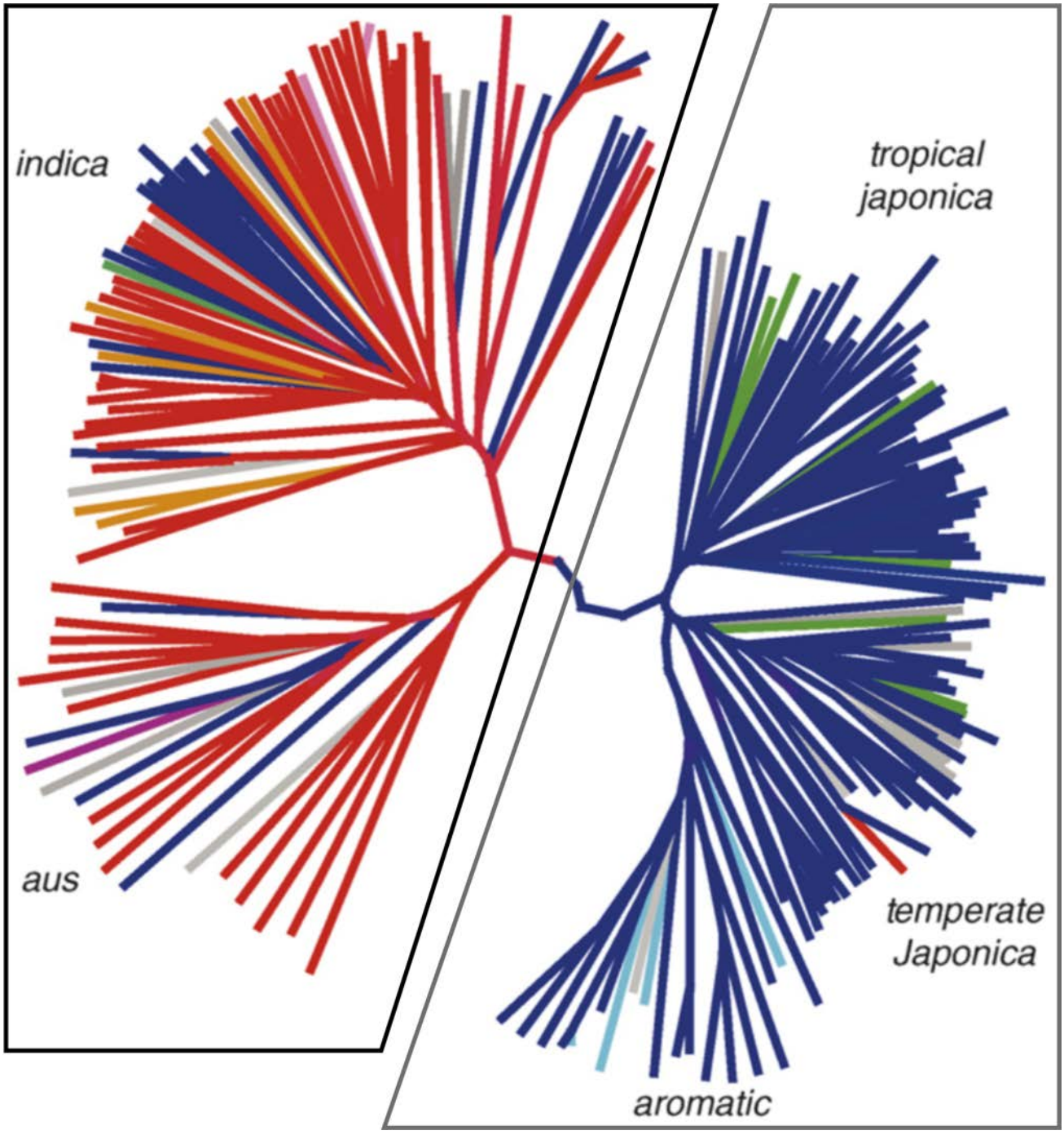


Figure 1. 2. Subspecies and subpopulation structure in *Oryza sativa*. *O. sativa* is characterized by the presence of deep genetic differentiation. This unrooted phylogenetic tree was constructed from data using 169 nuclear SSR (Simple Sequence Repeat) and two chloroplast markers on 234 landraces of O. sativa [17]. The branch structure reflects the phylogenetic relationships based on the nuclear SSR markers. The branch color corresponds to the chloroplast haplotype of each accession. This tree illustrates the major division between the two varietal groups (*japonica* enclosed in black and *indica* enclosed in grey), which are further subdivided into the five rice subpopulations: *indica*, aus, tropical *japonica*, temperate *japonica* and aromatic. Adapted from Kovach et al. (2007).

The rice plant is considered a semiaquatic annual grass although it can survive as a perennial. Its height varies between 50 to 500 cm depending on the species and geographic location since currently there are more than 3,000, being farmed *ca.* 75% of them and several were produced by rice research (McLean et al. 2013; 3000 rice genomes project 2014). Rice breeders and researches have proposed several classification systems including: Haun (1973), Zadoks et al. (1974), IRRI (1980) and the BBCH scale by Lancashire et al. (1991). Counce and colleagues published a new classification scale that exploits the presence and absence of distinct morphological criteria (Counce et al. 2000). In this system, rice morphological development has been divided into three phases which can be observed in Figure 1.3: seedling, vegetative and reproductive.

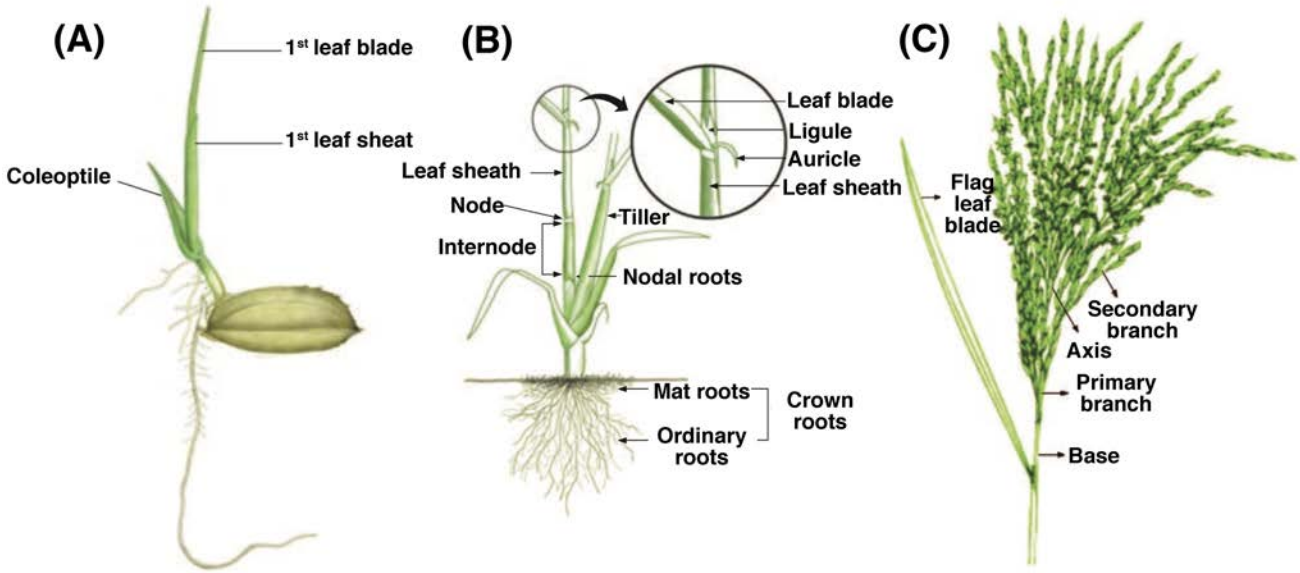


Figure 1.3. Representative external morphological structures of a rice plant in its seedling stage (A), vegetative stage (B) and reproductive stage (C). V1 rice plant relative to the growth staging system. Adapted from Counce et al. (2000) and McLean et al. (2013).

In the first stage, a dry seed imbibes water breaking dormancy and afterwards either the coleoptile or radicle emerges. Later, a prophyll (rudimentary leaf) emerges from the coleoptile

and followed by the emergence of the firs leaf (one with a blade and a sheath, Figure 1.3). After these events, the vegetative stage begins where the main stem comprises a series of nodes and internodes. Each of this node bears a leaf, with a sheath and a blade that can grow into a tiller. It is at this point when tillering occurs, which is the process of tiller production. At the end of the reproductive stage when plants are mature, they have one stem and several tillers (McLean et al. 2013). During this stage is when plants grow the most in terms of height and number of tillers. This stage culminates with the appearance of the final leaf, called flag leaf, which is crucial for the reproductive stage. Finally, reproductive stage begins when the shoot apex begins initiation of panicle structures (i.e. panicle initiation or more commonly known as booting). Then, these panicle branches differentiate leading to the florets that contains the male and female organs and the inflorescence is pollinized when anthesis occurs. Then, during the ripening period, each inflorescence develops into a grain that fills up with starch and slowly dries down to be fully mature developing into a rice grain. The whole process from dry seed to a fully mature rice grain passing through the seedling, vegetative and reproductive stage lasts from three to six months depending greatly on the variety (genetic background) and the environment. For example, it has been observed that the numbers of days to heading time can vary between 50 to 130 days (Xue et al. 2008; Kong et al. 2018).

1.1.2. Rice in the green revolution

The green revolution was a process that originated in the mid-1960s with a more efficient use of fertilizer, pesticides and water irrigation and the appearance of high-yielding varieties [HYVs: High Yielding Varieties, Zeng et al. (2014); Figure 1.4. This high-yielding varieties are

in fact the product of varieties that displayed semi-dwarf or dwarf traits, which allowed to allocate more efforts on grain production (Hedden 2003; Wang et al. 2017). This major technological outbreak led to a threefold increase crop production that was accompanied only by a 20% crop area increase, and by the growth of the world population from 3 to 7 billion (Figure 1.5).

Figure 1.4. Semi-dwarf rice cultivars and their tall isogenic lines. From left to right: Dee-geo-woo-gen (dwarf *indica* cultivar), Woo-gen (tall equivalent), Calrose76 (dwarf *japonica* cultivar), Calrose (tall equivalent). Retrieved from Hedden (2003).

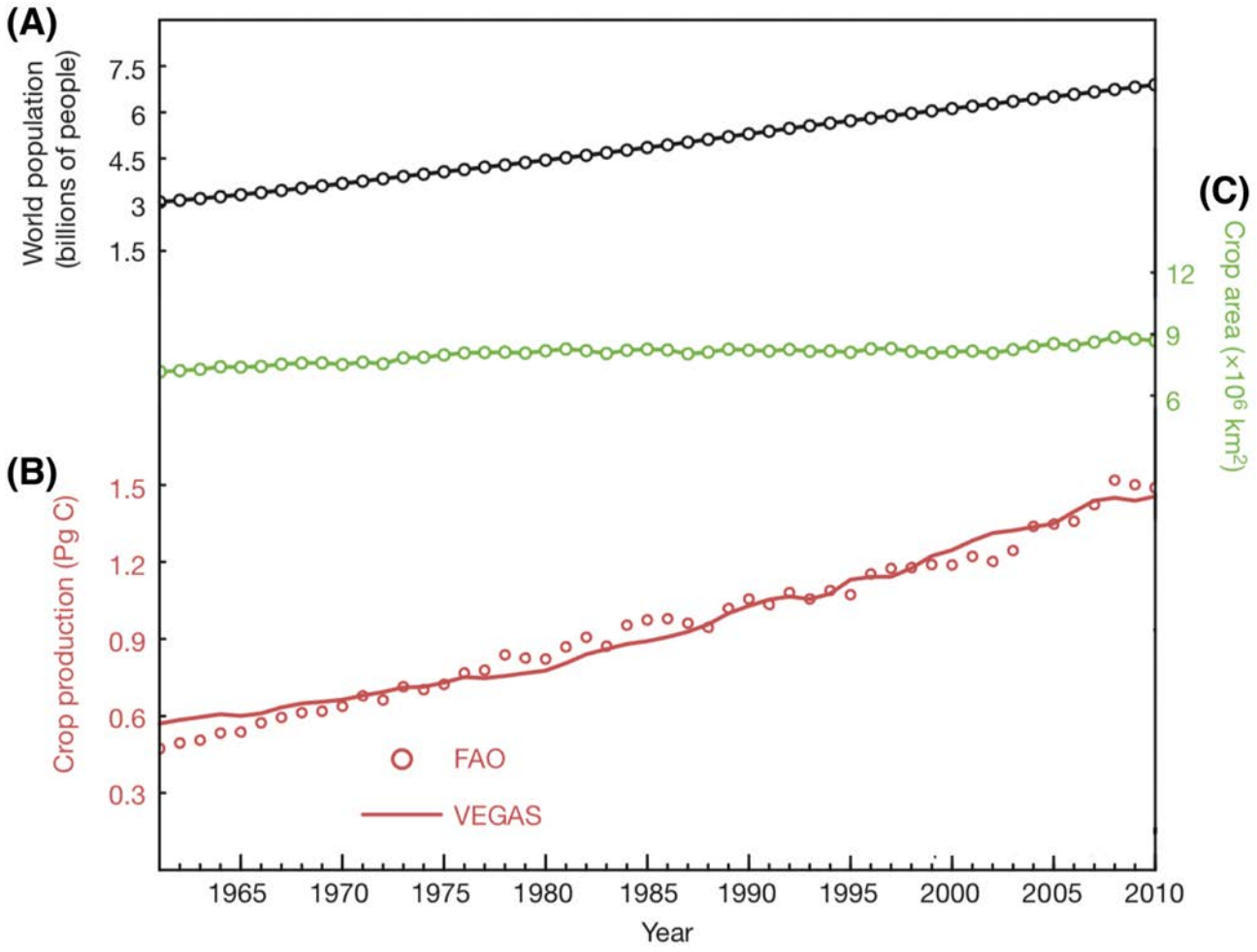


Figure 1.5. Changes in world population (A), crop production (B) and crop area (C) during the green revolution period. VEGAS is a model simulated crop production and FAO is the estimated statistics. Retrieved from Zeng et al. (2014).

For rice specifically, the first HYV was IR8, released on 1996 by the IRRI (Sasaki et al. 2002; McLean et al. 2013). Shortly after, several other semi-dwarf varieties were developed using gamma (γ) irradiation derived progenitors such as Jikkoku and Reimei in Japan and Calrose76 in USA (Sasaki et al. 2002; Hedden 2003). All the first semi-dwarf varieties contained mutations that affected GA production, which was called *sd1* mutation and was defined as a recessive one (Sasaki et al. 2002; Hedden 2003). Several research groups, in an independent manner, isolated the *sd1* gene and showed that it encodes for the GA20-

oxidase 2 (*GA20ox-2*), a gene involved in gibberellins biosynthesis (Ashikari et al. 2002; Monna et al. 2002; Sasaki et al. 2002; Spielmeyer et al. 2002; Hedden 2003). Nevertheless, the mutations found in the different semi-dwarf varieties vary between them as shown in Figure 1.6. Interestingly, the dwarfing alleles used in the *japonica* lines are generally weaker than in the *indica* varieties, indicating that the mutant enzymes might possess some activity.

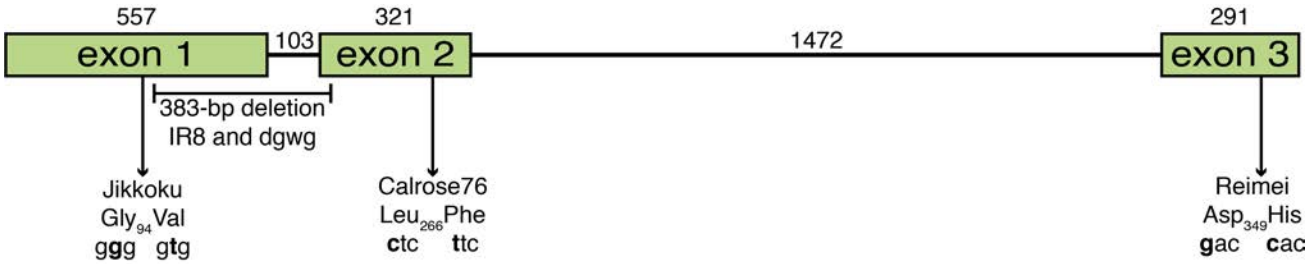


Figure 1.6. Mutation sites of the four *sd1* alleles in the GA20ox-2 genes. This gene consists of three exons (green boxes) and two introns (black lines), and the base pair length of each part is indicated. The deletion present in IR8 and dee-geo-woo-gen (dgwg) is indicated by a horizontal line and the single-nucleotide substitutions by arrows. Adapted from Ashikari et al. (2002) and Sasaki et al. (2002).

1.1.3. Rice in the human diet

Since its domestication, rice has fed world population continuously through the years in an increasing manner, and along with wheat and maize are the three most grown cereals crop worldwide (Hu and Wang 2016). In Figure 1.7, it can be observed that compared to wheat and maize (as coarse grains) rice production is lower, however, it stands out since more than 80% of its production is destined to direct human consumption according to the forecast for 2018/2019 (Trade and Markets Division 2018).

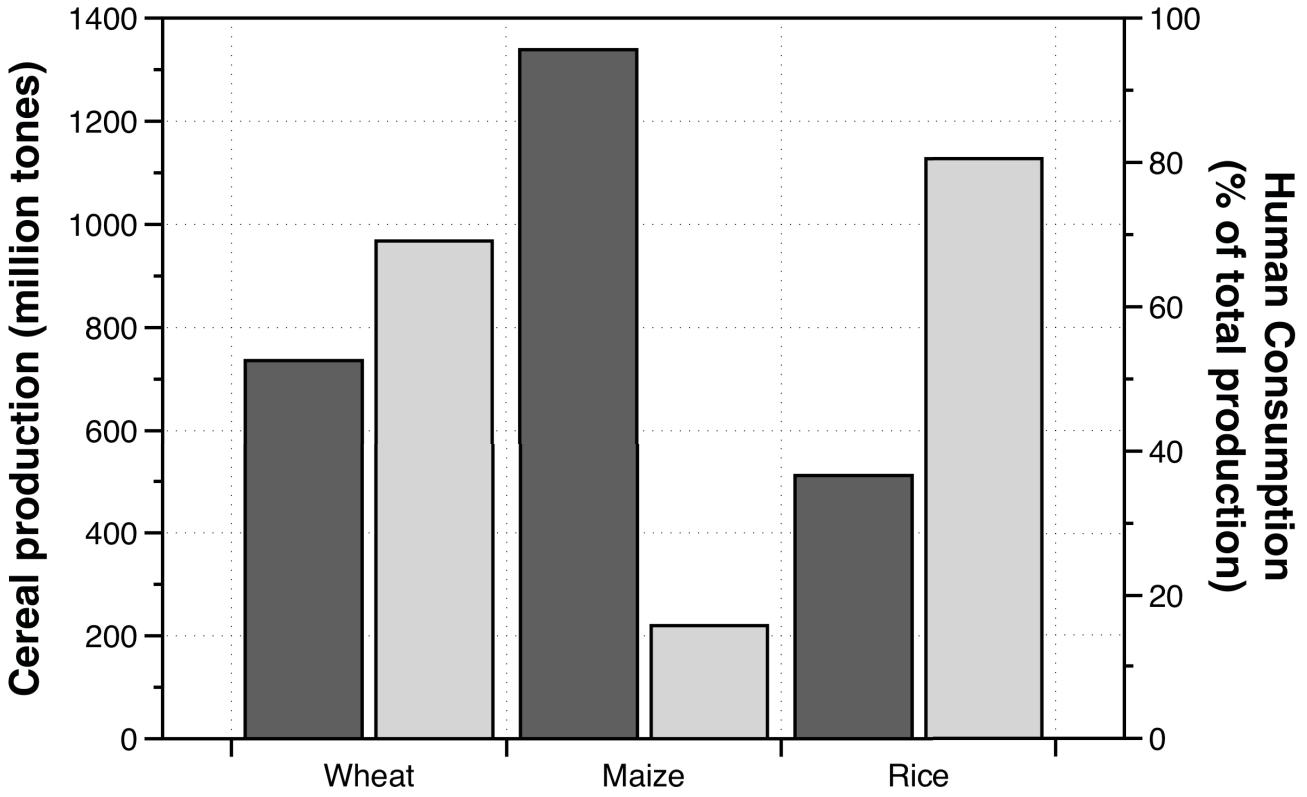


Figure 1.7. Wheat, maize (as coarse grains) and rice yearly production (left axis) and human consumption (% of total production, right axis) for the 2018/2019 forecast according to the Trade and Market Division of FAO (2018).

Moreover, rice consumption per capita has been increasing although in the last years it has been stabilizing (Figure 1.8). Despite this, food consumption has been continuously increasing due to a concomitant growth of the world population (Mohanty 2013; FAO 2018a). Production of rice (in milled equivalents) has also been increasing since yields were incremented during the green revolution, but are now stabilized. In the forthcoming years it will not sustain the growing population, which will lead to a production shortage (FAO 2018a). This will impact heavily on the low- and lower-middle-income countries in the forthcoming years, where rice is the main staple food providing the main calories supplies (McLean et al. 2013; FAO 2018b).

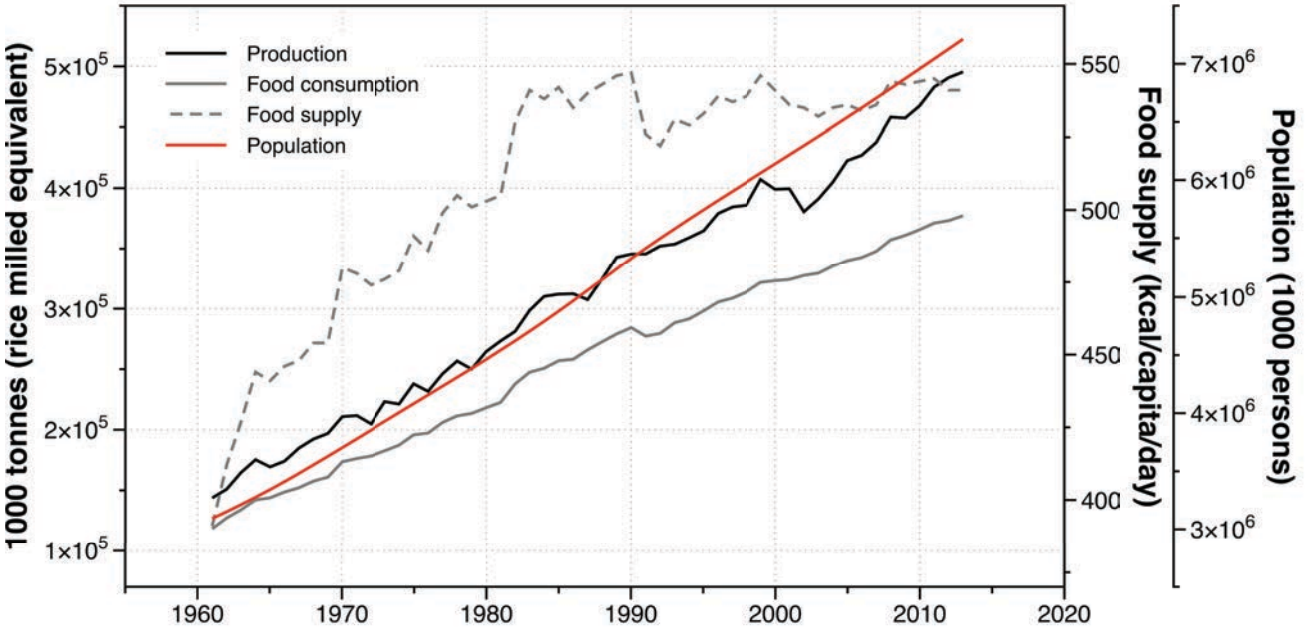


Figure 1.8. Rice production in milled equivalents (black line, left axis), food consumption in milled equivalents (grey line, left axis), food supply (grey dashed line, 1st right axis) and population (red line, 2nd right axis). Data retrieved from FAOSTAT (2018a).

1.1.4. Rice in Europe culture

Rice in Europe story began around the 9th century where it was cultivated in Guadalquivir marshy lowlands (called *marismas*), Guadiana estuary and *L'Albufera* lagoon in the Valencia coast. Afterwards, in the 12th century, Spanish took over the rice cultivation and in the 15th century it spread out through Italy, then France and later to other countries. During this 600 year period rice was adapted to the European climate, and nowadays it exhibits yields between 7,826 and 10,421 Kg/ha (Pla et al. 2017). Its production is mainly concentrated in deltas with Italy leading the production followed by Spain (Figure 1.9), with *ca.* 50 and *ca.* 30% of the total production, respectively (FAO 2018a). Interestingly, contrarily to the rest of the world where *indica* subspecies are the most cultivated, in Europe the predominating one is *japonica,* as it has been traditionally used for cooking paellas and risottos in Spain and Italy

respectively (Franquet Bernis and Borràs Pàmies 2004). Europe is not self-sufficient in rice, importing more than 40% of the total consumed rice in his continent (European Commission 2018).

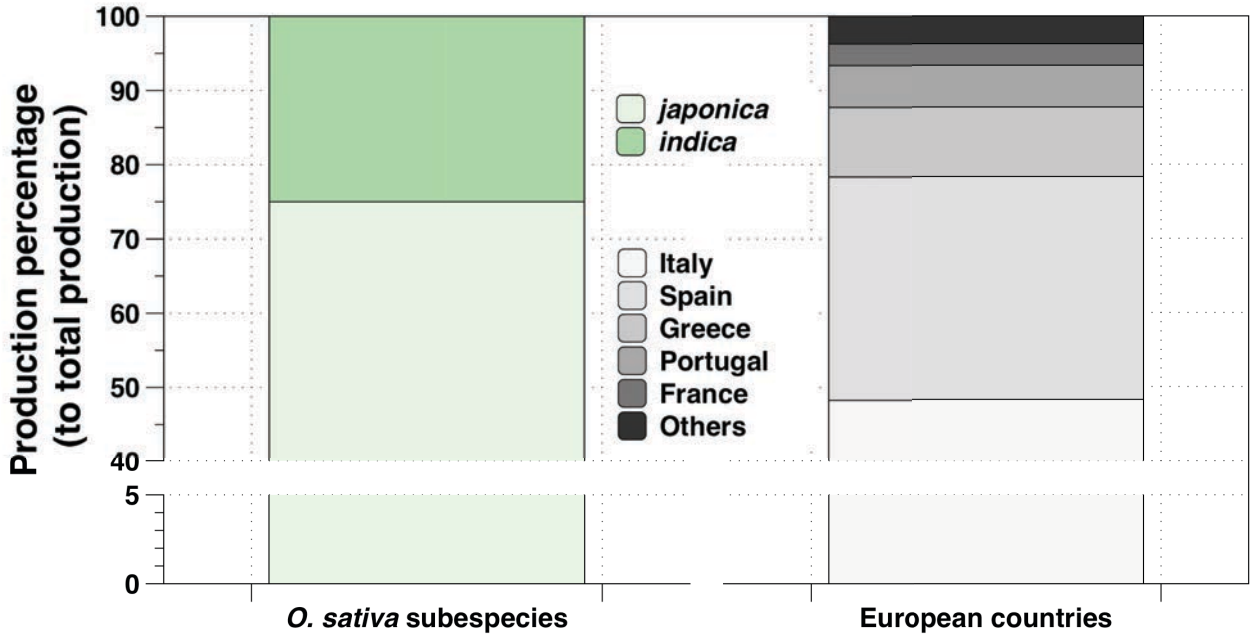


Figure 1.9. Rice production in Europe in terms of *O. sativa* subspecies (A) and country production (B). Retrieved from European Commission (2018).

1.2. Agriculture and rice

1.2.1. Current status and future perspectives

Agriculture was installed in the human society when we shifted to a relatively settled way of life from a nomadic hunter-gatherer life-style (Thrall et al. 2010). Nowadays, agriculture is facing problems worldwide since the majority of crops' yields are not increasing while we expect human population to reach 9 billion by 2050 according to a medium variant model (Figure 1.10). This means that the big gap reached with the HYVs in the green revolution in terms of yields is now at risk. In fact, some of the semi-dwarf varieties produced often display pleiotropic phenotypes that negatively affect productivity via altered tillering, stem physiology and fertility suggesting that the dwarfing phenotype does not fully depend on that trait (Liu et al. 2018). In addition, water irrigation techniques, pesticides and fertilizers are optimized at their best (Zeng et al. 2014), and at the same time there are global concerns in regard to reducing their environmental impact (Thrall et al. 2010). Agriculture has now entered a new era in which the key to success is the access to timely information and the elaborated decision making is crucial for farm management or smart farming (Fountas et al. 2015). However, it is most certain that conventional techniques, HYVs and smart farming cannot not ensure food security in the forthcoming years, therefore, plant biotechnology will be of great momentousness.

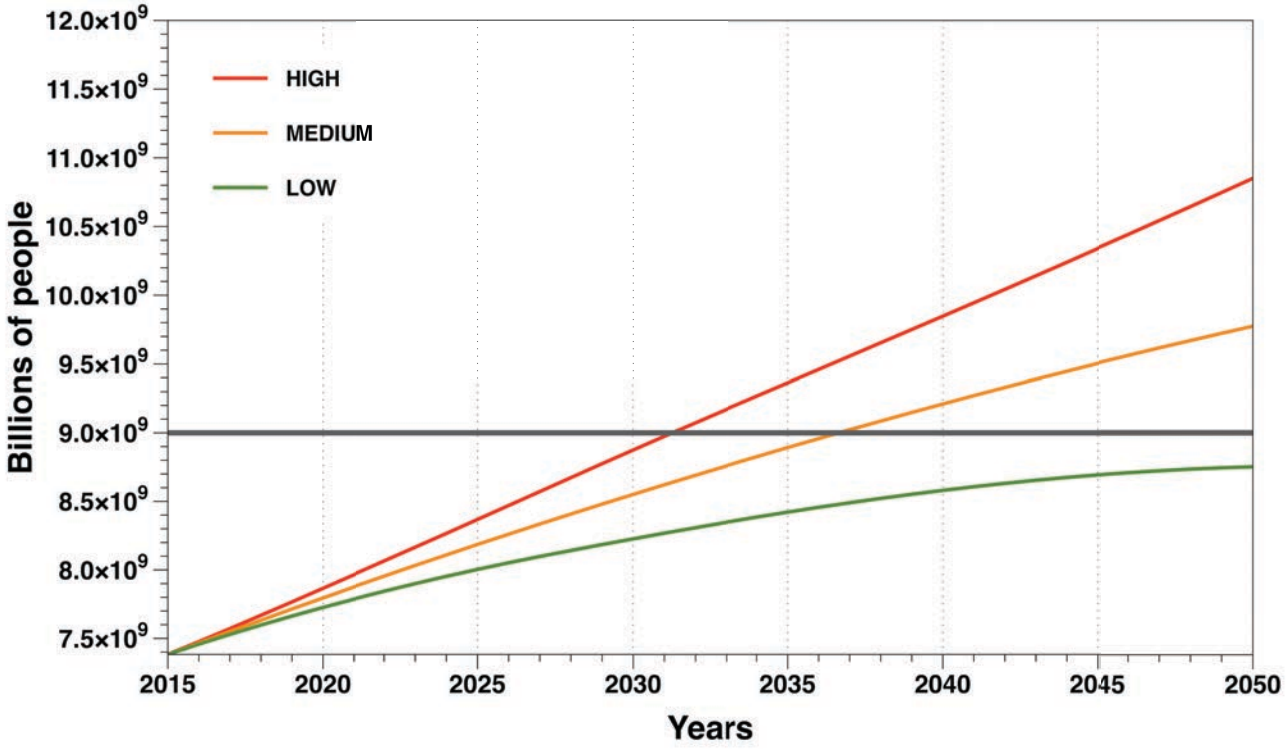


Figure 1.10. Growth population in the three models according to the World Populations Prospects 2017 of the United Nations until the year 2050 (United Nations 2017). Low variant model in green, medium variant model in orange and high variant model in red. Horizontal dark grey line correspond to the 9 billion people mark.

1.2.2. Climate change scenario

The 21st century is being marked by climate change, which is one of the most menacing phenomena that agriculture is facing as its spread worldwide which is mainly driven by an augment in the CO_2 concentrations (Figure 1.11A). The climate change translates into reductions in the quantity and quality of arable land due to salinization of soil as well as water scarcity which is the single most important factor that determines global crop yields (Dunwell 2010; Mickelbart et al. 2015; Shrivastava and Kumar 2015). Some of these effects are caused by the abnormalities that are correlated to temperature anomalies which in turn are increasing the sea level (Figure 1.11B and Figure 1.11C). There are several studies supporting the fact

that if CO_2 emissions are not halted or diminished, there is a considerable risk to the food supply. In fact, if the problems related to the climate change persist, they could lead to severe food shortages due to the increasing world population (Kumar Dubey et al. 2017).

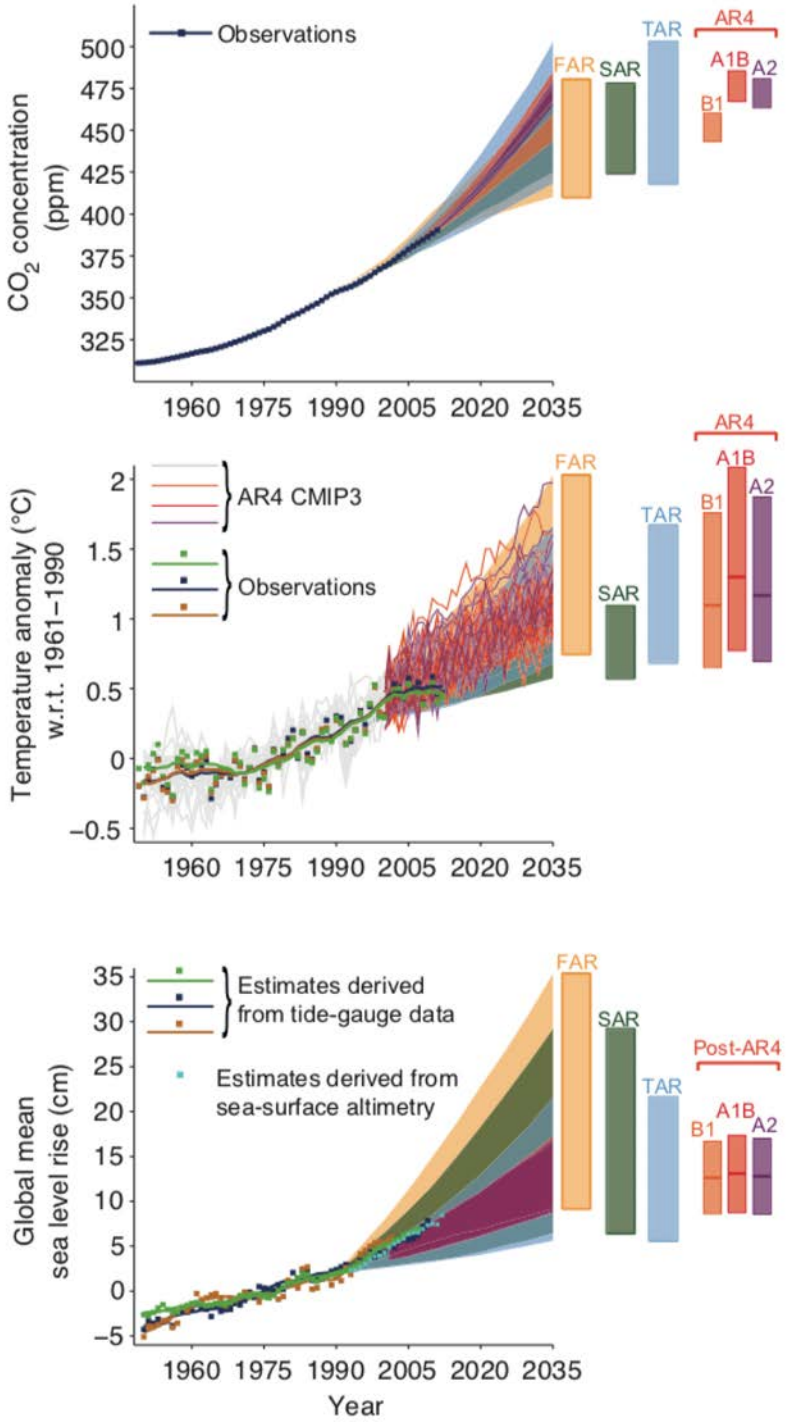


Figure 1.11. Observed global changes in the CO_2 concentration (ppm; A), temperature (ºC; B) and the sea level rise (cm; C) from 1950 compared with the previous IPCC projections (FAR, 1990; SAR, 1996; TAR, 2001) and the climatic model AR4 with three scenarios (B1, A1B y A2) from 2001 to 2035. Retrieved from IPCC (2013).

1.2.3. Abiotic stress: the case of salinity

As aforementioned, salinity is one of the most severe stress imposed by the climate change, as it is influenced by soil salinization and water scarcity. A saline soil is generally defined as one in which the electrical conductivity (EC) of the saturation extract (ECe) in the root zone exceeds 4 $dS \cdot m^{-1}$ [58.4 mM, according to the pseudo-linear approach by Sposito (2008)] at 25 ºC and has an exchangeable sodium of 15% (Sposito 2008; Shrivastava and Kumar 2015). Soil salinity imposes into plants both an osmotic and an ionic stress and rice is severely affected by it as it is the most salt-sensitive cereal worldwide (Sahi et al. 2006; Genua-Olmedo et al. 2016; Reddy et al. 2017; Lakra et al. 2018). The seedling and reproductive stages of rice are the most sensitive to salinity stress with exposure leading to yield losses (Heenan et al. 1988; Lutts et al. 1995; Sahi et al. 2006; Munns and Tester 2008; Kumar et al. 2013; Negrão et al. 2013; Abdollah Hosseini et al. 2015; Reddy et al. 2017). Salinity stress in rice triggers several adaptive responses at the molecular, cellular, metabolic, and physiological levels to cope with the osmotic and ionic stress (Kumar et al. 2013; Gupta and Huang 2014; Das et al. 2015). These responses are mainly related to ion homeostasis in the form of reduced salt intake through roots and by an efficient intra cellular compartmentation and transport of salts to vacuoles or to the external medium. Nevertheless, some generic responses to stress such as antioxidant metabolism activation, protein modifications and increases in energy and biomolecule metabolism are also activated (Abdollah Hosseini et al. 2015; Das et al. 2015; Lakra et al. 2017; Reddy et al. 2017). According to Roy et al. (2014) and Reddy et al. (2017), three main mechanisms can be considered, for rice and plants in general, which are illustrated in Figure 1.12: (i) tissue tolerance, (ii) osmotic tolerance or exclusion and (iii) ion exclusion.

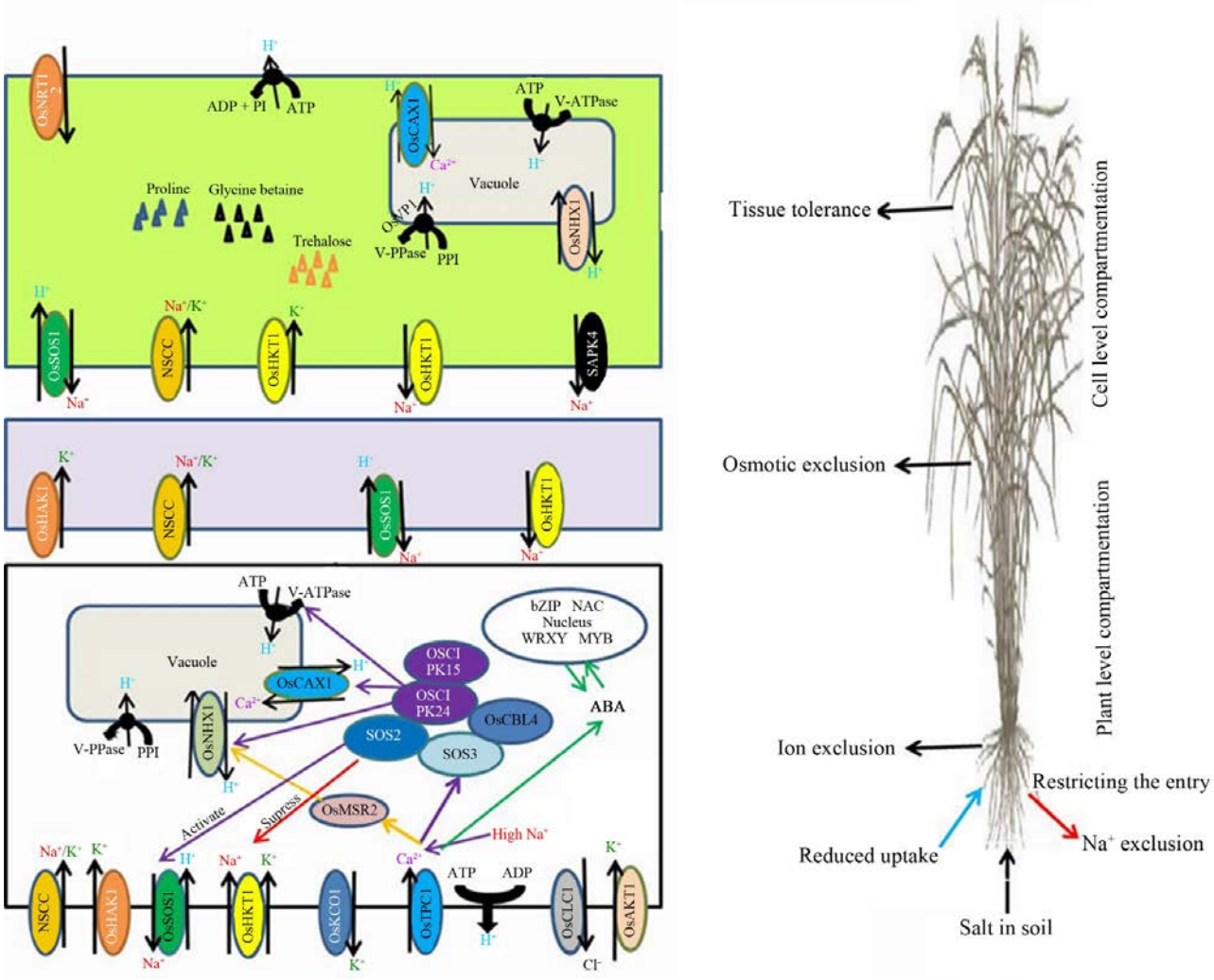


Figure 1.12. Rice salt tolerance mechanism-overview of important genes involved at root, shoot and leaf levels. Retrieved from Reddy et al. (2017).

As it could be expected, salt tolerance in rice has been profusely studied due to its agricultural importance and its high sensitivity to this stress. In fact, more than 70 QTLs related to salinity tolerance have been identified in rice but that only explain 10-30% of the trait (Lin et al. 2004; Singh et al. 2007; Kumar et al. 2015). A benchmark discovery occurred in 2002, when a major QTL named *Saltol* located in chromosome 1 explained between 64-80% of the salinity tolerance in a Pokkali (IRGC 108921)/IR29 (IRGC 30412) RIL population [Figure 1.13A, Bonilla et al. (2002)]. Likewise, a QTL named SKC (Shoot K^+ concentration) was also found

on Nona Bokra/Koshihikari crosses which explained more than 40% of the tolerance trait [Figure 1.13B, Lin et al. (2004)]. In fact, both QTLs are the same one which is mapped in the short arm of chromosome 1, in a region that is nowadays termed *Saltol* which is derived from salt-tolerant landraces. This region has been associated with a low Na^+/K^+ ratio, and therefore a high K^+ concentration in shoots and roots [i.e. Pokkali and Nona Bokra, López-Cristoffanini et al. (2019), Figure 1.13C]. This *Saltol* region has been inspected thoroughly and it contains at least 4 genes that have been annotated and functionally: *OsHKT1;5* (previously known as *OsHKT8* and SKC1), *SalT*, pectinesterase and peroxidase (PDX) (Ren et al. 2005; Kim et al. 2009b; Thomson et al. 2010; Kumar et al. 2015; Krishnamurthy et al. 2016). Although not fully proven, there is substantial evidence that the *OsHKT1;*5, which encodes for an HKT-type transporter, is the key gene in conferring salt tolerance as it could be involved in Na^+ unloading from the xylem (Ren et al. 2005; Platten et al. 2006, 2013; Thomson et al. 2010).

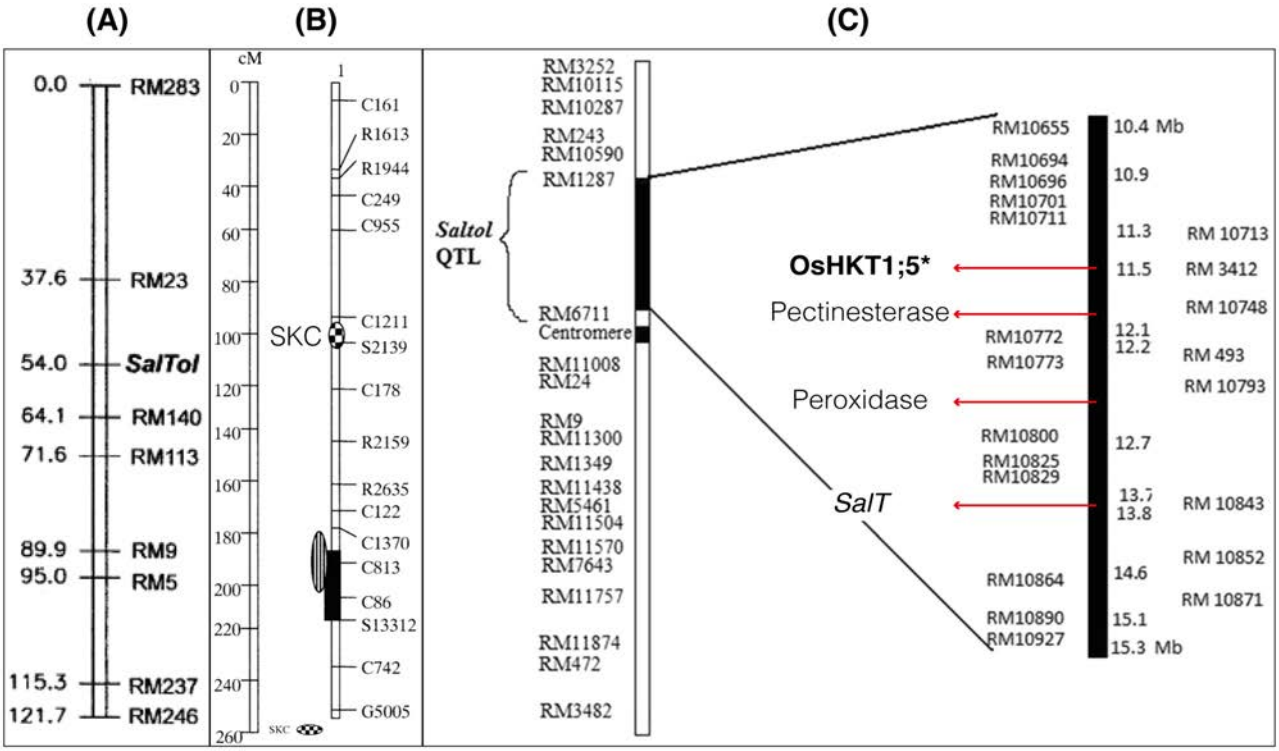


Figure 1.13. *Saltol* region identified by Bonilla et al. (2002) (A, distance between markers are given in cM based on Kosambi function), SKC QTL identified by Lin et al. (2004) (B, distance between markers are given in cM), and detailed mapping of the genes harboured in the *Saltol* region (C, distance between markers within the *Saltol* region are given in Mb). *: key gene for salinity tolerance in the *Saltol* region. Retrieved and adapted from Bonilla et al. (2002), Lin et al. (2004), Kim et al. (2009b), Thomson et al. (2010) and Krishnamurthy et al. (2016).

1.3. The omics and the high-throughput era for rice

Nowadays we have the technological and methodological advances for obtaining large scale-data in a high-throughput manner (Itoh et al. 2018). Moreover, the availability of the full genome sequence of rice in 2005 (IRGSP 2005), marked a milestone for rice research as the full length of the genes is at grasp and allows a full integration of the data obtained. Nowadays, researchers are analyzing large scale-data, biological entities or phenomena as a whole, an approach that has coined the term 'omics' (Sumner et al. 2003; Yadav 2007; Skirycz and Hannah 2012; Itoh et al. 2018). As expected, this suffix has been attached to many field of study of the classical approaches such as genomics, transcriptomics, proteomics, metabolomics and phenomics among others (Sumner et al. 2003; Oikawa et al. 2008; Langridge and Fleury 2011; Van Emon 2016; Itoh et al. 2018; Wing et al. 2018). It is important to highlight that neither omics nor high-throughput revolutionized the techniques used for studying traits and features in plants, but did make the leap in new ways to obtain, integrate and analyze data. Researchers have at the time several genomes (for both *O. sativa* L. spp *indica* and *japonica*) and transcriptome databases, as well as integrated databases such as the Oryzabase [https://shigen.nig.ac.jp/rice/oryzabase, Itoh et al. (2018)] that can inform of a plant behavior at one specific moment (Trewavas 2009). Phenomics and genomics are generally used in large-scale studies (high number of varieties or inbred lines) whereas transcriptomics, proteomics and metabolomics are used in medium- or small-scale studies. The bulk data that omics can analyze make them in a technique that is used for: (i) screening a genotype for new traits, (ii) comparing the behavior of several genotypes, and finally (iii) focus on key traits of interest with precision for breeding [(Oikawa et al. 2008; Gapper et al. 2014; Van Emon 2016), Figure 1.14]. In fact, all the huge amount of data that

has already been generated is being used to focus omics in agricultural research with aims to develop cultivable varieties with higher resistance to abiotic and biotic stress, higher yields, less water or fertilizers consumption and even for its product to have better texture or flavors (Langridge and Fleury 2011; Van Emon 2016).

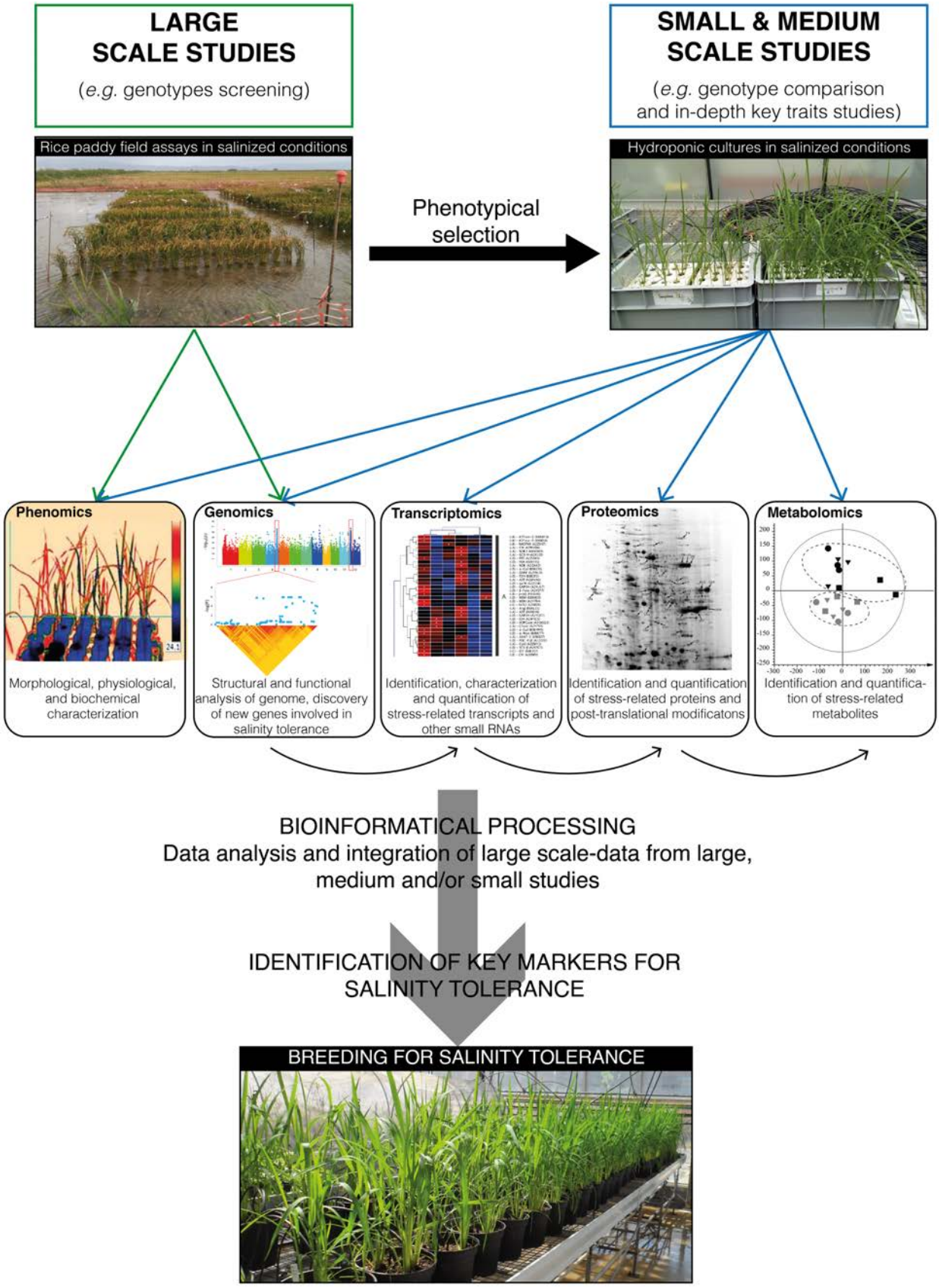


Figure 1.14. Omics approaches for developing new varieties with the focus on salt stress. Adapted from Das et al. (2015) and Reddy et al. (2017).

1.4. Rice proteomics

1.4.1. Methodological aspects of proteomics

Proteomics is the branch of the omics that focuses on analyzing the totality of proteins at one specific moment, may it be developmental, after abiotic stress or in one specific organelle. Independently of the study focus, proteins are of huge interest as they are responsible for many cell functions as they are the final product encoded by the genes (Komatsu and Tanaka 2005; Van Emon 2016). From 2000, considerable research efforts have been made allowing us to study the proteome of whole organisms, different tissues or even some subcellular compartments (Rakwal and Agrawal 2003; Komatsu and Tanaka 2005). For performing proteomics studies, we can consider four steps: (i) sample preparation, (ii) approach used (top-down or bottom-up), (iii) database search and bioinformatics, (iv) proteome visualization and interpretation (discussed in the next section; Figure 1.15).

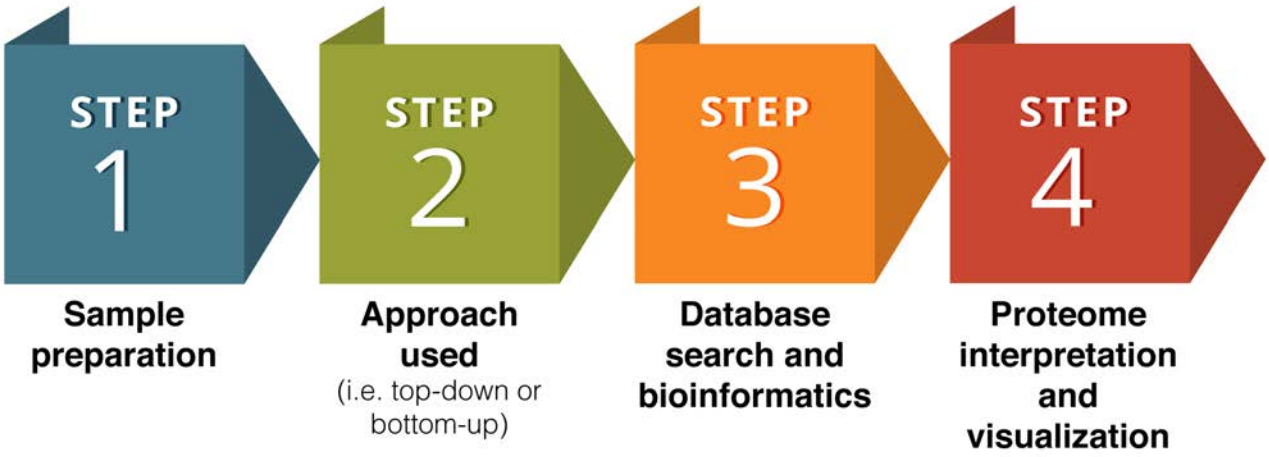


Figure 1.15. Four steps for performing a proteomic study.

Sample preparation, here referred to the protein extraction, is of uttermost importance as a high quality sample will allow to at the end obtain proper results (Rakwal and Agrawal 2003;

Haynes and Roberts 2007; Contreras-Porcia and López-Cristoffanini 2012). Detergents are routinely used for membrane destabilization, but some of them can be detrimental in the consequent steps therefore the correct one must be chosen (Shaw and Riederer 2003; Zhang et al. 2015; Wang et al. 2018). The widely used detergent SDS can interfere in the two-dimensional electrophoresis (2-DE) step as well as it can be detrimental to peptide LC-MS sensitivity (Zhang et al. 2015; Mehraj et al. 2018). Contrarily, nonionic zwitterionic detergents such as NP-40 or CHAPS are more compatible and easier to remove (Shaw and Riederer 2003; Feist and Hummon 2015). Then, protein extracts are ready to be analyzed, which can be performed in a top-down or bottom-up manner being a key difference that the latter does not need necessarily need a protein separation prior to the MS/MS analysis [Chait (2006), Figure 1.16A]. Independent of the approach used, mass spectrometry (MS) is the analytical method chosen for protein identification as it can precisely and accurately identify proteins at the femtomole level and their post-translational modifications [Li and Assmann (2000) and Hirano et al. (2004), Figure 1.16B]. Advances in MS analysis allow nowadays to perform bottom-up in a complex protein mixture, which is called shotgun proteomics, a term coined by the Yates lab because of its analogy to shotgun genomic sequencing (Zhang et al. 2015). Altogether, shotgun bottom-up proteomics has several advantages: (i) peptides are more easily fractionated, ionized and fragmented than whole proteins, (ii) hydrophobic and very low abundant proteins can by analyzed [in 2-DE it is not possible, Neilson et al. (2007) and Gupta et al. (2015)], and (iii) peptides from different samples can be simultaneously quantified (Chandramouli and Qian 2009). Once peptides have been identified by MS in a bottom-down approach, peptide identification is achieved by comparing the tandem mass spectra derived from peptide fragmentation with theoretical tandem mass spectra generated from *in silico* digestion of a protein database (Zhang et al. 2015). Novel technologies have made possible

to analyze different samples in one run by tagging each sample with a different identification marking. For example, iTRAQ technology uses isobaric tag for peptides allowing to analyze even 8 samples at the same time in a high-throughput manner obtaining relative quantification for all peptides (Chandramouli and Qian 2009).

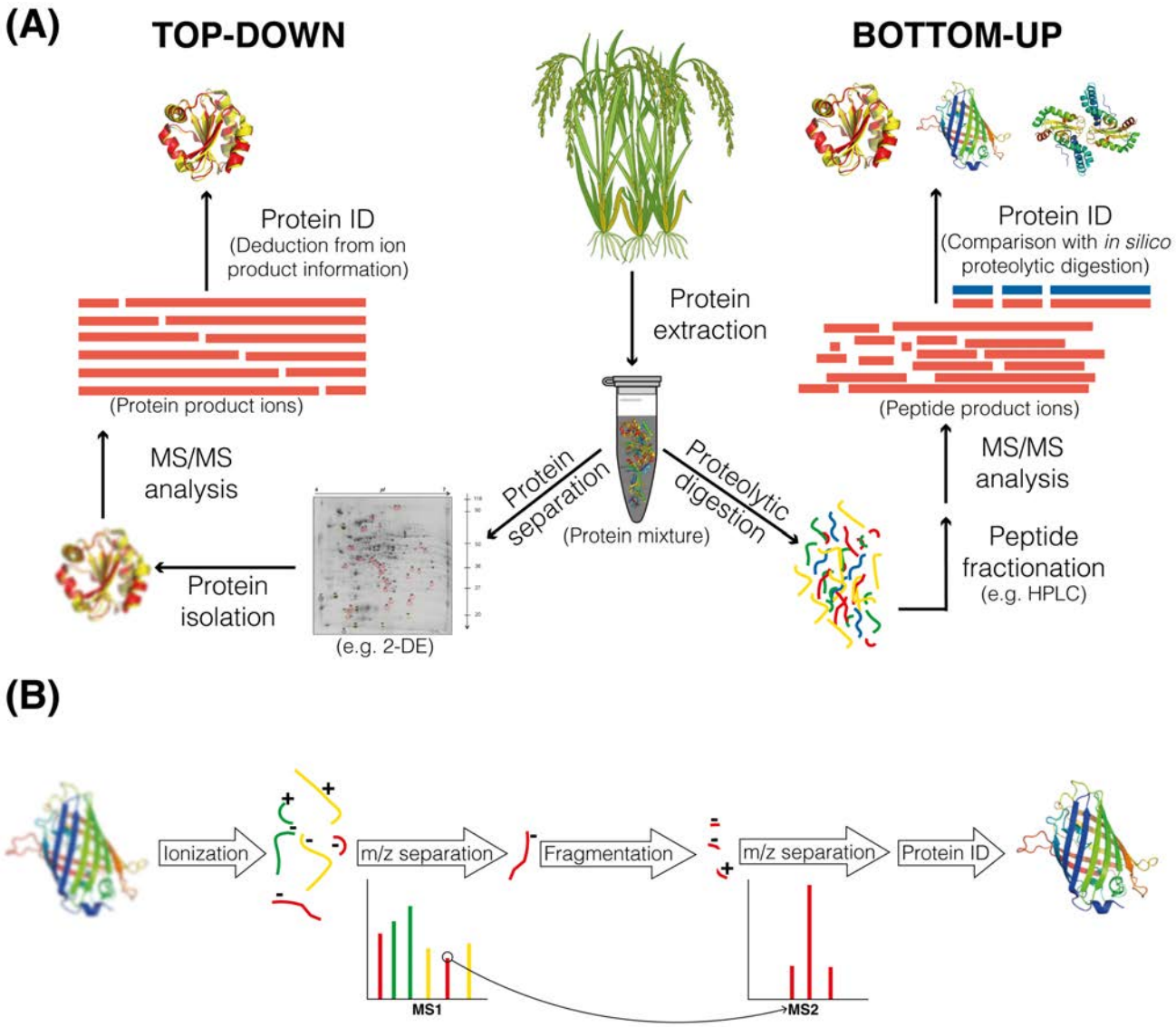


Figure 1.16. (A) Top-down and bottom-up approach. (B) Schematic representation of a protein identification using tandem mass spectrometry (MS/MS).

1.4.2. Proteome visualization and interpretation

Omics studies, like proteomics, and specially shotgun proteomics, generates huge datasets which are of tremendous importance but are not easily handled and interpreted (Nesvizhskii et al. 2007; Skirycz and Hannah 2012; Oveland et al. 2015; Wang et al. 2015). Vizcaíno et al. (2015) mentioned, in a special issue in PROTEOMICs entitled *Proteomics Data Visualisation* that data visualization has moved from being a secondary focus to being one of the key elements to facilitate the understanding, interpretation and validation of the data". Advancements in bioinformatics, software and hardware, along with increasing information in databases have made it possible to make this leap. We will focus on visualization of identified proteins that have been quantified or relatively quantified through the different approaches available [iTRAQ, free-labelling, ^{15}N metabolic labeling, SILAC and ^{18}O-Trypsin among others; Chandramouli and Qian (2009) and Thelen and Peck (2007)]. For a quantitative overview of the proteome, two good approaches are volcano plots and Venn diagrams [Oveland et al. (2015), Figure 1.17]. Volcano plots are graphs generated by plotting the fold change (using a $\log_2$ transformation) versus the *p*-value ($-\log_{10}$ transformed) of the selected quantified proteins, therefore both are used for comparing proteins relative abundances (Oveland et al. 2015; Wang et al. 2015; López-Cristoffanini et al. 2019). Venn diagrams are often used to illustrate number of proteins uniquely present in one condition and not in others, or even in two or more conditions (Oveland et al. 2015). Both approaches are good for an overview but overpass information, but for making in-depth analysis of data subsets heatmaps and network/pathways are more appropriated. These approaches allow to add more detailed information such as functional annotations, protein interactions and

pathways among others (Oveland et al. 2015; Wang et al. 2015; Szklarczyk et al. 2017); therefore providing more substantial information to the analysis.

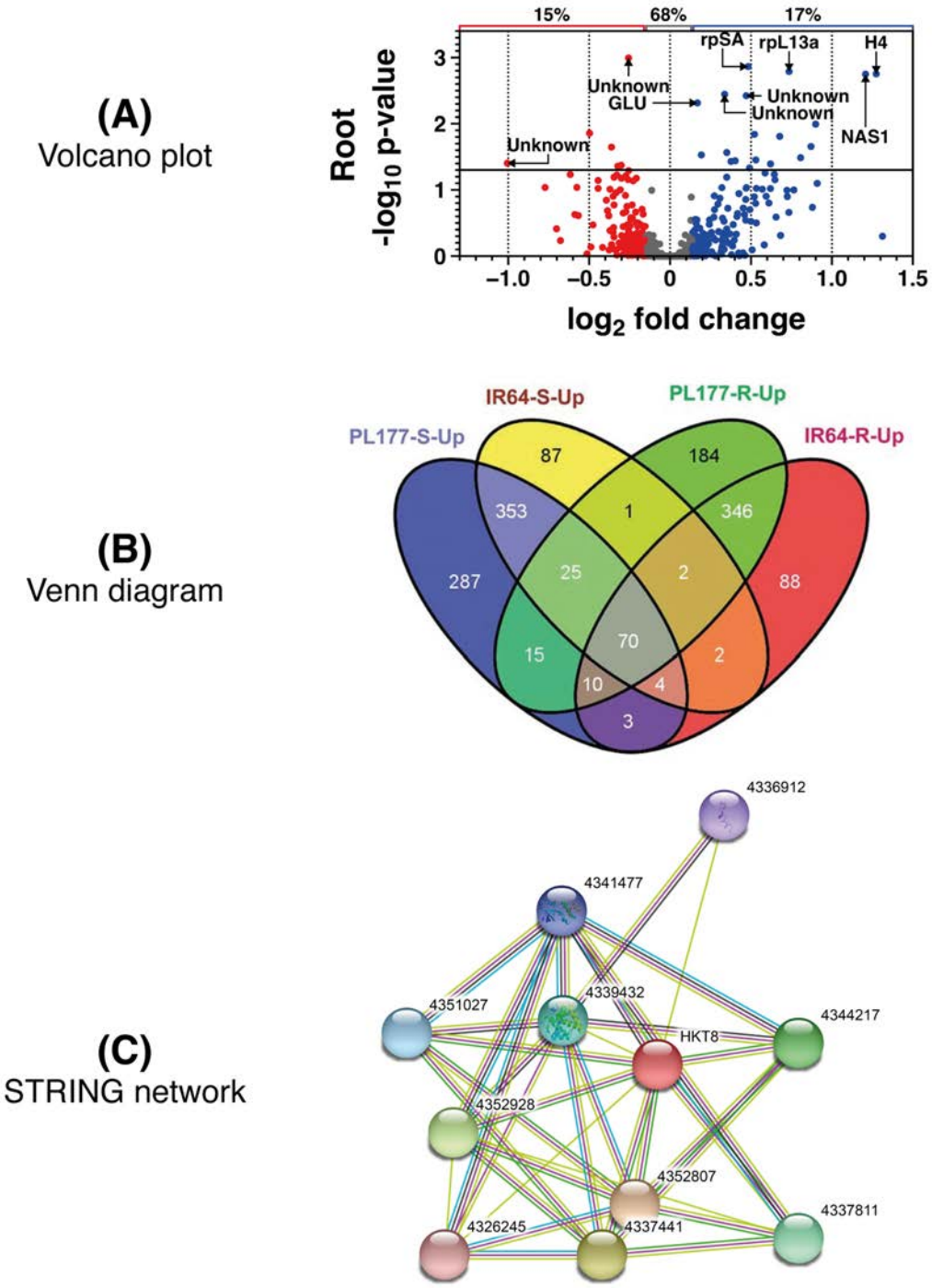


Figure 1.17. Examples of visualization approaches for proteomic data sets. (A) Volcano Plot, (B) Venn Diagram, (C) networks (STRING web interface).

1.4.3. An outlook on rice proteomics

The publication of the rice genome allowed to make great advancements in the proteomics area, *i.e.* it was possible to predict proteins from the genome or to determine a gene from a novel identified protein (Rakwal and Agrawal 2003). In fact, if a search is done in PubMed (https://www.ncbi.nlm.nih.gov/pubmed/), from 2006 onforward the number of articles concerning rice proteomics has increased considerably (Figure 1.18). In fact, a dedicated proteogenomics database for rice (OryzaPG-DB) was available from September 2010 to May 2017, and its most recent version can be downloaded at https://github.com/MoHelmy/oryza-PG/. Studies in rice proteomics have covered several areas of rice studies such as the effects of heavy metals, drought or tissue-specific and organelle-specific proteomics (Rakwal and Agrawal 2003). At first, as in other plant species, several studies were performed in order to tune up proteomics methods as the number of proteins that could be identified was low, mainly due to the lack of technological advancements and genomic information (Salekdeh et al. 2002; Rakwal and Agrawal 2003; Abbasi and Komatsu 2004). In fact, Abbasi and Komatsu (2004) published an article on 2004 where they only identified 8 proteins involved in salinity tolerance in rice leaves, whereas an article published just one year later by Kim et al. (2005) identify by MS more than 30 proteins. Afterwards, studies pointed at basic science for discovering proteins or for identifying and quantifying differential expressions in different conditions (Agrawal et al. 2009). Nevertheless, in rice 2-DE and subsequent MS analysis have been the trademark and novel techniques such as shotgun proteomics are not routinely used, a quick search on Pubmed displays 799 articles for “rice proteomics” and only 57 for “shotgun proteomics rice” (Agrawal and Rakwal 2011). Nowadays, as more genomic information is available, proteomic studies are moving into a new filed which is aimed at

understanding plant behavior at the proteomic level for generating new cultivars (Kim et al. 2014; Das et al. 2015; Hu and Wang 2016).

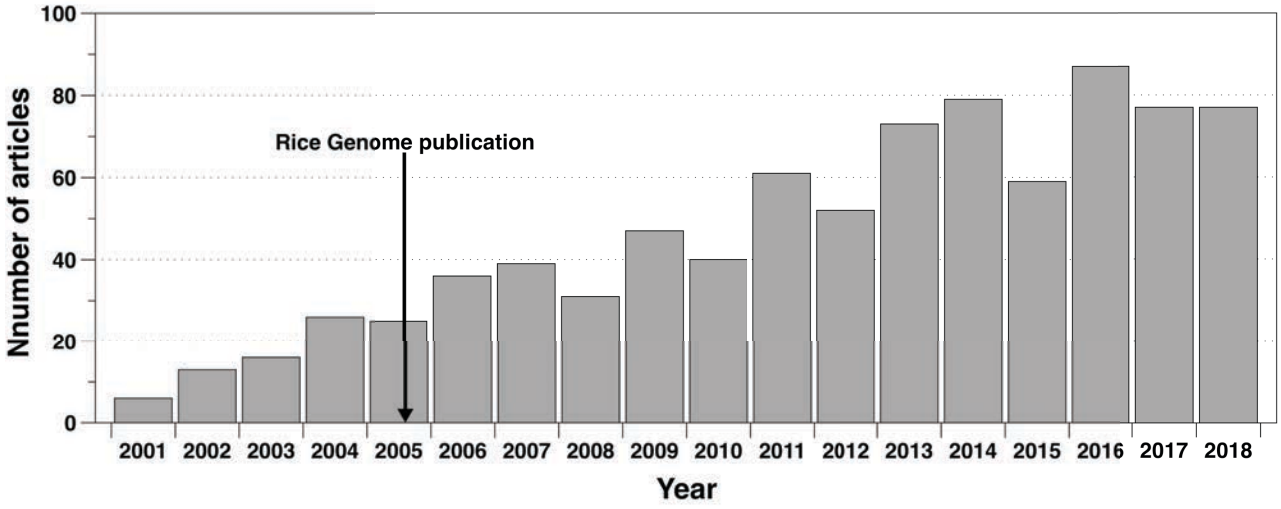


Figure 1.18. Rice proteomics publications (search performed in PubMed using the terms “rice proteomics”).

1.5. Rice and hormones

1.5.1. Hormones and abiotic stress

Plant hormones, or phytohormones, are small molecules that act at very low concentrations and that have concrete signaling functions. They are key participants in several growth and developmental processes (Davies 2010; Kudo et al. 2013). The five “classical” phytohormones include abscisic acid (ABA), cytokinin (CK), auxins (AUX), gibberellins (GAs) and ethylene (ET), whereas the “modern” hormones include jasmonates (JA) brassinosteroids (BS), salicylic acid (SA) and strigolactones [SL; Santner and Estelle (2009) and Peleg and Blumwald (2011)]. Nowadays, there is vast knowledge regarding phytohormone biosynthesis, regulation and most of their specific roles in the signaling pathways in the different processes (Peleg and Blumwald 2011). Besides this regulatory

functions during development, they are also critically involved in the responses to both abiotic and biotic stress [(Javid et al. 2011; Eyidogan et al. 2012; Khan et al. 2012; Fahad et al. 2015), Figure 1.19]. They have been also looked at to be engineered for abiotic stress tolerance in crop plants (Wani et al. 2016).

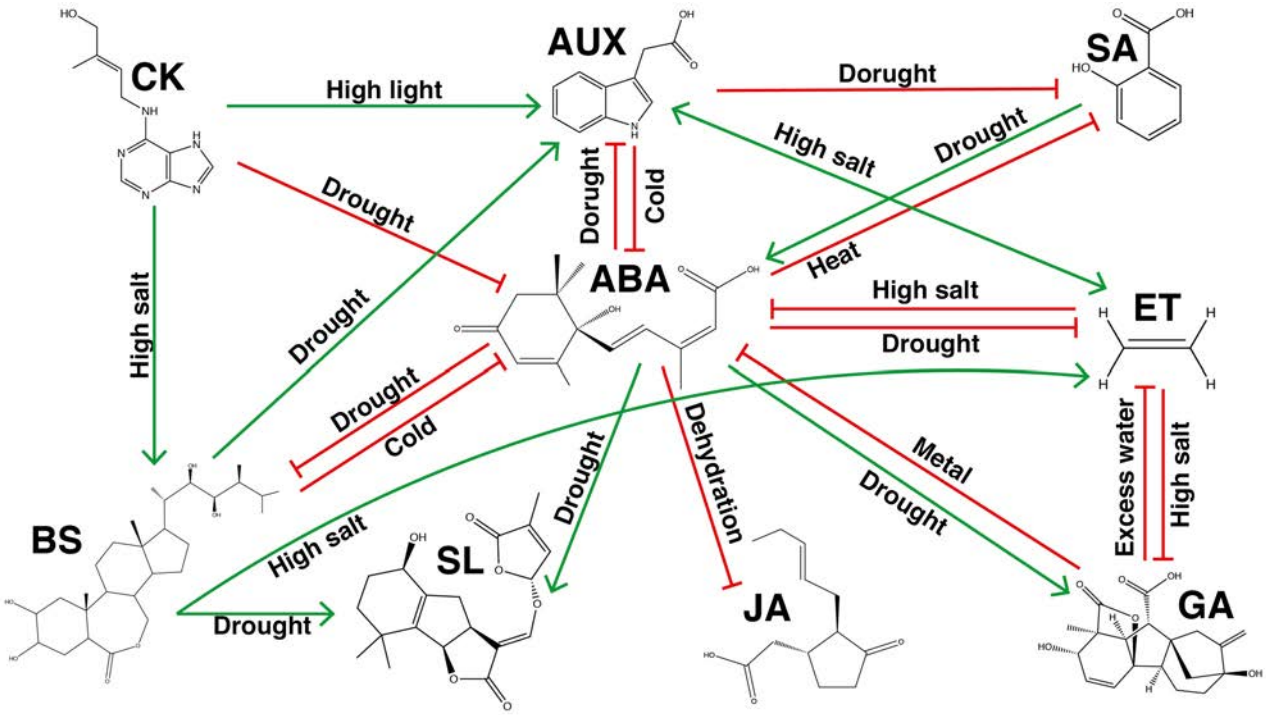


Figure 1.19. Phytohormones involved in abiotic stress and their cross-talk. ABA: abscisic acid; AUX: auxin (indole-3-acetic acid); BS: brassinosteroids (Brassinolide); CK: cytokinin (zeatin): ET: ethylene; GA: gibberellin (GA_3). Adapted from Anderson (2004), Gururani et al. (2015), Wani et al. (2016).

As explained in previous sections, salinity is one of the most menacing abiotic stress currently faced by agriculture (Parihar et al. 2015). This section will therefore focus on some of the roles and/or mechanisms that phytohormones have on plants when facing salinity stress. ABA is a key player in these responses as it regulates the plant's water status among other responses (Fahad et al. 2015). ABA is involved in signaling guard cells for stomata closure to avoid water loss through transpiration, which is caused by a downstream phosphorylation cascaded caused by a Ca^{2+} concentration increase in the cytoplasm (Munemasa et al. 2015).

In addition, ABA is also involved in decreasing Na^+ accumulation in leaves by: (i) Na^+-sequestering in the vacuole, (ii) Na^+ accumulation in shoots and (iii) Na^+ reduced xylem transport. In correlation with ABA increased contents, cytokinins (CK), an ABA antagonist, is known to decrease as well as its biosynthesis genes expression when water availability is low (Peleg and Blumwald 2011; Ha et al. 2012). It is well known that indole-3-acetic acid (IAA), member of the auxin family, also responds to salinity stress, however there is little information on its alleviating role facing this stress (Jain and Khurana 2009; Sangha et al. 2013; Fahad et al. 2015). Clearly, YUCCA genes family seems to be key for auxin response to abiotic stress (Bielach et al. 2017; Korver et al. 2018). SA responses to salinity stress are rather ambiguous: some researches have shown that SA induces oxidative stress and genes that are related to signaling pathways that lead to cell death, whereas others that its presence accumulates ABA and prolines [osmoprotectants; Shakirova et al. (2003), Horváth et al. (2007) and Khan et al. (2012)]. Finally, gibberellins are also involved in salinity stress as they regulate other phytohormone levels through hormone cross-talk (Peleg and Blumwald 2011). In fact, it was shown that *Arabidopsis thaliana* overexpressing a GA-biosynthesis gene (*FsGASA4*) showed improved salt tolerance (Alonso-Ramirez et al. 2009), and in rice GA_3 alleviated the effect of salinity stress and induced the accumulation of proteins involved in salt stress tolerance (Wen et al. 2010).

1.5.2. Gibberellins and height

Gibberellins are a large group of tetracyclic diterpenoid carboxylic acids involved in several developmental processes, although relatively few have intrinsic biological activity (Hedden and Phillips 2000). They were first identified as secondary metabolites of the fungus *Gibberella fujikuroi* (reclassified as *Fusarium fujikuroi*), but are known to be present ubiquitously in higher plants (Hedden and Thomas 2012). More than 136 different gibberellin structures have been found, but the majority are only precursors of the 4 known bioactive forms: GA_1, GA_3, GA_4 and GA_7 (Hedden and Phillips 2000; Yamaguchi 2008; Binenbaum et al. 2018). Gibberellin biosynthesis, in plants, starts in plastids where *trans*-geranylgeranyl diphosphate (GGPP) is converted in two steps to *ent*-kaurene (Figure 1.20). Then, this molecule goes to the endoplasmic reticulum where it is lastly converted into gibberellin GA_{12} which can be then synthesized to GA_{53} through and -OH addition by the GA 13-oxidase (*GA13ox*) (Yamaguchi 2008; Urbanová et al. 2013). Then, both GAs enter the cytoplasm where two pathways can be followed: (i) the GA_{12}- or non-hydroxylated gibberellins pathway or (ii) to follow the GA_{53}- or hydroxylated gibberellin pathway. Interestingly, both pathways have the same enzyme, GA20-oxidase (*GA20ox*), which produces GA_9 and GA_{20} for GA_{12}- and GA_{53}- pathways respectively (Yamaguchi 2008). Then, by the action of GA 3-oxidase (*GA3ox*) the bioactive gibberellins are produced: GA_1 and GA_3 (GA_{53}-pathway) and GA_4 and GA_7 (GA_{12}-pathway) (Hedden and Phillips 2000). All these findings have revealed that there are several steps for GAs biosynthesis regulation including genes for activation/deactivation and phytohormones interaction at several levels of the biosynthesis pathways (Wang et al. 2017).

The study of gibberellins is not an easy task as their concentration in plants is very low, varying between 0.89 to 16.8 ng g^{-1} of fresh weight (Chen et al. 2012). Therefore, efforts must be made for obtaining GA-enriched extracts (Urbanová et al. 2013). The majority of GAs extraction methods use the classic liquid-liquid extraction and solid phase extraction (SPE) with reverse phase C-18 cartridge for sample concentration and clean up (Macías et al. 2014). And as for quantifying, likewise for proteomics, HPLC-MS/MS is the standard and routine technique for GAs separation and detection (Urbanová et al. 2013; Macías et al. 2014) mainly using triple quadrupole instruments for their quantification at trace levels.

Gibberellins have been largely viewed as phytohormones involved in processes such as seed germination, vegetative growth, flowering, and fruit development (Olszewski et al. 2002; Binenbaum et al. 2018). This phytohormones gain a large focus in 1960 during the green revolution as it was previously mentioned, and it has been shown that a mutation in one of their biosynthesis genes causes dwarfism traits in plants (Hedden 2003). Nevertheless, not all semi-dwarf or dwarf mutants are of agricultural interest, as they can have undesirable secondary effects such as altered tillering, small grains, semi-sterility and malformed panicles (Liu et al. 2018). Independently of the *GA20ox-2* mutation that provokes height reduction, the gibberellins production pattern is disrupted as *sd-1* mutated plants show GA_{53} accumulation and a lower content of bioactive gibberellins (Ashikari et al. 2002; Sasaki et al. 2002; Spielmeyer et al. 2002). It is worth noting that the *GA20ox-2* mutation only causes biosynthesis disruption and that external GA applications allow to recover normal height (Hedden 2003). Moreover, a *slender* rice mutant has been found, which has a mutation in the *SLR1* gene which provokes a constitutive gibberellin response phenotype [Ikeda et al. (2001), Figure 1.21]. Finally, as there are critically involved in rice plant height control, their role in rice lodging has been well studied (Okuno et al. 2014; Plaza-Wüthrich et al. 2016).

Lodging is the bending over the stems which reduces yields and depends largely on plant height but also on the physical strength of culms (Ookawa et al. 2014; Wing et al. 2018). A detailed study by Okuno et al. (2014), showed that GA-deficient mutants have increased bending-type lodging resistance whereas high producing GA plants had increased breaking-type lodging resistance and higher biomass. Therefore, the involvement of GAs in rice variety improvement still has a long way to go and this calls for more studies on the subject.

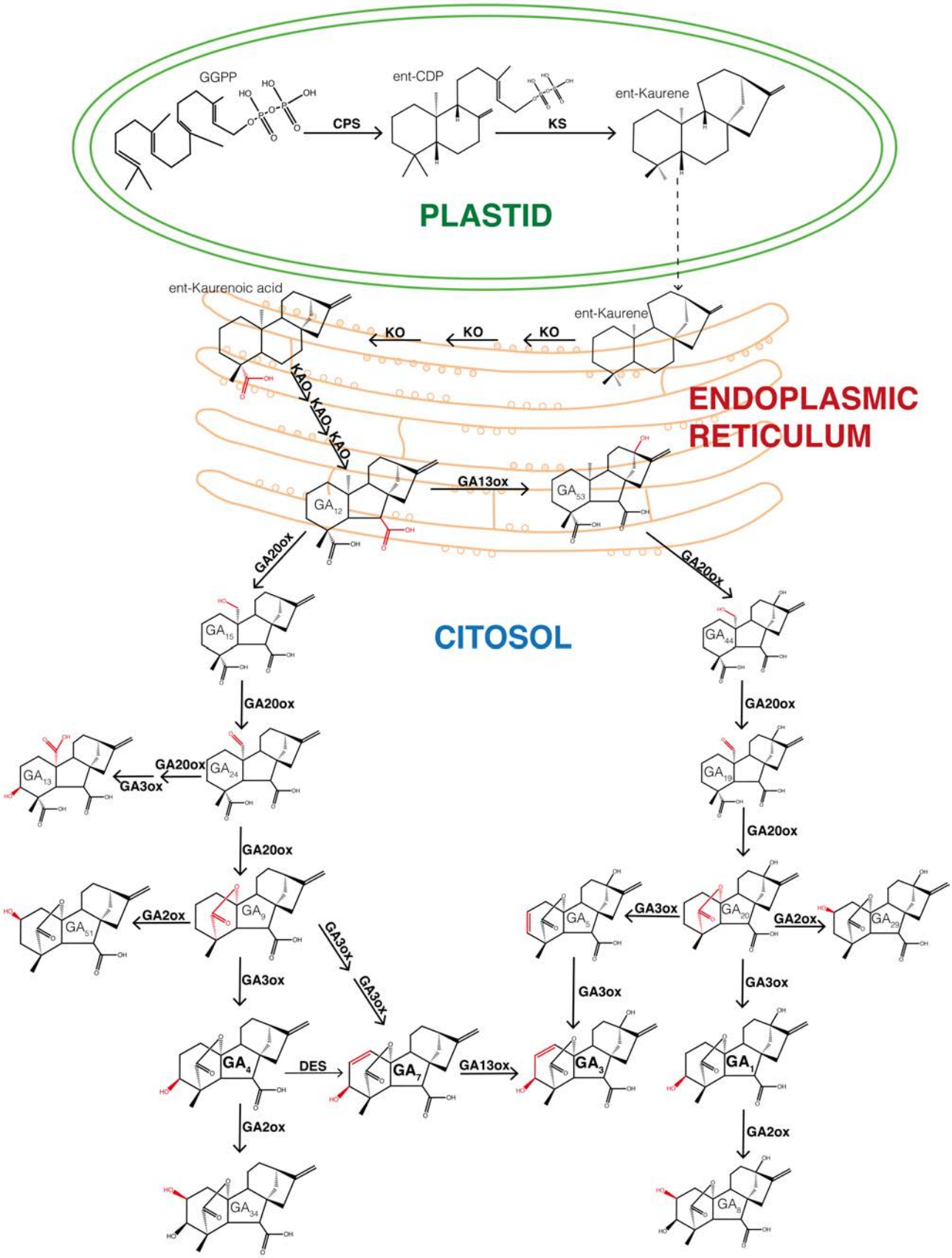


Figure 1.20. Gibberellins (GAs) biosynthesis in plants. Adapted from Hedden and Phillips (2000), Hedden and Thomas (2012) and Urbanová et al. (2013).

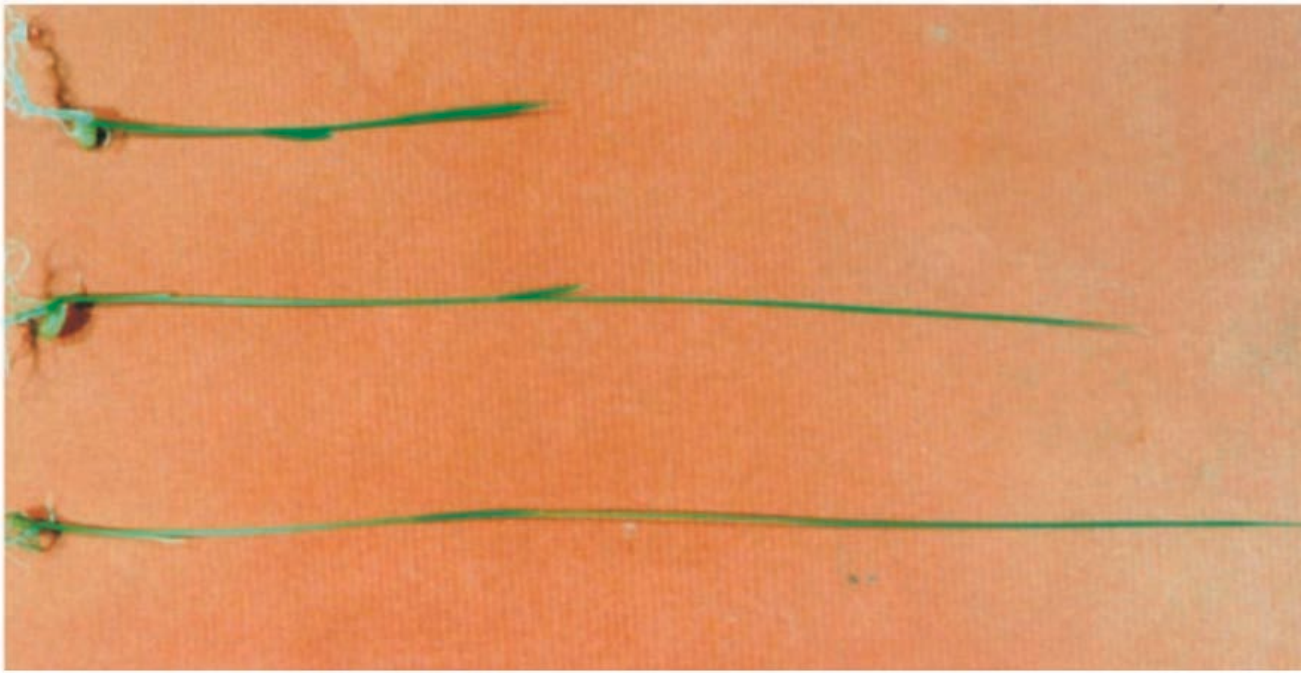


Figure 1.21. *Slender* phenotype caused by a mutation in *SLR1*. Up: wild-type plant, middle: wild-type treated with 10 µM GA_3; bottom: *slr1-1* mutant. Retrieved from Ikeda et al. (2001).

1.6. Variety improvement

Since the domestication of rice that lead to *O. sativa* and *O. glaberrima*, humans have been selecting traits on the rice plant. At first, traits were selected in an unconscious manner but always fulfilling human needs such as shorter time for maturity, higher yield and higher tolerance to biotic and/or abiotic stress. All these traits were selected due to it was possible to characterize them visually due to phenotypic differences (Sang and Ge 2007). In fact, improving varieties has always been one of human focuses in agriculture and domestication (see section 1.1.1) could be considered as the first attempts at variety improvement. Nowadays, we have more advanced techniques to evaluate traits in plants (as explained in section 1.4) for improvement of rice varieties.

1.6.1. Classical vs. modern breeding

The domestication process, although not a breeding process, correspond in fact to the varietal improvement as more tolerant or productive plants were deliberately chosen (Gepts 2002). Classical breeding, at its core, is the deliberate interbreeding (crosses) of different varieties to obtain better descendants which undergo a pedigree or post-breeding selection, i.e. artificial selection, for the desired phenotypically traits (Visscher et al. 1996; Wing et al. 2018). A widely used and efficient way to produce new varieties are the backcrosses programs, which aim is to introgress a gene/trait from a “donor” into the genomic background of a “recipient” (Hospital and Charcosset 1997; Hasan et al. 2015a), and for this the recipient variety is crossed again at each generation with the resulting backcrossed lines (Figure 1.22). The “foreground selection”, assessing the presence of the introgressed gene/trait, was greatly enhanced with the appearance of molecular markers, as those traits with no direct phenotypic evaluation could be easily followed. However, to ensure that the recipient variety keeps all his genes except for the donor one, a “background selection” along with the foreground selection is carried out as to only introgress the desired genomic region, QTL or gene (Hasan et al. 2015a). It has been empirically shown that the mark-assisted back-cross breeding programs are more efficient in retaining higher percentages (Figure 1.22). As for other branches of rice science, the availability of the rice genome also improved the techniques used in backcrosses. An example is the Kompetitive allele-specific PCR (KASP), a technique based on single nucleotide polymorphism (SNPs) markers for detecting variations between crosses (Smith and Maughan 2014). It greatly enhances background selection as it is possible to analyze in one single run a huge number of genomic regions to determine if it belongs to the donor or recipient variety by using the already available rice

varieties genome sequences (Lateef 2015). KASP technique is faster and cheaper than the SSRs microsatellite markers and more flexible than genotyping by sequencing (GBS) or array-bases genotyping (Lateef 2015; Steele et al. 2018). Moreover, Steele et al. (2018) designed a free software, KASP design generator, to aid breeders worldwide to implement this technique.

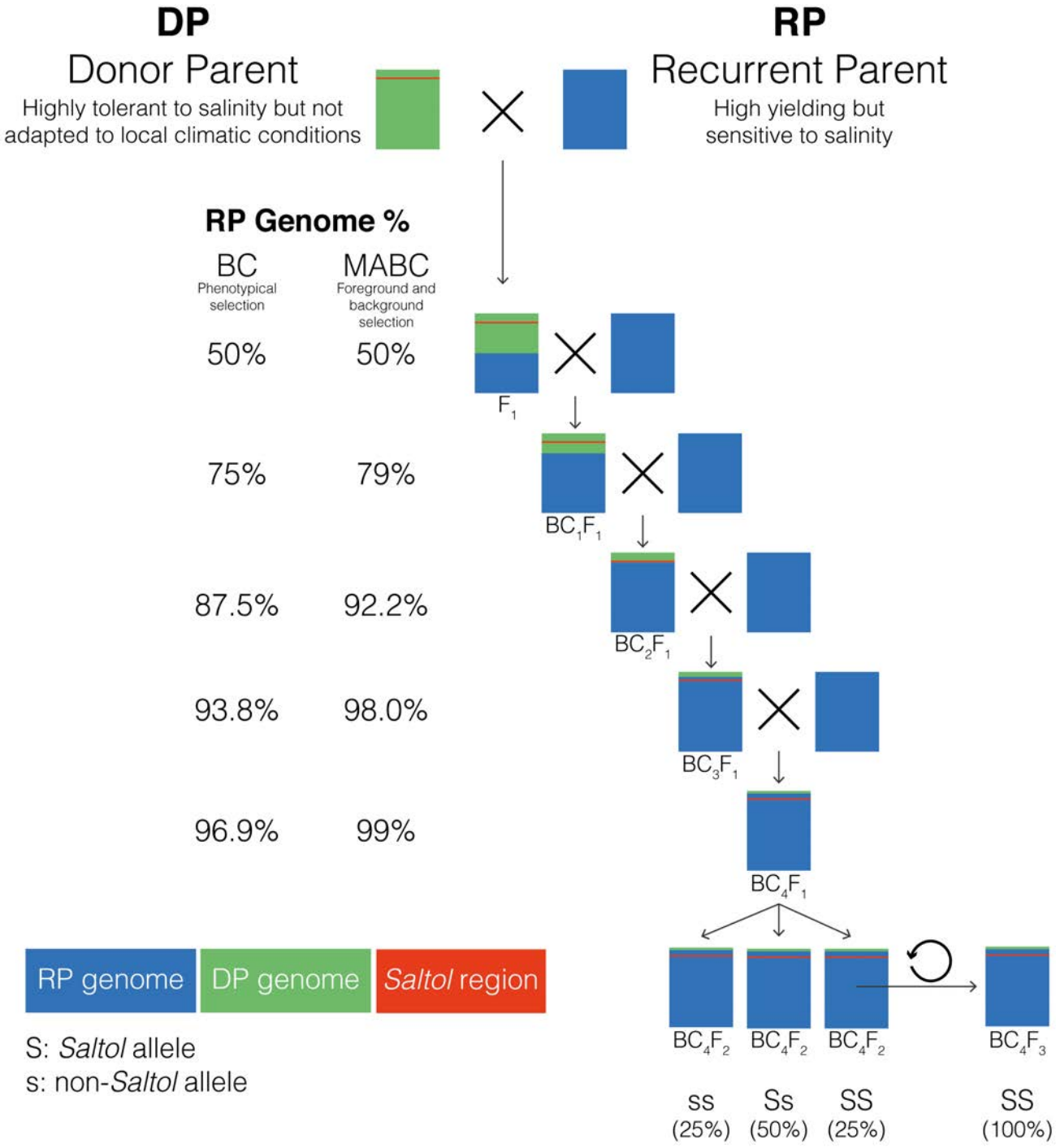


Figure 1.22. Backcross program scheme for the introgression of *Saltol* into Spanish rice varieties. BC: conventional BackCross, MABC: Marker-Assisted BackCross. Adapted from Hasan et al. (2015b).

1.6.2. Genetic engineering

Alongside breeding, nowadays we have the resources and techniques to perform modifications, that vary in precision, directly in the genome of rice plants (Eriksson 2018). The use of biological, physical or chemical mutations has been widely useful as more than 400 varieties have been produced this way (Ahloowalia and Maluszynski 2001; Ahloowalia et al. 2004). Despite the usefulness of this technique, the main problem is that mutation is random, which could lead to (i) undesired mutations that breeders could be unaware of, or (ii) to silent mutations that are not detected (Capilla-Perez et al. 2018). Another technique for genetic engineering that has been widely used, although primarily for basic science, is the production of genetically modified organisms through recombinant DNA. This process is called transgenesis and allows to introduce genes from unrelated and cross-incompatible species (Araki and Ishii 2015). Its major advantage is the fast production of new varieties through the introgression of cloned genes, which suppose an added value, into commercial varieties (Zhang et al. 1998). Nevertheless, likewise for induced mutations, the transgene during the process is inserted randomly in the genome in a nonspecific manner (Ray and Langer 2002; Britt and May 2003). This can cause problems since its insertion could provoke deleterious or undesired phenotypes depending on where it is inserted. Thus, conventional genetic engineering is labor-intensive and requires time-consuming screens to identify the desired plant mutants (Araki and Ishii 2015). In contrast to induced mutations or transgenesis, genome-editing is an advanced genetic engineering tool that can more directly modify a gene within a plant genome (Araki and Ishii 2015). Although some of these techniques have been available since the 90's, they have been only recently become widespread. This inflection point occurred when researchers noted that using site-directed nucleases (SDNs) they

engineering of the nuclease allows for highly specific targeting to any given gene of interest (Wolt et al. 2016). In fact, nowadays several techniques using SDNs are available, such as such as CRISPR (Clustered Regularly-Interspaced Short Paloindromic Repeats), ZFN (Zinc Finger Nuclease) and TALEN [Transcriptional Activator-Like Effector Nuclease; Belhaj et al. (2015) and Wolt et al. (2016)]. Moreover, as seen in Table 1.1, several applications using these techniques have been reported including several applications in rice like the *OsBADH2* for fragrance improvement and the simultaneous edition of three homoalleles of *TaMLO* in the hexaploidy bread wheat (Araki and Ishii 2015).

Table 1.1. Examples of reported genome-editing-mediated gene modifications in major crops. Adapted from Araki and Ishii (2015) and Bortesi and Fischer (2015).

Species	Target locus	Genome-editing technique	Modification type	Efficiency of modification	Off-target mutation	Genotyped subject
Rice	*ZmIPK*	TALEN	Indel	39.1%	N.D.	Plants
	ZmIPK	CRISPR-Cas9	Indel	13.1%	N.D.	Protoplasts
	OsSWEET14	TALEN	Biallelic indel	6.7-27%	N.D.	Plants
	OsPDS-SP1 *OsBADH2* *OsMPK2*	CRISPR-Cas9	Biallelic indel	3.1% (OsPDS-SP1) 0% (OsBADH2) 0% (OsMPK2)	No (OsPDS-SP1) Yes (OsMPK2) N.D. (OsBADH2)	Plants
	OsSWEET11 *OsSWEET14*	CRISPR-Cas9	Indel	91% (OsSWEET11)[a] 90% (OsSWEET14)[a]	N.D.	Protoplasts
	OsBADH2 *OsCKX2*	TALEN	Biallelic indel	12.5% (OsBADH2) 3.4% (OsCKX2)	N.D.	*Calli*
	OsBEL	CRISPR-Cas9	Biallelic indel	2.2%	No	Plants
	OsPDS	CRISPR-Cas9	Introducing KpnI + EcoRI sites	6.9%	No	Protoplasts
Barley	*HvPAPhy_a*	TALEN	Indel	16-31%	N.D.	Plantlets
Maize	*ZmIPK1*	ZFN	Inserting PAT	3.4-22.1% (autonomous)[b] 16.7-100% (non-autonomous)[b]	No	*Calli*
Soybean	*DCL4b*	ZFN	Biallelic indel	25%	N.D.	Plants
	FAD2	TALEN	Biallelic indel	33.3%	No.	Plants
Tomato	*PROCERA*	TALEN	Biallelic indel	2.5%	N.D.	Plants
Wheat	*TaMLO*	CRISPR-Cas9	Indel	28.5%	N.D.	Protoplasts
	TaMLO	TALEN	Heterozygous indel for all three homoeoalleles	3.7%	N.D.	Plants

[a]Indicates the results of sequencing after the enrichment of mutated alleles.

[b]Two different donor constructs containing short homology arms were used: one with an autonomous herbicide tolerance gene expression cassette (PAT), the other with a non-autonomous donor that relied on precise trapping of the endogenous *ZmIPK1* promoter for expression of the marker.

N.D: not determined.

1.6.3. In-vitro techniques: anther culture

In-vitro techniques refer to those of plant cell, tissue and organ culture, which are literally performed “in glass” where they are grown (Hussain et al. 2012). All the *in-vitro* techniques depend upon two principal concepts or properties of plant cells: cell totipotency and cell plasticity (García-Gonzáles et al. 2010; Ikeuchi et al. 2016). Although at first they were predominantly used for answering important fundamental questions and identifying mechanisms in plant biology, they are currently and widely employed as useful biotechnological tools (Loyola-Vargas and Ochoa-Alejo 2012; Thorpe 2012). In the case of the genetic engineering techniques previously discussed, for most of them if not all, an *in-vitro* step must be employed at one moment of each different method or procedure. In fact, in Table 1.2 the genotyped subject is detailed and it can be observed that not only plants were used as some study used *calli* [an unorganized mass of differentiated plant cells, Hussain et al. (2012)] and protoplasts [plant cells without cell walls, Thorpe (2012)]. Moreover, even if a whole plant is used for the procedure, it is then subcultured using *in-vitro* techniques and a plant is regenerated from an explant [an excised piece or part of a plant used to initiate a tissue culture, Hussain et al. (2012)].

In addition to the applications aforementioned, *in-vitro* culture is also used for commercial micropropagation of different plant species, generation of disease-free plant materials, generation of novel hybrid plants through the rescue of hybrid or production of haploid and double-haploid plants among others (Hussain et al. 2012; Loyola-Vargas and Ochoa-Alejo 2012; Ikeuchi et al. 2016). One of this applications has been extensively used in rice breeding programs, the production of double haploid plants through *in-vitro* anther culture (Courtois 1993; Serrat et al. 2014; Mishra and Rao 2016; Hooghvorst et al. 2018; López-Cristoffanini

et al. 2018). In rice, the first anther culture was developed by Niizeki and Oono (1968), and its applicability has been demonstrated as several rice commercial varieties have been produced using this technique since the 80's [Serrat et al. (2014) and Mishra and Rao (2016); Table 1.2]. Rice anther culture is a two-step process of initial development of *calli* and subsequent regeneration of green plants from embryogenic *calli* [Mishra and Rao (2016), Figure 1.23]. Although several improvements have been made, there is still need to optimize the efficiency of this technique. The androgenesis (microspore or pollen embryogenesis) is affected by endogenous factors (genotype, the developmental stage of the anther or donor plant physiology) and exogenous factors [anthers pre-treatment, culture medium, growth regulators and antimitotic agents; Trejo-Tapia et al. (2002a), Mishra and Rao (2016), Hooghvorst et al. (2018) and López-Cristoffanini et al. (2018)]. The auxin 2,4D (2,4-dichlorophenoxyacetic acid (2,4-D) and NAA (naphthaleneacetic acid) are among the most used phytohormones for the first step of rice anther culture [i.e. *calli* induction from anther; Trejo-Tapia et al. (2002a)]. Regarding the second step, antimitotic agents such as colchicine and oryzalin have been used for increasing the rate of anther *callus* induction and green plantlets (and also green double haploid plantlets) regeneration in several species but rarely assessed in rice (Hansen and Andersen 1998; Hooghvorst et al. 2018). Despite the need for improving the anther culture, this technique is highly valued as it bypasses the long inbreeding process of the pedigree selection allowing to obtain pure and stable descendants (i.e. homozygous lines) in only one year (Miah et al. 1985; Agache et al. 1989).

Table 1.2. List of released commercial rice varieties generated through anther culture. Adapted from Serrat et al. (2014) and Mishra and Rao (2016).

Variety	Characteristic	Country
Huayu I, Huayu II, Xin Xiu, Late Keng 959, Tunghua 1, Tunghua 2, Tunghua 3, Zhonghua 8, Zhonghua 9, Huahanzao, Huajian 7902, Tanghuo 2, Shanhua 7706, Huahanzao 77001, Nanhua 5, Noll, Hua 03	High yielding varieties with superior grain quality; resistant to blast and bacterial blight diseases	China
Guan 18	Early maturity; good quality and disease resistance	China
Huayu 15	Resistant to lodging and diseases; good quality	China
Milyang 90	Good grain quality; resistant to brown planthopper and stripe virus disease	China
Hwacheongbyeo, Joryeongbyeo, Hwajinbyeo	Resistant to brown planthopper, rice stripe tenuivirus, blast and bacterial blight	South Korea
Bicol (IR51500AC11-1)	Salt tolerant	the Philippines
Parag-401	Superior grain quality and resistant to iron chlorosis	India
Risabell	High milling and cooking quality; resistant to blast	India
Janka	Drought tolerance; good grain quality	India
Abel	Cold tolerance at early stage	India
CR Dhan 10 (CRAC2221-43), Satyakrishna	Resistant to neck blast, sheath-rot and yellow stem borer	India
CR Dhan 801 (CRAC2224-1041, IET 18720), Phalguni	Resistant to leaf blast, gall midge; moderately resistant to sheath rot, rice stripe tenuivirus, yellow stem borer, brown spot and sheath blight	India
NRVC20110077	High yielding variety	Spain

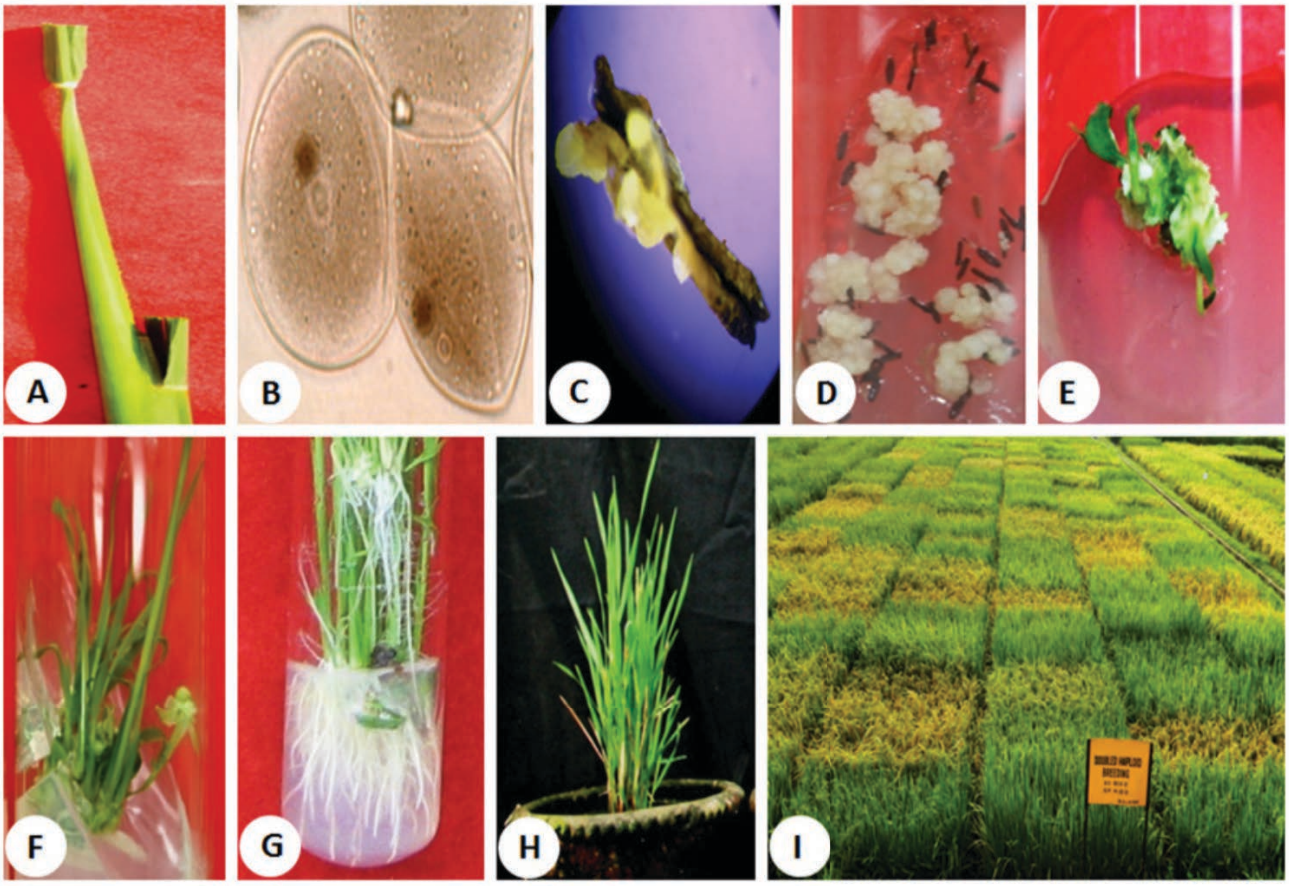


Figure 1.23. *In-vitro* androgenesis in rice. A, boot leaf collected having microspores at mid- to late-uninucleate stage; B, cytological confirmation of mid- to late-uninucleate stage; C, microscopic view of anther with multiple *calli*; D, *callus* induction; E, *callus* regeneration; F, green plant regeneration; G, rooting; H, anther derived plants in net-house condition; I, anther derived plants in field for agronomic evaluation. Retrieved from Mishra and Rao (2016).

2

AIMS & OBJECTIVES

The general aim of this PhD thesis was to study different techniques for evaluating and producing new improved rice varieties. The three specific objectives of this PhD thesis are:

1. To characterize the salinity tolerance of the *Saltol*-harbouring variety FL478, in both shoot and root, subjected to differential salinized hydroponic cultures.
    1.1. Development of a suitable method for protein extraction for a shotgun proteomic analysis.
    1.2. Identification and discovery of proteins involved in the salt tolerance mechanisms through bioinformatics.
    1.3. Characterization of the physiological performance of FL478

2. To develop a phytohormonal profiling for rice and to study the effect of the *GA20ox* mutation in Mediterranean rice varieties with contrasting heights.
    2.1. Evaluation of the performance of different phytohormone extraction protocols, varying in the extraction medium and the SPE approach used, in the phytohormone recovery rate.

2.2. Evaluation of the growth of three Mediterranean rice varieties: NRVC980385, Bomba and *dwarf*-Bomba.

2.3. Analysis of the phytohormonal profiling of the three different varieties in several developmental stages (from coleoptile emergence until flower development).

3. To apply and improve an anther culture protocol in Mediterranean rice varieties.

3.1. Improvement of an anther culture protocol to Mediterranean rice varieties by determining the optimum cold-pretreatment duration.

3.2. Analysis of the antimitotics and hormones effects on green double haploid plantlets production in Mediterranean rice varieties.

3.3. Field evaluation of anther-produced Mediterranean rice varieties.

REPORT ON THE IMPACT FACTOR OF THE PUBLISHED ARTICLES

Dr. Marta López-Carbonell and Prof. Salvador Nogués Mestres as supervisors of the thesis entitled: "**Variety improvement in rice (*Oryza sativa* L.): proteomic, hormonal and *in vitro* studies**" that has been written by Camilo López-Cristoffanini report on the impact index and participation of the doctoral student in the articles included in his doctoral thesis.

Chapter 1 – "PROTEOME PROFILING IN SHOOTS AND ROOTS OF THE FL478 GENOTYPE OF RICE (*ORYZA SATIVA* L. SSP. *INDICA*) BY SHOTGUN PROTEOMICS DURING EARLY SALINITY STRESS" by Camilo López-Cristoffanini, Mireia Bundó, Xavier Serrat, Javier Moros, Blanca San Segundo, Marta López-Carbonell, Salvador Nogués; submitted to the journal *PROTEOMICS* (3-year impact on 2017: 3.657) with reference pmic.201800337.

In this study the proteome of a highly salinity tolerant was evaluated in shoots and roots in the early responses to salt stress. In this work, a huge database for salt stress-related proteins in root was obtained. The doctoral student was involved in the study design, experimental work, result analysis and finally the article writing.

Chapter 2 – "PHYTOHORMONE PROFILING METHOD FOR RICE: EFFECTS OF *GA20ox* MUTATION ON THE GIBBERELLIN CONTENT OF MEDITERRANEAN *JAPONICA* RICE VARIETIES" by Camilo López-Cristoffanini, Xavier Serrat, Olga Jáuregui, Salvador Nogués,

Marta López-Carbonell; submitted to Frontiers in Plant Science – Technical Advances in Plant Science (4-year impact on 2017: 4.541) with reference 448885.

In this chapter, the doctoral student deepened in the effect of the GA20ox mutation on three Mediterranean rice varieties. Moreover, to our knowledge, he developed for the first time a method that allows quantifying 13 gibberellins in one run in rice samples of different tissues. The doctoral student was deeply involved in this study, which took more than one year from plant growth until the last gibberellin analysis, including the method tune-up. Finally, he was the main involved in the article writing.

Chapter 3 – *In vitro* anther culture studies

Chapter 3.1 **–** **"**AN IMPROVED ANTHER CULTURE PROCEDURE FOR OBTAINING NEW COMMERCIAL MEDITERRANEAN TEMPERATE *JAPONICA* RICE (*ORYZA SATIVA*) GENOTYPES" by Camilo López-Cristoffanini, Xavier Serrat, Eduardo Ramos-Fuentes, Isidre Hooghvorst, Roser Llaó, Marta López-Carbonell, Salvador Nogués; published in the journal *Plant Biotechnology* (4-year impact on 2017: 1.094) in April 2018, volume 35, issue 2, pages 161-166, doi: https://doi.org/10.5511/plantbiotechnology.18.0409a.

In this chapter, the student was involved in all the tasks of this study. This work in addition of improving the anther culture protocol efficiency, included agronomical assays of the lines produced through this *in-vitro* technique. This showed that the student is capable of performing diverse tasks in all the research areas. The doctoral student was involved in the design, *in vitro* work and writing of the study.

Chapter 3.1 **–** **"**ANTIMITOTIC AND HORMONE EFFECTS ON GREEN DOUBLE HAPLOID PLANT PRODUCTION THROUGH ANTHER CULTURE OF MEDITERRANEAN JAPONICA RICE" by Isidre Hooghvorst, Eduardo Ramos-Fuentes, Camilo López-Cristoffanini, Mirari Ortega, Raimon Vidal , Xavier Serrat, Salvador Nogués; published in the journal *Plant Cell, Tissue and Organ Culture (PCTOC;* 4-year impact on 2017: 2.333) in August 2018, volume 134, issue 2, pages 205-215, doi: https://doi.org/10.1007/s11240-018-1413-x.

In this chapter, the student was mainly involved in the experimental design and the writing of the article. This work supposed a great experience for the doctoral student as he was partly in charge of supervising other students' work, which is of crucial importance for a doctoral student's training. Moreover, in this published article we showed improvements in the anther culture at several stages as well as a post anther culture treatment. This is the first time this work has been used in a doctoral thesis and is not pending to be used in another doctoral thesis. The doctoral student was mainly involved in the design and writing of the study, but also in the *in vitro* work.

Dr. Marta López-Carbonell
Thesis supervisor

Prof. Salvador Nogués Mestres
Thesis supervisor

3

RESULTS

CHAPTER 1 - Proteome profiling in shoots and roots of the FL478 genotype of rice (Oryza sativa L. ssp. *indica*) by shotgun proteomics during early salinity stress

Camilo López-Cristoffanini[1], Mireia Bundó[2], Xavier Serrat[1], Javier Moros[1], Blanca San Segundo[2,3], Marta López-Carbonell[1], Salvador Nogués[1]

[1]Departament de Biologia Evolutiva, Ecologia i Ciencies Ambientals, Secció de Fisiologia Vegetal, Universitat de Barcelona, 08028 Barcelona, España.

[2]Centre for Research in Agricultural Genomics (CRAG), CSIC-IRTA-UAB-UB. Edifici CRAG, Campus de la UAB, 08193 Bellaterra, Barcelona, España.

3Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, España.

This chapter has been submitted as a research paper to PROTEOMICS and is currently under major revisions, manuscript reference: pmic.201800337.

ABSTRACT

Climate change is affecting crop production due to soil salinization and water scarcity, and is predicted to worsen in the coming years. Rice is a major staple food and the most salt-sensitive cereal. High salinity in the soil triggers several adaptive responses in rice to cope with osmotic and ionic stress. A major QTL for salinity tolerance, named *Saltol*, is present on chromosome 1 of Indian rice landrace varieties. In this study, we characterized the early proteomic and physiological responses to salinity in FL478, an inbred rice line harboring the *Saltol* region. Roots showed a faster and more coordinated proteomic response than shoots, with a marked increase in transcription- and translation-related proteins. Moreover, roots exhibited a higher accumulation of stress-related proteins in response to salinity treatment, like peroxidase and *SalT*, which are both present in *Saltol*. At the physiological level, salinity significantly reduced shoot length after 48 h, whereas root length significantly increased. Moreover, the Na^+/K^+ ratio was maintained at lower levels in the shoots compared to the roots FL478 plantlets have roots that respond in a highly adaptive manner to salinity stress, which suggests that this tissue is critical to the tolerance observed in this inbred line.

KEYWORDS: FL478, shotgun proteomic, rice, salinity, *Saltol*.

INTRODUCTION

The 21st century is being marked by climate change, which is translating into reductions in the quantity and quality of arable land due to salinization of soil and water scarcity [1,2]. These problems are directly affecting crop production, which could lead to severe food shortages due to the increasing world population [3]. Among the crops cultivated and consumed worldwide, rice stands out because it is a major staple and one of the most economically important foods for more than half of the world's population [4–6]. Moreover, it is estimated that more than 80% of its production will be destined for direct human consumption in 2017/2018 [7].

Therefore, the increase in soil salinity in the coming years is one of the main concerns for rice production as this cereal is severely affected by the severe osmotic and ionic stresses posed by salinity [4,5,8]. A saline soil is generally defined as one in which the electrical conductivity (EC) of the saturation extract (ECe) in the root zone exceeds 4 $DS \cdot m^{-1}$ (58.4 mM, according to the pseudo-linear approach by Sposito (2008)) at 25 ºC and has an exchangeable sodium of 15% [1,9]. Rice is the most salt-sensitive cereal worldwide, and is thus classified as a glycophyte [10]. The seedling and reproductive stages of rice are the most sensitive to salinity stress with exposure leading to yield losses [8,10–16].

Salinity stress in rice triggers several adaptive responses at the molecular, cellular, metabolic, and physiological levels to cope with the osmotic and ionic stress that excess salt implies [12,17,18]. These responses are mainly related to ion homeostasis in the form of reduced salt intake through roots and by an efficient intra cellular compartmentation and transport of salts to vacuoles or to the external medium. Other typical responses to salinity stress are antioxidant metabolism activation, protein modifications and increases in energy and biomolecule metabolism [4,10,15,18]. According to Roy et al. (2014) and Reddy et al. (2017),

three main mechanisms can be considered: (i) tissue tolerance, (ii) osmotic tolerance and (iii) ion exclusion [10,19]. All these mechanisms allow plants to successfully cope with the stress. In fact, Djanaguiraman et al. (2003) showed that salt-tolerant rice varieties have higher rates of germination, greater shoot and root lengths, and a higher vigor index [20]. It has also been observed that landraces, which are naturally occurring varieties, have greater tolerance to salinity due to their height which allows them to dilute the Na^+ content in their cells, even if the net transport of Na^+ is comparable to high-yielding varieties [10,18,21].

Rice tolerance to salinity has been widely studied and more than 70 QTLs have been identified with this trait in several varieties [22–24]. Moreover, because the majority of salinity tolerance mechanisms regulate ion homeostasis, the majority of QTLs have been identified in relation to Na^+/K^+ transport, exclusion and compartmentation [22,24,25]. Zhang et al. (1995) detected a QTL involved in salt tolerance on chromosome 7 of a mutant line, M-20, which originated from plating anthers of a semi-sensitive variety, 77-170, on a medium containing NaCl [26]. Later, Gong et al. (1999) and Prasad et al. (2000) mapped QTLs for salt tolerance on chromosomes 1 and 6, respectively [27,28]. In 2001, one QTL for Na uptake, two QTLs for Na+ concentration, and one QTL for the Na^+/K^+ ratio were identified in a mapping population designated IR55178 (cross between IR4630- and IR15324-) [29]. Finally, Bonilla et al. (2002) found in a Pokkali(IRGC 108921)/IR29(IRGC 30412) RIL population that a QTL explained more than 70% of the variation in salt uptake during salt stress, this being caused by high K^+ and low Na^+ absorptions and therefore a low Na^+/K^+ ratio [8,30–32]. From this RIL population, the FL478 (*Oryza sativa* L. ssp. *indica*) line was developed, which has high levels of seedling-stage salinity tolerance, lacks photoperiod sensitivity, and is shorter in height and life cycle than the original salt-tolerant Pokkali landrace [33]. Similar to the RIL population, Lin et al. (2004) found in crosses between Nona Bokra

and Koshihikari that a QTL explained more than 48% of the phenotypical variances caused by an accumulation of K^+ in shoots during salinity stress [8]. In fact, the QTLs from both the RIL and Nona Bokra/Koshihikari populations can be mapped very closely on rice chromosome 1, in a genomic region named *Saltol*, which is derived from landraces known to be salt-tolerant, such as Pokkali and Nona Bokra [8,18,21,22,32]. Within this region, *OsHKT1;5*, *SalT*, peroxidases (PDXs), wall associated kinases and protein kinases have been identified alongside several transcription factors [34–38]. There is substantial evidence that the key gene for conferring high tolerance to salinity in this region is the *OsHKT1;5* gene, which encodes an HKT-type transporter (previously known as *OsHKT8* and SKC1) [21,39,40].

The benchmark discovery of the *OsHKT1;5* gene has led to an increase in studies of varieties that carry this salinity tolerance allele such as Pokkali, Nona Bokra and the RIL, FL478. The approaches taken to explore this gene's involvement in salinity tolerance have included physiological studies, transcriptomics and proteomics [4,15,21,41–44]. This gene was isolated and studied in detail by Ren et al. (2005) to understand its molecular basis [42]. Their conclusion, combining rice mRNA expression and voltage clamping of *Xenopus laevis* oocytes, was that *OsHKT1;5* acts in the recirculation of Na^+ by unloading it from the xylem, where the gene is mainly expressed, and delivering the Na^+ to the roots in order to avoid its accumulation in shoots. *OsHKT1;5* physiology was studied in detail in two T-DNA insertional mutants where it was shown to be present in roots and that its major role is to prevent Na^+ accumulation in shoots, as also observed by Ren et al. (2005) [45]. Using an Affymetrix rice genome array containing 55,515 probe sets, Walia et al (2005) found no evidence for the gene's expression in shoots [44]. Similarly, Lakra et al (2017) only detected expression of a similar gene, *OsHKT1;1*, using a qPCR approximation [4]. On the other hand, *Saltol* was

introgressed into Pusa Basmati 1121, and OsHKT1;5 expression was detected in the generated lines through qPCR approximation showing differential patterns between lines [46].

Therefore, the main objective of this study was to characterize the proteome of FL478, a salt-tolerant elite variety that carries the *Saltol* region, during salinity stress via a shotgun proteomic approach, which allows identification of hydrophobic proteins and proteins of very low abundance [47,48]. In addition, a physiological characterization was performed. Although *OsHKT1;5* protein was not detected, we reported changes in *SalT* and peroxidases abundances, genes contained in the *Saltol* region. Finally, use of the bottom-up proteomics technique allowed us to identify several other proteins related to salinity stress responses in this *Saltol*-harboring variety, FL478.

MATERIALS AND METHODS

PLANT MATERIAL AND HYDROPONIC CULTURES

Seeds of the salt-tolerant variety FL478 (IR66946-3R-178-1-1; *Oryza sativa* L. ssp. *indica*) used in this study were obtained from IRRI (International Rice Research Institute, Los Baños, Laguna, Philippines). To perform the hydroponic assays, seeds were first externally sterilized in 70% ethanol for 3 minutes, then in 40% sodic hypochlorite solution supplemented with 0.02% Tween-20 for 30 minutes and finally washed 5 times in sterile water. Afterwards, seeds were germinated on a Petri dish containing a sterile filter that was previously soaked with sterile water for 7 days at 28 ± 0.5 ºC and 166.05 µmol m^{-2} s^{-1} fluorescent light under a 12h/12h light/darkness photoperiod. Each seedling was carefully placed on one pre-cut piece of foam (2*2*1 cm, width*length*height) that was inserted into a rectangle (17*25*2 cm, width*length*height) of extruded polystyrene (XPS) with 24 pre-made holes (2 cm diameter), which served as a floating platform. Two squares of XPSs were positioned on containers filled with 10L of modified Yoshida solution (Table SM.1.1) [49]. A total of 6 containers with 40 seedlings were positioned in a greenhouse (25 ± 3 ºC and 50 ± 10% RH) for 1 week for plantlet acclimatization. Then the hydroponic solution was supplemented with 100 mM NaCl (salinity treatment) in three containers and the other non-supplemented containers were used as a mock treatment (0 mM NaCl). Salinity in each container was measured with a DiST 4 Waterproof EC Tester (Hanna Instruments, Rhode Islands, USA) with initial conductances of 1.13 ± 0.05 and 9.28 ± 0.10 mS cm^{-1} being registered in the mock and salinity treatments, respectively. Samples were collected at three time points (6, 24 and 48 h) after the salinity treatment using a randomized block design for all six containers. At the time of sample collection, shoots and roots were separated and four plantlet pools were quickly rinsed with

MilliQ water to remove excess salt and rapidly frozen *in situ* in liquid nitrogen, and then stored at -80 ºC until required.

PROTEIN SAMPLE PREPARATION

A RuBisCO depletion protocol that employs protamine sulfate to selectively precipitate this protein was used, as removing this enzyme can improve the proteome coverage and could lead to the identification of novel unidentified low-abundant proteins [47,50,51]. The RuBisCO depletion step by protamine sulfate was only performed when extracting total shoot proteins because it has been shown that RuBisCO is not present in rice roots [52]. Protein concentrations were determined with the Bradford reagent (Bio-Rad Laboratory, USA). As suggested by Kim et al. (2013), extracted shoot and root proteins were stored in 80% acetone at -20ºC until further use.

PROTEIN DIGESTION

Samples stored in 80% acetone were centrifuged to efficiently remove acetone and compact the precipitate. This precipitate was resuspended in 700 µL 8 M urea supplemented with 50 mM ammonium bicarbonate (AB), and disaggregated with ultrasonic probe disagregation-solubilization (UP200S ultrasonic processor, Hielscher Ultrasonics, 20% Amplitude, 0.1 cycles, 45 minutes). Disaggregated samples were cleaned in a particle filter (Corning® Costar® Spin-X® centrifuge tube filters, cellulose acetate membrane, pore size 0.45 µm, non-sterile; 9000 g, 2 min, RTº), and the resulting filtrate was designated as the soluble protein fraction for analysis. Subsequently, the volume of the soluble protein fraction was reduced to 150-200 µL with an Amicon Ultra filter (3 KDa, 0.5 mL, Millipore) and then quantified with the Pierce™ 660 Protein assay (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

A total of 35 µg of each sample was made up to 400 µL with 50 mM AB/8 M urea (pH 8.0-8.5) for digestion using the FASP (Filter-Aided Sample Prep) approach. Samples were reduced with 5.3 mM Tris-(2-Carboxyethyl) phosphine (TCEP) (90 min, 30 ºC) and alkylated with 27.3 mM iodoacetamide (RTº, 30 min in the dark, 30 ºC). To remove interfering agents, samples were then loaded onto an Amicon Ultra filter (10 KDa, 0.5 mL, Millipore) and washed with 2 rounds of centrifugations with 8 M urea supplemented with 50 mM AB (13600 g, 25 min, RTº, 400 µL), and a final wash with 50 mM AB (13600 g, 25 min, RTº, 400 µL). Protein samples were then digested on the filter in 400 µL of 1M urea supplemented with 50 mM BA plus 2.8 µg of trypsin/sample (Sequence grade modified Trypsin, Promega) for 3 h (32 ºC, pH 8.0), and re-digested again for 16 h with 1.4 µg of trypsin/sample (32 ºC, pH 8.0). The resulting peptide mixtures were recovered by 3 rounds of centrifugation/washing of the filter with (2x) 50 mM AB (300 µL) and (1x) 20% acetonitrile (ACN)/50 mM AB (200 µL) (13600 *g*, 25 min). The volume of the peptide solutions was reduced to 300 µL on a SpeedVac (Thermo Fisher Scientific) and then acidified with formic acid (FA) (1% final concentration). Acidified peptide solutions were desalted in a C18 tip (P200 Toptip; PolyLC) - as per the manufacturer's instructions - then dried in a SpeedVac and kept at -20 ºC until subsequent steps. Moreover, a pool of the analyzed samples (PAS) for shoots and roots was prepared in the same way as the samples used for LC-MS normalization purposes to allow the multiple comparison throughout the multi-iTRAQ (Isobaric Tags for Relative and Absolute Quantification) experiment.

iTRAQ LABELING OF PROTEIN SAMPLES

For shoot and root protein samples, a total of 3 iTRAQ experiments were performed, each one containing six treatments (two concentrations: 0 and 100 mM NaCl; three time points: 6,

24 and 48 h) and the PAS. Therefore, each iTRAQ experiment consisted of a biological replicate.

Digested and washed samples were resuspended in 30 µL of 500 mM TEAB (tetraethylammonium bromide) to perform iTRAQ labeling (iTRAQ™ 8plex Multiplex kit) according to the product specifications. Briefly, 70 µL of isopropanol were added to each vial of iTRAQ labeling reagent, the vials were vortexed for 60 seconds and spun. The contents of the label vials were transferred to each sample tube, and sample-iTRAQ mixtures were mixed and incubated at room temperature for 2 h to allow the iTRAQ labeling reaction to run. An aliquot of each reaction was cleaned up with a homemade C18 tip and analyzed with LC-MS/MS to ensure complete labeling before combining the seven samples in each batch. To each reaction mixture, a volume of 100 µL of water was added in order to quench the iTRAQ reaction and labeled samples were combined and dried down in a SpeedVac (Thermo Fisher Scientific).

Before LCMS/MS analysis, the combined iTRAQ-labeled samples were washed in two steps and then fractionated into 11 fractions, including flow through and wash, with a high pH reversed phase spin column (Pierce™, Thermo Fisher Scientific, Waltham, USA). In the first clean-up step, the sample was resuspended in 100 µL 1% formic acid (FA) solution, desalted in a C18 tip (P200 Toptip, PolyLC), following the manufacturer's instructions, and dried in a SpeedVac. In the second clean-up step, dried peptides were resuspended in 100 µL 20% acetonitrile (ACN) supplemented with 0.1% FA (pH 2.7-3.0), cleaned in a strong cationic exchange tip (P200 toptip, PolySULFOETHYL A; PolyLC), according to the instructions, and dried in a SpeedVac. The sample was then subjected to high pH fractionation with a high pH reversed-phase peptide fractionation kit (Pierce™, Thermo Fisher Scientific, Waltham, USA) following the manufacturer’s instructions. Briefly, the samples were loaded onto a spin

column in 0.1% trifluoroacetic acid (TFA), washed and buffer exchanged with high pH buffer and then eluted in 9 fractions of increasing acetonitrile (ACN) concentration (f1 = 10% ACN; f2 = 12.5% ACN; f3 = 15% ACN; f4 = 17.5% ACN; f5= 20 % ACN; f6 = 22.5% ACN; f7= 25% ACN; f8 = 50% ACN; f9 = 75% ACN). The flow through and wash fractions were pooled together, as well as fractions one and eight and two and nine, which along with fractions three to seven resulted in eight fractions that were dried down in a speed-vacuum centrifuge.

LC-MS/MS ANALYSIS

The 8 dried-down fractions of each iTRAQ experiment were analyzed in a nanoAcquity liquid chromatograph (nanoAcquity, Waters, Milford, Massachusetts, USA) coupled to an LTQ-Orbitrap Velos (Thermo Fisher Scientific, Waltham, USA) mass spectrometer. The tryptic labeled peptides of each fraction were resuspended in 2% ACN supplemented with 1% FA solution and an aliquot of 10 µL was injected into the chromatograph for separation. Peptides were trapped on a Symmetry C18TM trap column (5 µm 180 µm x 20 mm; Waters) and separated using a C18 reverse phase capillary column (Acquity UPLC M-Class; 75 µm Øi, 25 cm, 1.7 µm BEH column; Waters). The gradient used for peptide elution was 2% to 35% B for 155 minutes, followed by a 35% to 45% gradient in 20 minutes (A: 0.1% FA; B: 100% ACN, 0.1% FA), with a 250 nL min^{-1} flow rate. Eluted peptides were subjected to electrospray ionization in an emitter needle (PicoTipTM, New Objective, Scientific Instrument Services Inc., New Jersey, USA) with an applied voltage of 2000 V. Peptide masses (m/z 300-1800) were analyzed in the data-dependent mode where a full-scan MS in the Orbitrap was performed with a resolution of 30,000 FWHM at 400 m/z. Up to the 15th most abundant peptide (minimum intensity of 2000 counts) was selected from each MS scan. They were fragmented by HCD (Higher Energy Collision Dissociation) in the C-trap using nitrogen as the collision gas, with 40% normalized collision energy and analyzed in the Orbitrap with a

resolution of 7,500 FWHM at 400 m/z. The scan time settings were: Full MS: 250 ms (1 microscan) and MSn: 300 ms (2 microscans). The generated *.raw* data files were collected with Thermo Xcalibur V2.2 software (Thermo Fisher Scientific, Waltham, USA).

PROTEIN IDENTIFICATION

A database was created by merging all entries for *Oryza sativa* ssp. *indica* present in the public database, UniProt (http://www.uniprot.org), with a database containing common laboratory contaminant proteins (Uniprot_Osativa_subs_indica_170405_cont.fasta). The Thermo Proteome Discover software V1.4.1.14 (Pierce™, Thermo Fisher Scientific, Waltham, USA) was used to perform the database search using SequestHT as a search engine. For each iTRAQ experiment, 8 *.raw* files from the MS analyses, corresponding to the 8 fraction injections, were used to perform a single search against this database (enzyme specificity: trypsin; maximum miscleavage sites: 2; fixed modifications: carbamidomethyl of cysteine, iTRAQ8plex (N-term) carbamidomethyl; variable modifications: oxidation of methionine, iTRAQ8plex, iTRAQ8plex; peptide tolerance: 10 ppm and 0.1 Da [respectively for MS and MS/MS spectra]). A database searching against both a target and a decoy database was made to obtain a false discovery rate (FDR), and thus estimate the number of incorrect peptide-spectrum matches that exceed a given threshold. Moreover, a manual search in databases (NCBI, UniProt and EBI) was performed to determine the identity of proteins with putatively unknown identity (e.g. uncharacterized proteins) but having a treatment/no-treatment ratio above 2.0 and below 0.5 in at least one of the time points (6, 24 and 48 h), which represents either a two-fold abundance or half the abundance decrease in plantlets subjected to 100 mM NaCl, respectively. Finally, only those putatively unknown proteins with known regions or domains were added to the identified proteins. Additionally, to improve the sensitivity of the database search, the Percolator algorithm (semi-supervised

learning machine) was used in order to aid the discrimination of correct and incorrect peptide spectrum matches (Target FDR (Strict): 0.01; Validation based on: q < 0.01). Percolator assigns a q-value to each spectrum, which is defined as the minimal FDR at which the identification is deemed correct. These q-values are estimated using the distribution of scores from the decoy database search. A quantification method for iTRAQ™ 8-plex mass tags optimized for Thermo Scientific Instruments was applied to obtain the reporter ion intensities.

QUANTITATIVE ANALYSIS AND FUNCTIONAL ANNOTATION

Reporter intensities from the Proteome Discoverer quantitation file were used to perform iTRAQ quantitation. Within each iTRAQ™ 8plex experiment, reporter ion intensities of each individual peptide from each fraction/LC-MS run were summed. Only unique peptides appearing in all samples of all iTRAQ experiments for either shoots or roots were considered for the analysis. To normalize the report ion intensities of each label between iTRAQ experiments a LOWESS correction (Locally Weighted Scatterplot Smoothing) was applied using the PAS as an internal standard within each iTRAQ experiment. According to Callister et al. (2006), the LOWESS span value was fixed to 0.4 [53]. Then the LOWESS normalized reporter ion intensities were divided to their PAS peptide intensities to allow comparison throughout the multi-iTRAQ experiment, for shoots and roots respectively. Normalized report intensities belonging to a given protein were then averaged to obtain the protein abundance. Subsequently, proteins were manually categorized into functional categories according to the GO (Gene Ontology Project, http://www.geneontology.org) and KO (KEGG Orthology, http://www.genome.jp/kegg/ko.html) databases [54,55].

PROTEOMIC DATA VISUALIZATION

Shotgun proteomics generates large sets of data that are not easily interpreted [56]. Accordingly, in this study several data visualization graphs were used: treemaps, volcano plots, protein-protein interaction displays (Cytoscape coupled with String-DB) and heatmaps. Treemaps is a method for displaying hierarchical data using nested figures that also display quantities via area size [57–59]. We used this approach to illustrate the functional categorization of identified proteins for shoots and roots. Volcano plots are graphs generated by plotting the fold change (using a $\log_2$ transformation) versus the p-value ($-\log_{10}$ transformed) of the selected quantified proteins. Volcano plots were used for depicting the relative abundance of all the proteins detected in the iTRAQ experiments. In addition, for each plot a line crossing the y-axis at 1.301 ($-\log_{10}$ value of the $p = 0.05$, used in the ANOVA analysis) was included [56]. Proteins with a $p < 0.05$ and a $q < 0.15$ for the 100mM/0mM (salinity/mock treatment) ratio were displayed through protein-protein interactions, which helps to elucidate the involvement/action of non-identified proteins. For this, we used Cytoscape V3.6.0 software coupled with stringAPP 1.3.0 (available at the Cytoscape App Store; http://apps.cytoscape.org/apps/stringapp) [56,60]. We imported the network using UniProt accessions, a confidence (score) cutoff of 0.4, and no additional interactors. Moreover, for each protein we displayed its abbreviation and the relative abundance of the 100mM/0mM ratio averaging the three time points in a $\log_2$ scale (downregulated (red) to upregulated (blue) in the salinity treatment). Finally, to visualize quantitative patterns across proteins and the different treatments we used heatmaps, which organize data sets as matrices without having to summarize the data [56,61].

PHYSIOLOGICAL CHARACTERIZATION

All the characterization was assessed at 6, 24 and 48 h after salinity treatment. Relative chlorophyll content (SPAD units) in rice seedlings was assessed with a SPAD-502 (SPAD MCL502, Minolta, Japan). Growth was determined using whole plantlet length (pL) and whole plantlet fresh weight (pFW), as well as shoot fresh weight (sFW), root fresh weight (rFW), shoot length (sL) and root length (rL). Fresh weight (pFW, sFW and rFW) was recorded before MilliQ rinsing and in groups of four plantlets. Prior to freezing samples, a photograph was taken for each group, which was then used to measure the length (pL, sL and rL) with ImageJ V1.50i (National Institute of Health, Bethesda, Maryland). Water content (reported as Water Content (WC, %) = fresh sample - dried sample) was determined for shoots (sWC) and roots (rWC), which enabled comparison of the osmotic effect of the salinity treatment between samples. For this, samples were dried for 72 h in an oven at 70 ºC and measuring the weight every 24 h to ensure complete water evaporation. Finally, dried samples were used to quantify Na^+ and K^+ using an acid digestion in shoots (sNa^+, sK^+) and roots (rNa^+, rK^+). This procedure consisted of a complete digestion with 2 mL of 67-69% HNO_3 (J.T.Baker - Fisher Scientific, Pittsburgh, USA) and 1 mL of 30% H_2O_2 (Merck Millipore, Darmstadt, Germany) for 12h at 90 ºC. Afterwards, samples were reconstituted with 25 mL of H_2O and quantified with an Optima 8300ICP-OES spectrometer (PerkinElmer, Wellesley, USA) at a wavelength (λ) of 589.592 nm for Na^+ and 766.490 nm for K^+.

STATISTICAL ANALYSIS

For shotgun proteomic analysis, we used the same procedure for shoot and root samples. Proteins identified with at least 2 unique peptides were considered for further statistical analysis. From the resulting proteins, variations in abundance were tested by a two-way ANOVA (p-value) and corrected for multiple comparisons (q-value) using the false discovery

rate (FDR) method [62]. Tukey's Honest Significant Difference (HSD) test was applied to determine where the ANOVA significant difference was within the multiple comparisons across tested factors (salinity and time). R packages were used for data analysis (R Development Core Team, 2011).

Prior to statistical analysis, physiological data were tested for normality and homoscedasticity using the Shapiro–Wilk and Levene's robust tests, respectively. All parameters with the exception of sNa^+, rNa^+ and SPAD units were suitable for performing ANOVA. For this, a two-way ANOVA (salinity, time and salinity*time) was performed, which was followed by a post-hoc Tukey's multiple comparison test. For non-homoscedastic data, a Kruskal-Wallis test followed by a Conover-Iman multiple non-parametric pairwise test was applied. For sWC, rWC, sNa^+, sK^+, rNa^+ and sK^+ a total of 3 biological replicates were used for the statistical test. A total of 6 and 36 biological replicates were used for tFW, sFW, rFW and for ChlQ, pL, sL and rL respectively for the corresponding tests. For all tests, differences were considered to be significant at a probability of 5% ($p < 0.05$). All statistic results for the physiological parameters can be observed in Table SM.1.2..

RESULTS

IDENTIFICATION AND FUNCTIONAL CATEGORIZATION OF THE PROTEINS OF THE FL478 EARLY PROTEOME UNDER SALINITY STRESS

SDS-PAGE was performed in order to test the protamine sulfate RuBisCO depletion protocol. The results of this were satisfactory because the RuBisCO large subunit band was absent in the depleted samples, and the rest of the bands were more intense (Figure SM.1.1). In fact, only two proteins in shoots, s148 and s315, of the total 990 proteins tested were identified with the RuBisCO large subunit and RuBisCO small subunit. After the depletion step, a total of 990 and 1122 proteins were identified in shoots and roots, respectively, by HPCL-MS/MS, of which 99.20% and 99.55% matched entry proteins in *Oryza sativa* L. ssp. *indica*.

Regarding the shoots, 37.17% of the matched proteins corresponded to annotated proteins, while the rest were uncharacterized (full annotated protein list available in Table SM.1.3). Similarly, in roots, annotated proteins comprised 34.31% of the total matched proteins (full annotated protein list available in Table SM.1.4). During protein identification, subcellular localization was also considered and the majority of proteins were assigned to the cytosol (85.33% and 91.95% for shoots and roots, respectively). In addition, regarding the genes present in the *Saltol* QTL region, SalT protein was found only in roots whereas peroxidase (*PDX*) proteins were found in both shoots and roots. In contrast, the *OsHKT1;5* transporter (previously named *OsHKT8* and SKC1), was not detected in either of the organs.

Finally, a functional categorization was undertaken that considered the 368 and 385 annotated proteins for shoots and roots, respectively (Figure 3.1.1, Table SM.1.3 and Table SM.1.4). A total of 5 functional categories were defined: (i) energy and biomolecule metabolism, (ii) genetic and environmental information processing, (iii) antioxidant and defense functions, (iv) cytoskeleton-related, and (v) unassigned function. Moreover, each

category was further divided into different subcategories as can be observed in Figure 3.1.1. For shoots and roots, the majority of the proteins were allocated to energy and biomolecule metabolism with an average of 57.56% of the total annotated proteins. This category was followed by proteins involved in genetic and environmental information processing and antioxidant and defense functions for both parts of the plant. Nevertheless, it was observed that for roots more proteins were allocated to the antioxidant and defense functions compared to the shoots. In addition, it is important to highlight that in comparison to shoots, more root proteins were found and assigned to the category of unassigned functions; probably because as observed in the literature, fewer studies have been performed on this tissue.

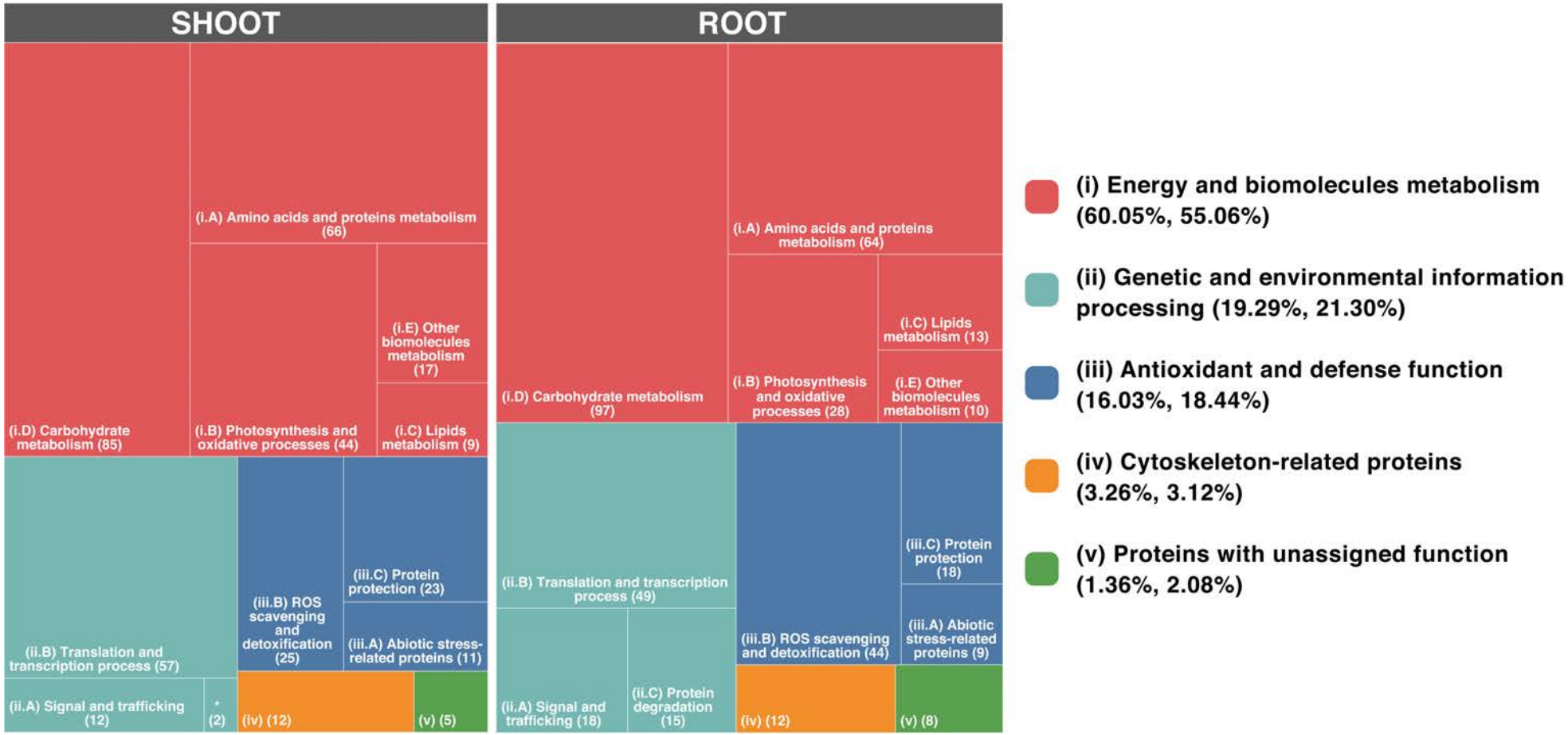


Figure 3.1.1. Treemap showing the functional categorization and subcategorization of proteins for shoots (left) and roots (right). The five functional categories are shown in the legend, where percentages correspond to proteins allocated to each category with respect to total proteins for shoots and roots respectively. Square sizes are directly proportional to the number of proteins for each subcategory, which are shown between parentheses. *: (iii.C) protein degradation in shoots.

When looking at shoot subcategories within (i) energy and biomolecule metabolism, the majority of proteins were involved in (i.D) carbohydrate metabolism (mainly glycolysis and the TCA cycle) and (i.A) amino acid and protein metabolism, representing respectively 38.46% and 29.86% of the proteins in this category (Figure 3.1.1). Within category (i) the next largest groupings included proteins involved in photosynthesis and oxidative respiration, with 19.91% and the remaining 11.76% including proteins involved in the metabolism of (i.C) lipids and (i.E) other biomolecules (Figure 3.1.1). Regarding genetic and environmental information processing proteins, the majority were centred on (ii.B) translation and transcription processes, representing more than 80.28% of the total within this category. Finally, within the antioxidant and defense functions category, 42.37% and 38.98% were assigned to (iii.B) ROS scavenging and detoxification and (iii.C) protein protection, respectively

For root proteins, a similar scenario was observed where the subcategories of the main category included carbohydrate metabolism followed by amino acid and protein metabolism, with 97 and 64 proteins, respectively. Similar to shoots, a high number of proteins were allocated to translation and transcription processes in roots. In addition, the number of root proteins allocated to (ii.C) protein degradation was 7 times that in shoots. As expected, roots had fewer proteins allocated to subcategory (iii.B), photosynthesis and oxidative processes, than the shoots. Finally, the number of proteins involved in (iii.B) ROS scavenging and detoxification was higher in roots than shoots (almost twice the number) which indicated that this tissue was better able to tolerate salinity stress.

GLOBAL ANALYSIS OF THE EARLY SALINITY STRESS PROTEOME OF FL478

For this analysis, the 982 and 1117 shoot and root proteins matched to ssp. *indica* were used and their fold change as a salinity/mock ratio (100mM/0mM NaCl) was calculated for each of the evaluated time points (6, 24 and 48 h) and plotted as volcano plots (Figure 3.1.2). This

established that a fold change below 0.9 (downregulation) and above 1.1 (upregulation) corresponded to proteins with differential abundances, with shoots and roots, having 43 and 36 proteins, respectively, with significant differential abundances ($p < 0.05$). More in detail, and in terms of the average of the differentially abundant proteins, in shoots 49.57% and 50.43% were up- and downregulated, respectively, whereas in roots the percentages were 57.48% and 42.52%.

Looking at the volcano plots, it is clear that a faster response occurred in roots, which are in direct contact with the high salinity, than in shoots, due to the substantially larger number of proteins that showed a significant increase after only 6 h of exposure (Figure 3.1.2). Only seven proteins had significant differential abundances in shoots at both 6 and 24 h and more than 50% of the proteins were allocated to the region indicated no differential abundance (gray region: fold change between 0.9-1.1, Figure 3.1.2). This situation was reversed at 48 h, when a very large number of differentially abundant proteins had accumulated in shoots in comparison to roots. In fact, the number of significantly differential proteins increased to 131 and only 28% of the proteins were allocated to the gray region. Moreover, the fold-change values at 48 h in shoots reached lower than -4 and higher than 3, whereas in roots the fold changes remained -1 and 1, as had occurred at 6 and 24 h. In accordance, root protein percentages in each fold-change region were similar between the three time points. Moreover, the number of proteins with significant differential abundance ($p > 0.05$, black horizontal line in the volcano plots) was also similar at the three time points evaluated in roots.

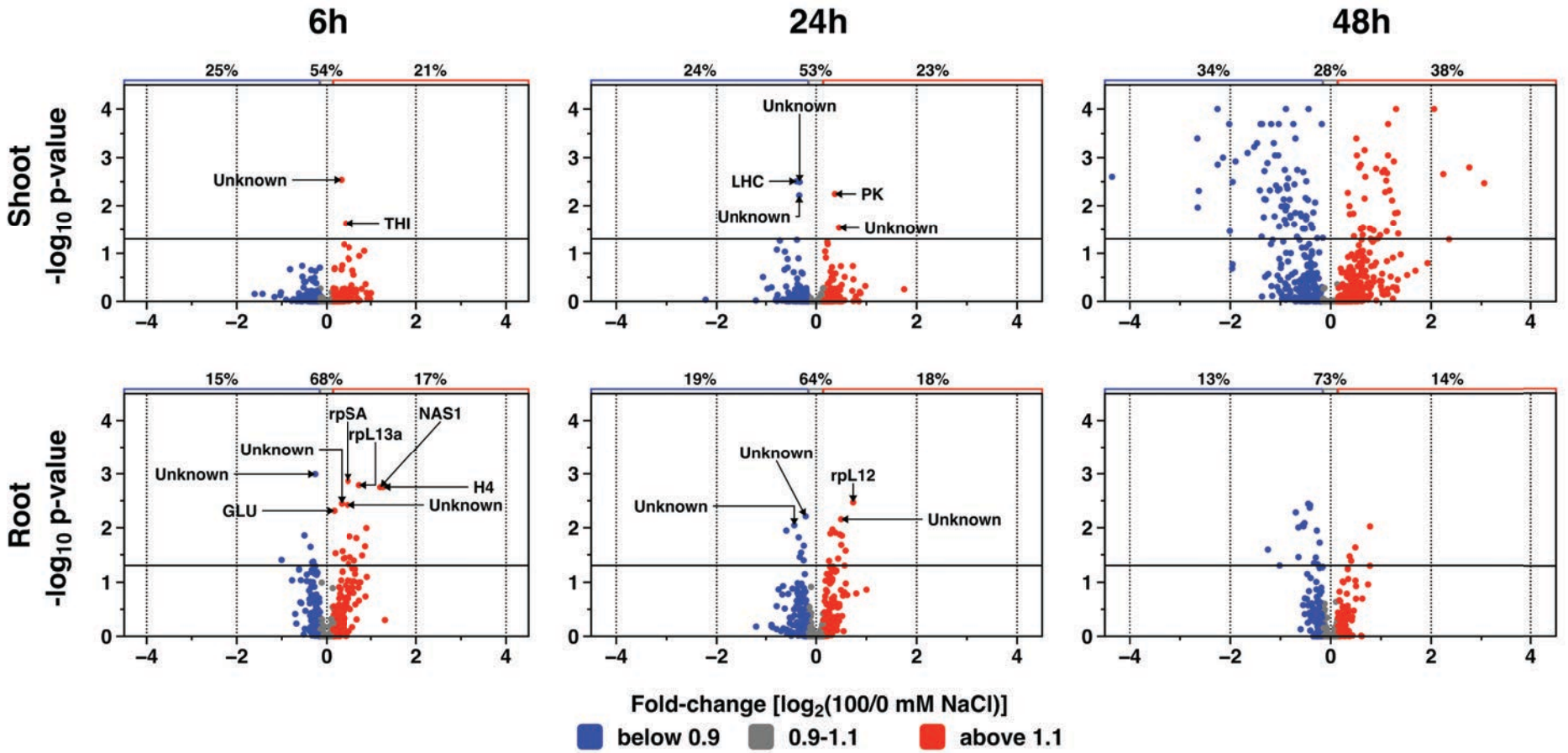


Figure 3.1.2. Volcano plot depicting the differential expression patterns of proteins in shoots and roots of FL478 plantlets at different time durations of salinity stress. Brackets at the top of each plot indicate the percentage of proteins in each fold-change region. A total of 982 and 1116 proteins are shown in all the time points for shoots and roots, respectively. Values of fold-change are reported as the $\log_2$ of the salinity/mock treatment (100mM/0mM NaCl) ratio for the three times evaluated (6, 24 and 48 h) where zero corresponds to a salinity/mock ratio of one. The horizontal black line crossing the Y-axis corresponds to 1.301 ($-\log_{10}$ value of the p = 0.05), and values above that line are significantly different.

IDENTIFICATION OF NOVEL CANDIDATE PROTEIN BIOMARKERS IN SALINITY TOLERANCE

Within the proteins identified with *O. sativa*, 85 shoot and 64 root proteins had significant differential abundances ($p < 0.05$) and a low false discovery rate ($q < 0.15$), considering the average of the fold changes (100mM/0mM NaCl; Table SM.1.5). In this subset, some proteins were unknown (not annotated), and this did not permit substantial analysis. To analyze them, we used Cytoscape software coupled with string-DB (for detail of the data visualization methods please see the Materials and Methods) because this enables establishment of networks of protein-protein interactions based on their genomic and proteomic data available in public databases. Therefore, network proteins can help elucidate the role of some of the unknown proteins (UnkS and unkR) and thus identify them as novel candidates involved in the salinity responses of FL478. The networks, which indicate the fold-change between salinity and mock treatments, can be seen in Figure 3.1.3 and Figure 3.1.4 for shoots and roots, respectively. As in the volcano plots, this analysis also demonstrates that the abundance changes in roots were more controlled than in the shoots because the fold-change represented in $\log_2$ form has a lower span compared to shoots.

The shoot network is interconnected with several clusters of proteins showing substantial abundance changes (spanning from a -2.7 to a 1.27-fold change), with the majority of proteins being downregulated under salinity (Figure 3.1.3). PLAT_plant_stress and a protein of the plant dehydrin family (DHN), both involved in tolerance to stress, had high abundances in the salinity treatment, and especially in the case of DHN. Moreover, two ribosomal proteins (rpuS5 and rpS30) were highly upregulated during salinity stress, as was UnkS-37, which may be involved in amino acid biosynthesis due to its close links with betaine aldehyde dehydrogenase 2 (BADH2), lysine-tRNA ligase (LysRS) and glutamine amidotransferase (GATase). In addition, two unknown proteins, UnkS-13 and UnkS-14, were markedly

upregulated in the salinity treatment. In contrast, three proteins related to photosynthesis (psbQ, psb27 and LCH) were downregulated, as expected, but another protein involved in this process was upregulated (PsbP). Two unknown proteins were significantly less accumulated under salinity (UnkS-2 and UnkS-35) but there were no other interconnected proteins.

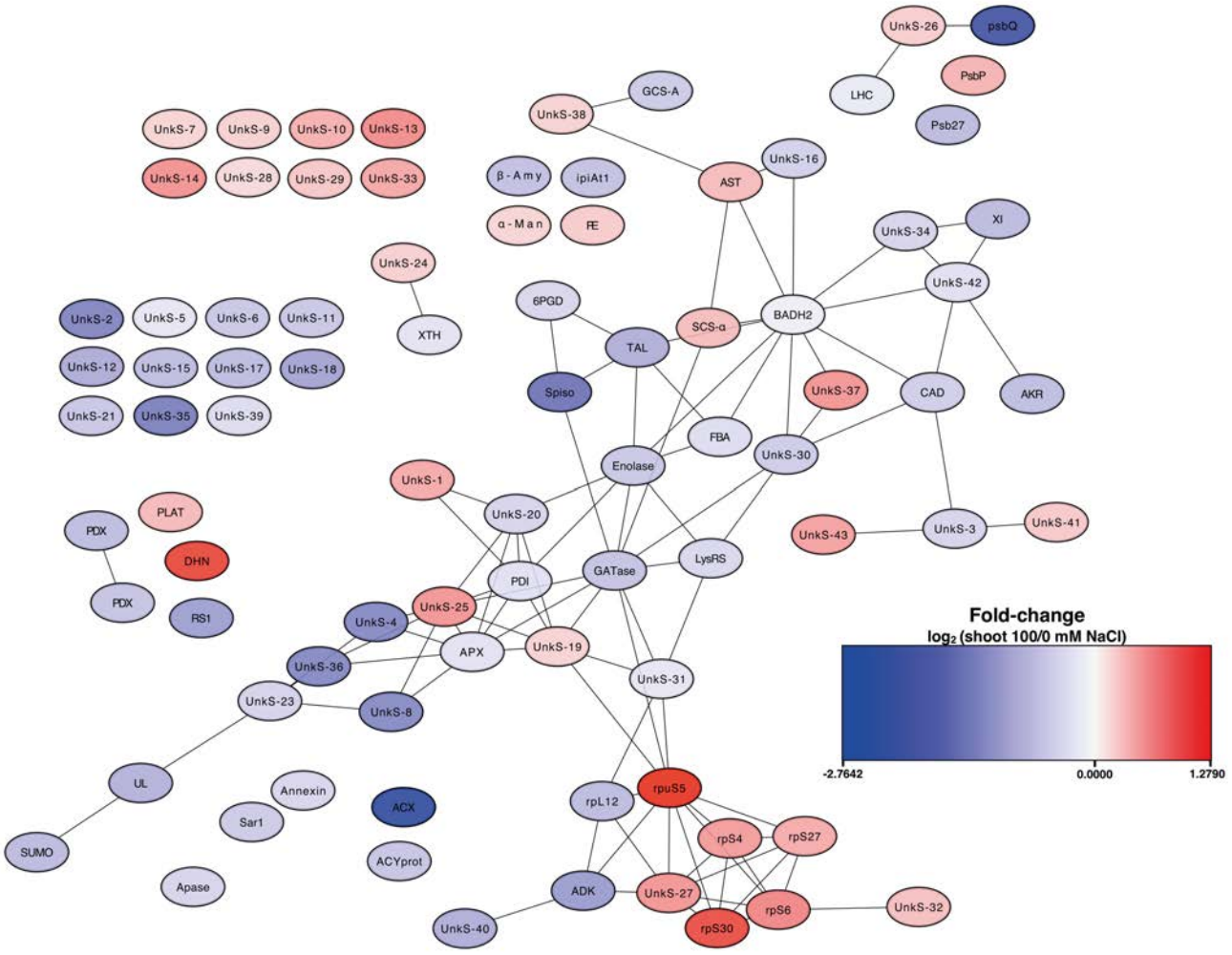


Figure 3.1.3. Shoot protein-protein interactions displayed using Cytoscape and Cytoscape's stringApp. The proteins shown have a $p < 0.05$ and a $q < 0.15$ for the salinity/mock treatment (100mM/0mM NaCl) ratio. The color of each protein corresponds to the 100mM/0mM ratio in a $\log_2$ scale, which is shown in the colored box of each network. As defined by the software, the distance of edges (relationships) between nodes (proteins) indicates the proximity of the protein-protein interactions.

For the root network analysis, we observed one highly interconnected (i.e. coordination) cluster containing several ribosomal proteins upregulated in the salinity treatment, as well as several unknown proteins that should also be involved in protein translation due to protein-protein interactions (Figure 3.1.4). Notably, UnkR-17 was highly accumulated under salinity but its function is completely unknown because it is not related to other proteins, so it should be investigated further as a potential candidate for salinity tolerance. UnkR-19 and UnkR-26, which were upregulated in the salinity treatment, might be involved in transcription as they are linked to Histone 2A and 4 (H2A and H4). Contrarily to this, UnkR-9, UnkR-10 and UnkR-24 displayed a marked low salinity/mock treatment ratio. Peroxidases (PDXs), of which one gene is contained within the *Saltol* region, displayed lower abundances in the salinity treatment.

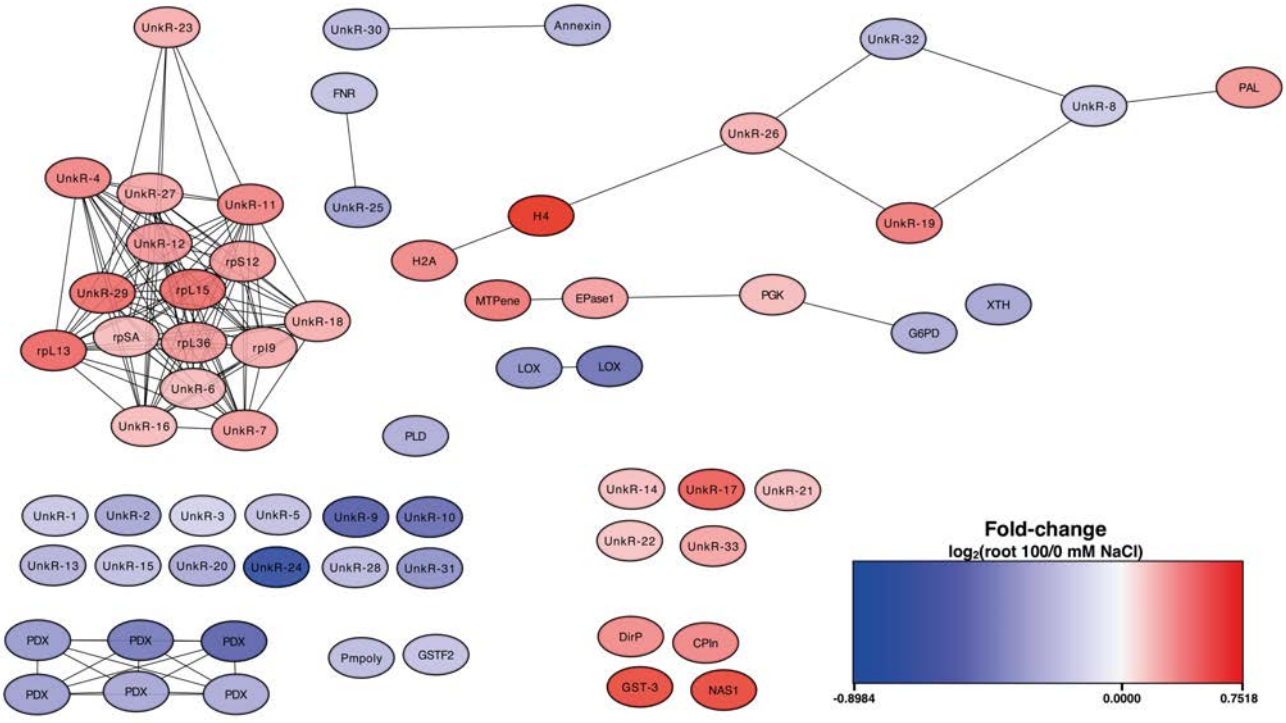


Figure 3.1.4. Root protein-protein interactions displayed using Cytoscape and Cytoscape's stringApp. The proteins shown have a $p < 0.05$ and a $q < 0.15$ for the salinity/mock treatment (100mM/0mM NaCl) ratio. The color of each protein corresponds to the 100mM/0mM ratio in a $\log_2$ scale, which is shown in the colored box of each network. As defined by the software, the distance of edges (relationships) between nodes (proteins) indicates the proximity of the protein-protein interactions.

ABUNDANCE PATTERNS OF THE EARLY PROTEIN STRESS RESPONSES OF FL478 UNDER SALINITY STRESS

Finally, all the proteins identified and listed in Table SM.1.3 and Table SM.1.4, were visually represented in heatmaps grouped by the 5 functional categories, without considering their sub-functional category, for shoots and roots (Figure SM.1.2 and Figure SM.1.3, respectively). As observed in Figure SM.1.2, for category (i), energy and biomolecule metabolism, the treatments (0 and 100 mM NaCl) at 48 h clustered separately, evidencing the drastic changes experienced by shoots during the salinity treatment. Moreover, for this category, 5 protein clusters were defined in which clusters 1 and 2 displayed lower abundances and cluster 4 higher abundances in the salinity treatment compared to mock at 48 h. Notably, several proteins involved in photosynthesis (psbQ, psb27, TSP9 and PSI-N among others) were grouped in clusters 1 and 2. In contrast, proteins involved in carbohydrate (e.g. GAPDH and PGK) and amino acid and protein (e.g. CysS and GCS-H) metabolism were within cluster 4. Similar patterns can be observed in category (ii), genetic and environmental information processing, where at 48 h the treatments also clustered separately. In addition, proteins involved in signaling (CaM-1 and annexin among others) displayed the higher abundance at 48 h during the mock treatment (cluster 1), whereas the majority of proteins associated with translation and ribosomes exhibited higher abundance in the salinity treatment (cluster 2). As expected for proteins involved in antioxidant and defense functions, category (iii), the mock and salinity treatments were clustered separately with increasing abundances as time progressed the three time points which incremented accordingly. Within this category, there were marked differences at 48 h between the mock and salinity treatments. Strikingly, in the case of cluster 3 where an increased abundance at 0 mM was observed for several enzymes involved in antioxidant stress and protein protection,

there was no upregulation of these enzymes at 48 h under 100 mM NaCl. On the other hand, there was a reverse of these dynamics for cluster 2 and 4 the dynamics with these proteins having increased abundance during the salinity treatment. Regarding the heatmaps of functional categories (iv) and (v), cytoskeleton-related and unassigned function, respectively, there were no clear patterns related to the stress experienced by the plants, but interestingly in both categories the treatments (0 and 100 mM NaCl) were grouped separately after the hierarchical clustering, as seen for category (ii).

Regarding the root heatmaps, 5 clusters were also defined within category (i), energy and biomolecule metabolism, which can be observed in Figure SM.1.3. Contrarily to the scenario observed for shoots, at 48 h both treatments were grouped together indicating that there were no large differences in this proteome subset in roots during the salinity treatment. In fact, the biggest differences were observed when comparing both treatments (0 and 100 mM NaCl) at 24 h in all clusters (Figure SM.1.3). Moreover, in cluster 5 a huge increase in protein abundance was observed for carbohydrate and amino acid and protein metabolism, but the abundances of the majority of these proteins were the most distant among the six conditions (white circles within the squares). As observed for category (i), in category (ii), genetic and environmental information processing, both the mock and salinity treatments at 48 h were grouped together showing similar abundances especially in cluster 2, which includes the majority of proteins associated with translation and ribosomes (Figure SM.1.3). In the case of shoots, annexin also displayed lower abundance when compared to the mock treatment at 48 h. In addition, there were marked differences between the 6 and 24 h time points in the salinity treatment for clusters 2 and 4, where proteins involved in the translation process decreased in abundance from 6 to 24 h. Unlike shoots, in the functional category (iii) the salinity and mock treatments did not cluster together in the roots. Despite this, it should be

highlighted that protein abundance patterns between treatments at 48 h for clusters 1 and 2, which included several antioxidant proteins, were very similar. In contrast, clusters 3 and 4, which contained a majority of proteins involved in protein protection (e.g. PPI and chp60), there was a higher abundance at 48 h under the 100 mM NaCl treatment. For the last two functional categories, (iv) and (v), the majority of proteins in all the samples assayed had an abundance close to 1:1 (black color) which denoted a low differential abundance when compared to other treatments, and moreover, the treatments were clustered together.

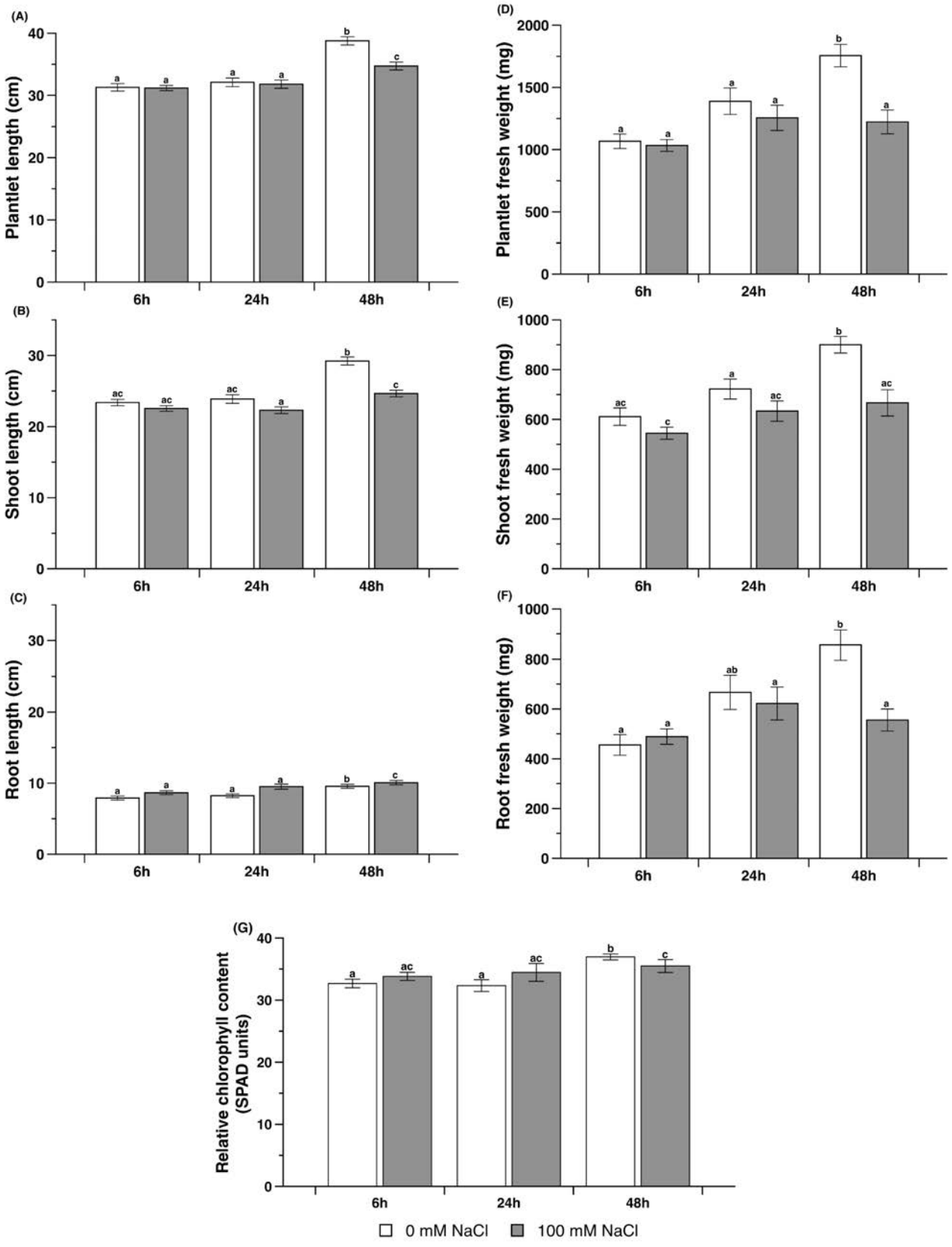
PHYSIOLOGICAL CHARACTERIZATION OF FL478 DURING SALINITY STRESS

Salinity treatment significantly reduced the overall growth, length (Figure 3.1.5A; Salinity: Table SM.1.2.) and fresh weight (Figure 3.1.5D; Table SM.1.2.) of FL478 plantlets. Shoot length (sL) increased during the experiment in the two treatments (0 and 100 mM NaCl), however, salinity treatment significantly reduced by 15.65% (Figure 3.1.5B, Table SM.1.2.). In contrast, root length (rL) increased in both treatments, being significantly higher at 48 h compared to 24 and 6 h. Moreover, rL in the salinity treatment was significantly higher than in the mock treatment (Figure 3.1.5C, Table SM.1.2.). Regarding shoot and root fresh weight (sFW and rFW, respectively), salinity treatment caused significant decreases in both parameters (Figure 3.1.5E and 3.1.5F, Table SM.1.2.). For sFW, significant differences had already appeared after only 6 h of treatment, whereas for rFW they started to appear after 24 h. Moreover, for both sFW and rFW, there were significant differences at 48 h between 0 and 100 mM NaCl (Figure 3.1.5E and 3.1.5F), corresponding to only 74% and 64% of their respective mock fresh weights. Relative chlorophyll content (SPAD units) seemed to increase initially in the salinity treatment compared to mock treatment, however, at 48 h the situation was reversed with relative chlorophyll content being significantly lower in plantlets grown at 100 mM NaCl (Figure 3.1.5G, Table SM.1.2.). In addition, relative chlorophyll content was

significantly higher by 48 h under 0 mM when compared to all the other treatments (Figure 3.1.5G).

Shoot water content (sWC) was maintained on average at 84.05 ± 0.51% for the mock treatment at each time point (Figure 3.1.6A). sWC was lower at all times in the salinity treatment, and the difference was significant at 24 and 48 h when compared to the mock treatment (Figure 3.1.6A, Table SM.1.2.). However, rWC did not vary significantly during the salinity treatment, being maintained at an average 92.28 ± 0.51 at all time points and in both treatments as observed in Figure 3.1.6B, and it was not influenced by salinity, time or their interaction (Table SM.1.2.). The Na^+/K^+ ratio in shoots and roots was significantly influenced by the salinity*time interaction, as their concentration changed significantly due to salinity and with time (Figure 3.1.6C, Table SM.1.2.). In the mock treatment, there were significant differences in the Na^+/K^+ ratio in the shoots, but remained at a low level with an average of 0.025 ± 0.006. In contrast, in the salinity treatment the Na^+/K^+ ratio was significantly higher at 48 h compared to all other conditions (Figure 3.1.6C). With the mock treatment in roots, no significant differences were observed, but in the salinity treatment there was a similar pattern to the shoots, where the Na^+/K^+ ratio increased during the salinity treatment (Figure 3.1.6D), but at a different magnitude. Moreover, for roots, at all-time points the Na^+/K^+ ratio was significantly different between the treatments (salinity and mock). Finally, it is noteworthy that the Na^+/K^+ ratio was higher at all-time points in roots when compared to shoots under the salinity treatment.

Figure 3.1.5 (page 129). Physiological characterization of the FL478 plantlets in response to different times and concentrations of salinity stress. Fourteen-day-old rice seedlings were subjected to salinity (100 mM NaCl) and mock (0 mM NaCl) treatment for 6, 24 and 48 h. Growth was monitored following two parameters: length and weight. (A) Plantlet (pL), (B) shoot (sL) and (C) root length (rL) values are the mean ± SE of 36 replicates. (D) Plantlet (pFW), (E) shoot (sFW) and (F) root (rFW) fresh weight values are the mean ± SE of six replicates. (G) Relative chlorophyll quantity (SPAD units) values are the mean ± SE of 36 replicates. Letters above pL, sL, rL, pFW, sFW and rFW and letters above SPAD units indicate significant differences at $p = 0.05$ using Tukey test and Conover-Iman test, respectively.


(A)
Plantlet length (cm)
(B)
Shoot length (cm)
(C)
Root length (cm)
(D)
Plantlet fresh weight (mg)
(E)
Shoot fresh weight (mg)
(F)
Root fresh weight (mg)
(G)
Relative chlorophyll content (SPAD units)
6h
24h
48h
0 mM NaCl
100 mM NaCl

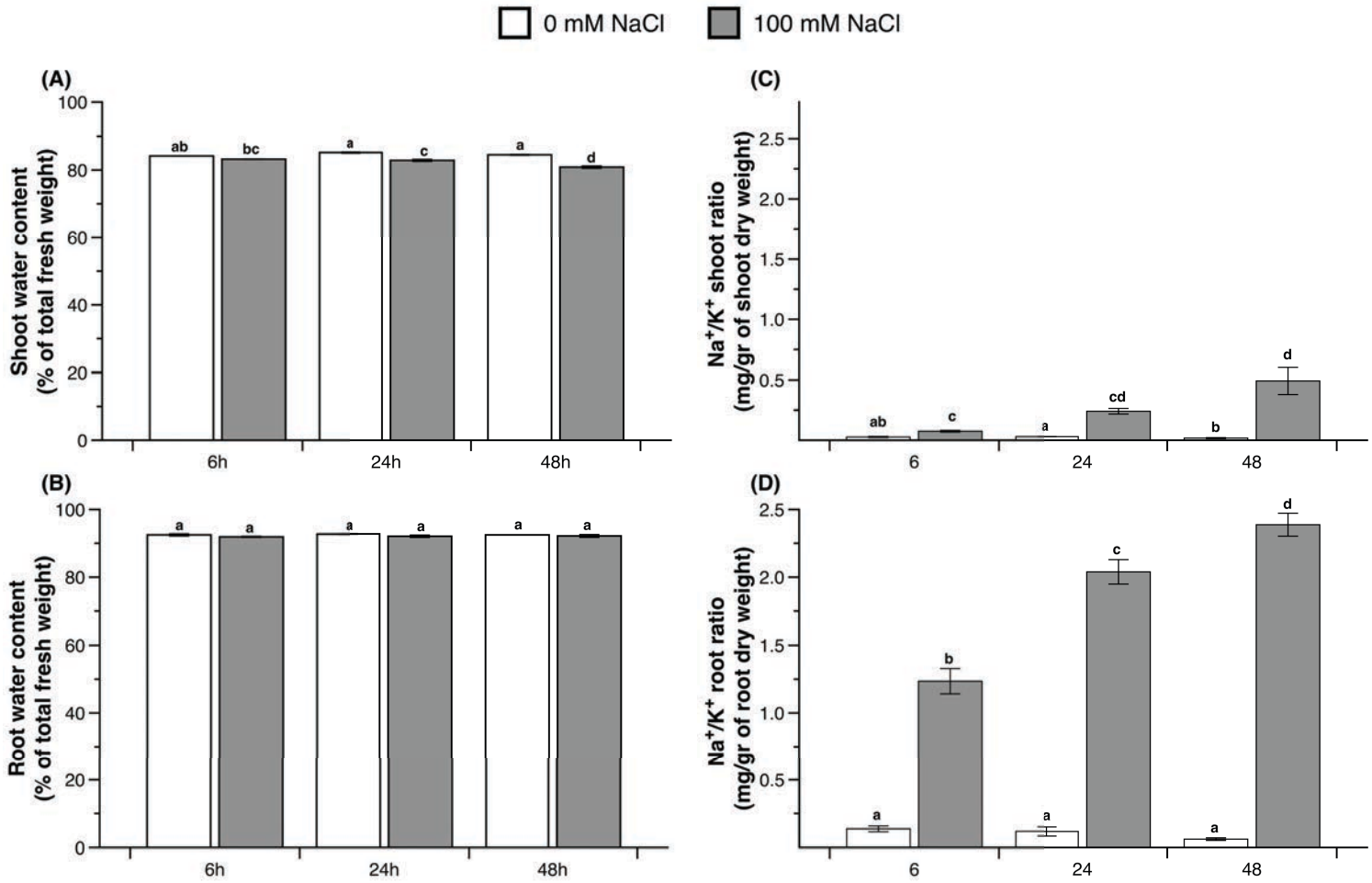


Figure 3.1.6. Shoot (A) and root (B) water content (SWC and RWC), and Na^+/K^+ ratio in shoots (C) and roots (D) (Na^+/K^+shoot and Na^+/K^+root). Values are the mean ± SE of three replicates. Letters above bars indicate significant differences (Tukey test for SWC, RWC and Na^+/K^+root); Conover-Iman test for Na^+/K^+shoot) at a p=0.05.

DISCUSSION

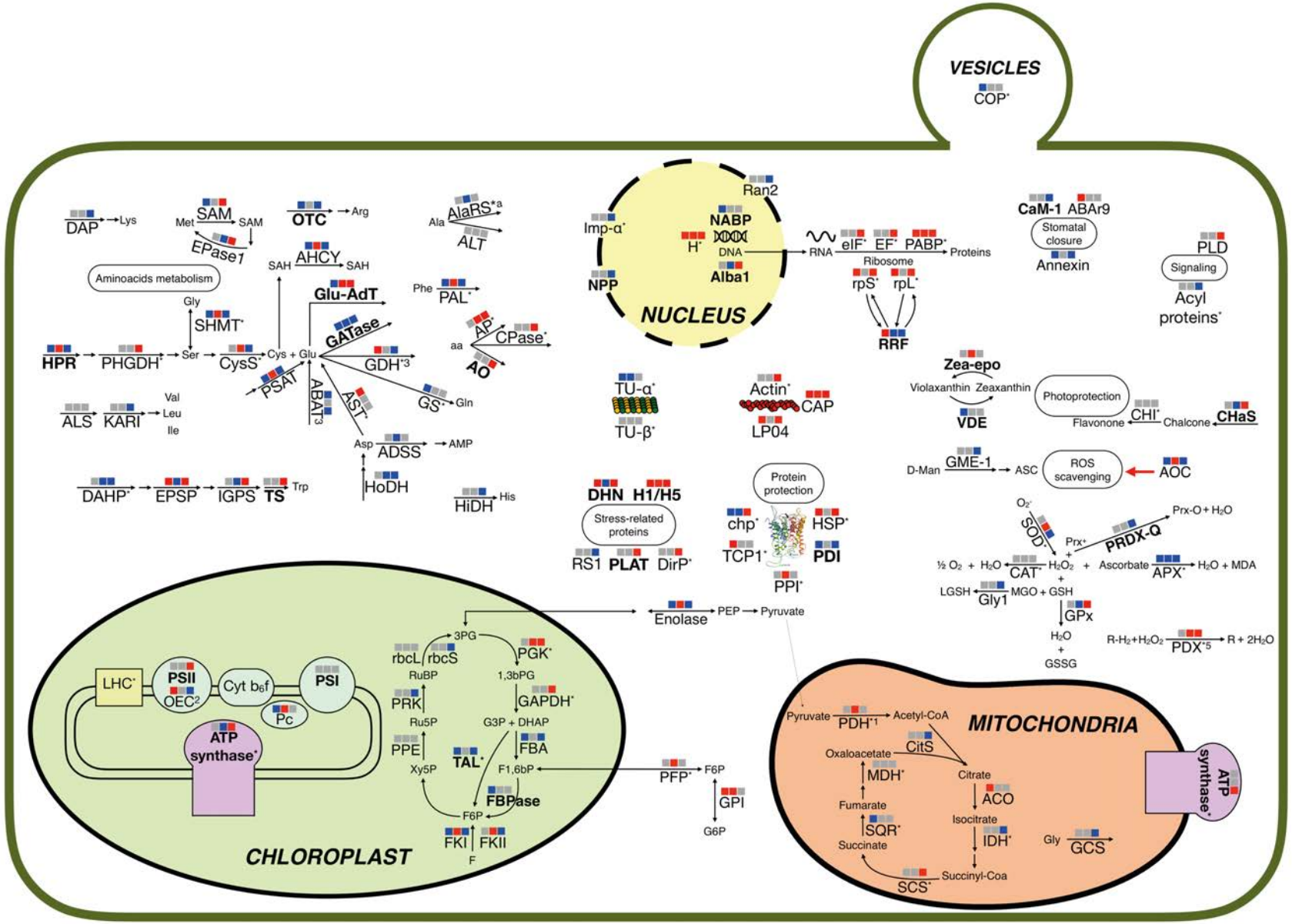
It has become well established that high salinity causes changes at the physiological, cellular, proteomic and transcriptomic levels in *Oryza sativa* plants [4,8,10,15,18]. Nevertheless, salt-tolerant rice varieties should be studied in detail to identify key proteins or mechanisms that facilitate this tolerance so that it may be incorporated into the breeding of commercial salt-tolerant varieties. In the present work we used the rice variety FL478, which carries the *Saltol* region on chromosome 1, a QTL known to bestow high salinity tolerance on rice [4,63–65]. Moreover, to our knowledge there is only one study of this rice variety's proteome under salinity stress where the authors examined the changes after 16 days of exposure [15]. Therefore, to expand our knowledge on the tolerance of *Saltol* varieties, we performed proteomic and physiological analysis during salinity stress at early stress stages in both shoots and roots (6, 24 and 48 h after salinity treatment). We found that changes at the proteomic and physiological level were more organized in roots, suggesting that this tissue is better able to tolerate the stress and therefore is crucially involved in salinity tolerance.

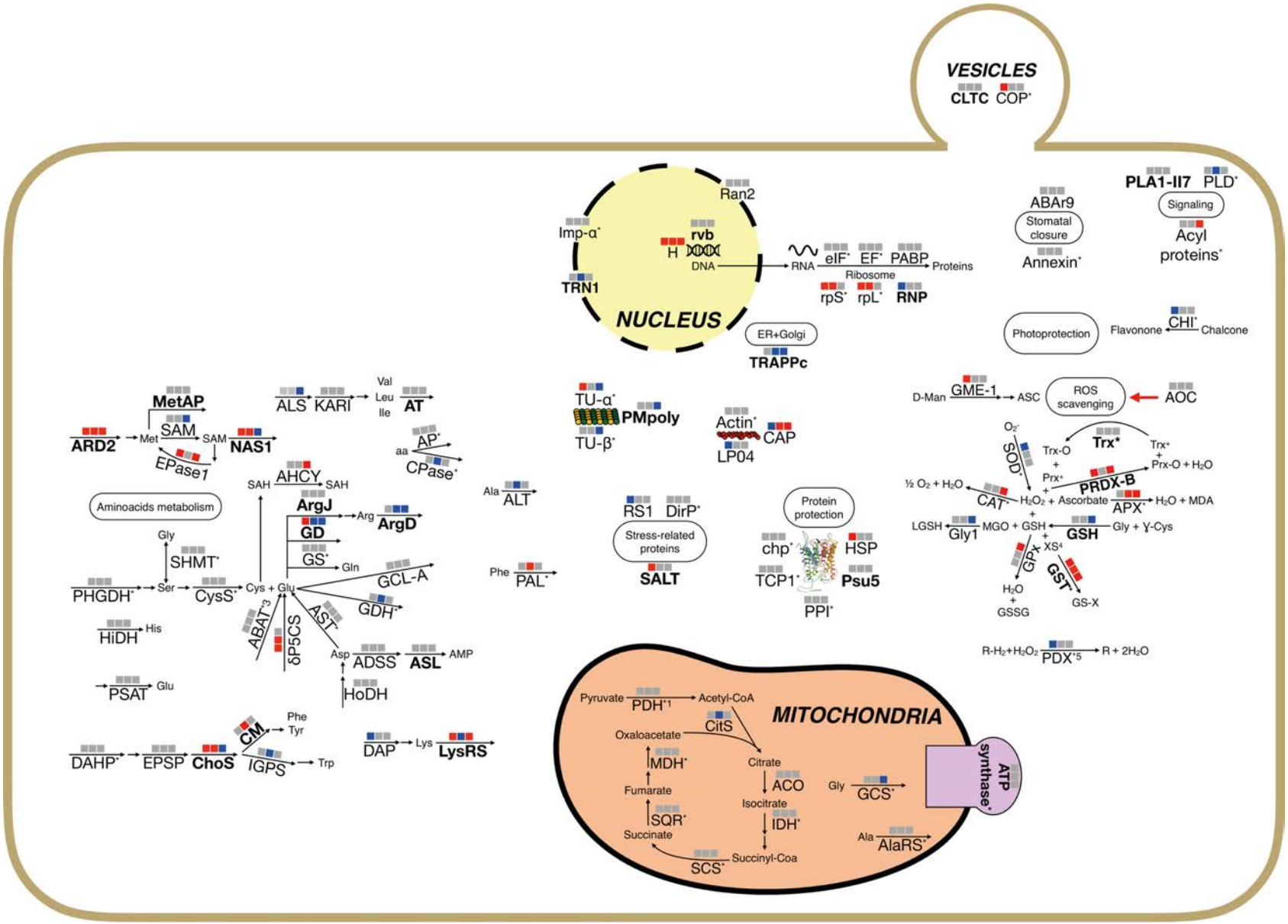
To characterize the shoot and root proteome we chose a shotgun proteomics approach, which allows large scale and high-throughput protein identification to be achieved via a bottom-up analysis, and this identifies proteins through analysis of the peptides released from the proteins by proteolysis [66,67]. Moreover, this technique overcomes the limitations of 2D-PAGE, which include low representations of proteins with high molecular weights, high isoelectric points (pIs), hydrophobic domains and those of low abundance [48]. Indeed, we detected a higher number of proteins than other studies that have analyzed the proteomes of rice varieties under salinity [4,15,68]. Compared to the work by Lakra et al. (2017) and Abdollah Hosseini et al. (2015), which used *Saltol* varieties and classic 2D-PAGE techniques, we found approximately 5 to 17 times more proteins in the shoot [4,15]. Therefore, this work

is a considerable expansion of knowledge of the shoot proteome in varieties harboring the salinity-tolerant *Saltol* QTL and it also serves as a major database for the root proteomes of these varieties.

All of the proteomics data that was generated is summarized in a schematic of key mechanisms for shoots and roots in Figure 3.1.7A (page 134) and Figure 3.1.7B (page 135), respectively. As a general overview, it can be observed that roots are more responsive to salinity than shoots because their response is faster and more organized. In fact, protein activation in roots starts at 6 h whereas in shoots it changes drastically at 48 h after treatment. These events are not unexpected as roots are the first organs that sense the salinity stress [69]. Nevertheless, our results contrast with those of Yan et al. (2005), who showed that upregulation of root proteins under salinity stress was maximized after 72 h of stress, which could be explained by the higher NaCl concentration that they used (150 mM). Nevertheless, in the present work the root protein abundances between the mock and salinity treatments were very similar, especially for the amino acid biosynthesis pathways, suggesting that their normal functions were not disrupted. In addition, the activation of proteins involved in stress tolerance was higher in roots than in shoots. Hence, we propose that the more efficient responses displayed by roots in the *Saltol* variety are explained by a better adaptation to the excess salt present in the growing medium [19,70].

Figure 3.1.7. Cell diagram of the proteins involved in salinity tolerance in shoots (A, page 134) and roots (B, page 135) of the salt-tolerant rice variety FL478 (abbreviations are according to Table SM.1.3 and Table SM.1.4). Bold abbreviations correspond to proteins exclusively in shoots or roots. The three squares above each protein correspond to the abundance at each time (6, 24 and 48 h), calculated as the fold change in the salinity/mock (100/0 mM NaCl) treatments. Colors in the square correspond to the fold-change: blue denotes down-regulated proteins (<0.9), grey denotes no change (0.9-.1.1) and red denotes up-regulated proteins (<1.1). *abundance corresponds to the average of proteins with the same abbreviation; [1]corresponds to the average of proteins comprising the pyruvate dehydrogenase complex; [2]corresponds to proteins comprising the oxygen evolving complex; [3]corresponds to a mitochondrial protein; [4]corresponds to xenobiotic substrates; [5]corresponds to the peroxidase reaction with its optimal substrate (H_2O_2).


VESICLES
COP
NUCLEUS
Ran2
NABP
H
DNA
Alba1
Imp-α
NPP
RNA
eIF
EF
PABP
Proteins
Ribosome
rpS
rpL
RRF
CaM-1
ABAr9
Stomatal closure
Annexin
PLD
Signaling
Acyl proteins
DAP
Lys
Met
SAM
EPase1
OTC
Arg
Ala
AlaRS
ALT
SAH
AHCY
Aminoacids metabolism
Gly
SHMT
Glu-AdT
GATase
Phe
PAL
AP
CPase
aa
AO
HPR
PHGDH
Ser
CysS
Cys + Glu
PSAT
ABAT
AST
GDH
GS
Gln
ALS
KARI
Val
Leu
Ile
Asp
ADSS
AMP
HoDH
HiDH
His
DAHP
EPSP
IGPS
TS
Trp
TU-α
TU-β
Actin
CAP
LP04
Zea-epo
Violaxanthin
Zeaxanthin
VDE
Photoprotection
Flavonone
CHI
Chalcone
CHaS
D-Man
GME-1
ASC
ROS scavenging
AOC
DHN
H1/H5
Stress-related proteins
RS1
PLAT
DirP
Protein protection
chp
HSP
TCP1
PDI
PPI
O2
SOD
Prx
PRDX-Q
Prx-O + H2O
½ O2 + H2O
CAT
H2O2
Ascorbate
APX
H2O + MDA
LGSH
Gly1
MGO + GSH
GPx
H2O + GSSG
R-H2+H2O2
PDX
R + 2H2O
Enolase
PEP
Pyruvate
LHC
PSII
OEC
Cyt b6f
PSI
Pc
ATP synthase
3PG
rbcL
rbcS
RuBP
PGK
1,3bPG
PRK
GAPDH
Ru5P
G3P + DHAP
PPE
FBA
Xy5P
TAL
F1,6bP
FBPase
F6P
FKI
FKII
F
PFP
F6P
GPI
G6P
CHLOROPLAST
MITOCHONDRIA
Pyruvate
PDH
Acetyl-CoA
CitS
Oxaloacetate
MDH
Citrate
ACO
Fumarate
SQR
Isocitrate
IDH
Succinate
Gly
GCS
SCS
Succinyl-Coa
ATP synthase


VESICLES
CLTC COP*
NUCLEUS
Ran2
Imp-α*
rvb
H
DNA
TRN1
RNA
eIF* EF* PABP
Ribosome
Proteins
rpS* rpL* RNP
ABAr9
Stomatal closure
Annexin*
PLA1-II7 PLD*
Signaling
Acyl proteins*
ER+Golgi
TRAPPc
Photoprotection
Flavonone CHI* Chalcone
MetAP
ARD2
Met
SAM
NAS1
EPase1
ALS KARI
Val Leu Ile
AT
AP*
aa
CPase*
SAH AHCY SAH
Aminoacids metabolism
ArgJ
GD
GS*
Arg
ArgD
Gln
GCL-A
GDH*
Gly
SHMT*
PHGDH*
Ser
CysS*
Cys + Glu
ABAT*3
δP5CS
AST*
Asp
ADSS
ASL
AMP
HoDH
HiDH
His
PSAT
Glu
CM
Phe
Tyr
DAHP*
EPSP
ChoS
IGPS
Trp
DAP
Lys
LysRS
TU-α*
PMpoly
TU-β*
Actin*
CAP
LP04
Ala
ALT
Phe
PAL*
RS1 DirP*
Stress-related proteins
SALT
Protein protection
chp*
HSP
TCP1*
Psu5
PPI*
D-Man
GME-1
ASC
ROS scavenging
AOC
O2-
SOD*
Trx-O
Trx*
Trx+
Prx-O + H2O
Prx+
PRDX-B
½ O2 + H2O
CAT
H2O2 + Ascorbate
APX*
H2O + MDA
LGSH
Gly1
MGO + GSH
GSH
Gly + γ-Cys
GPx*
XS4
GST
H2O + GSSG
GS-X
R-H2+H2O2
PDX*5
R + 2H2O
MITOCHONDRIA
Pyruvate
PDH*1
Acetyl-CoA
CitS
Oxaloacetate
MDH*
Citrate
ACO
Fumarate
Isocitrate
IDH*
SQR*
Succinate
SCS*
Succinyl-Coa
Gly
GCS*
Ala
AlaRS*
ATP synthase*

The majority of proteins related to photosynthesis were downregulated in the salinity treatment, concomitant with a reduction in the relative chlorophyll content values, and this suggests a halt in photosynthetic processes. In accordance to our findings, Moradi et al. (2007) showed that photosynthetic CO_2 fixation, stomatal conductance (gs) and transpiration decreased substantially in rice plants exposed to salt stress [71]. Photosynthesis-related proteins were identified in roots and showed no significant changes between treatments as expected because this tissue is non-photosynthetically active [52]. Despite the photosynthetic processes being downregulated, a high number of proteins involved in energy and biomolecule metabolism were upregulated. Greater changes in abundance were observed in shoots compared to roots, implying that shoots require more intense activation of cell machinery than roots. The upregulation of these processes does not greatly enhance the tolerance to the salinity stress itself, but is essential in the activation of pathways involved in producing compatible solutes (e.g. proline, glycine betaine), triggering antioxidant responses and aiding in signaling cascades for sensing salinity among others. For this, a highly coordinated response is required in rice plants, which include the rapid upregulation of initiation and elongation factors at the transcription and translation levels, as well as a high number of ribosomal proteins needed to activate the salinity tolerance pathways.

Concerning strategies and mechanisms for coping with excess salt, all plants, even glycophytes (non-salinity tolerant plants), have developed the ability to sense the hyperosmotic component and the ionic Na^+ component of the salinity stress [70,72]. This sensing ability depends on a multigene response, and even though several genes are already known, the molecular identities of these sensors are not yet fully elucidated [10,19,70]. With our results we have contribute towards understanding the sensing pathways, as we have identified proteins such as calmodulin, annexin, phospholipases and ABA receptors. High

salinity stress rapidly leads to cytosolic Ca^{2+} spiking [12], which could strongly influence low affinity channels called nonselective cation channels that allow Na^{+} to enter cells, and if not adequately regulated could drastically increase Na^{+} levels and perturb the Na^{+}/K^{+} ratio [10]. Calmodulin is a ubiquitous protein that binds Ca^{2+} and is highly conserved in eukaryotes [73], and which in our work as well as the work others has shown a low abundance in leaves during salinity treatments [4]. It has also been demonstrated that the loss of function of the *SOS* gene in Arabidopsis, which encodes a Ca^{2+} binding protein, produces mutants that are oversensitive to salt stress [74]. Annexin, a Ca^{2+}-dependent phospholipid binding protein, was found to have higher abundance under salinity compared to the mock treatment at 48 h in both roots and shoots, and it has been reported that high transcriptional activity of this protein is related to increased stress tolerance [75,76]. In addition, it has been proposed that downstream Ca^{2+} kinases could be activated that transduce the signal to gene transcription, such as calcium-dependent protein kinases (CDPKs) [70]. In the present work, several translation initiation and elongation factors concomitant with a high number of ribosomal proteins were upregulated in both shoots and roots. This increase in proteins related to gene expression has also been reported in rice varieties harboring *Saltol* [4,15]. On the other hand, phospholipase D was detected in both shoots and roots but it had its highest abundance in shoots at 48 hours. This enzyme is involved in the main regulation of phospholipid-based signaling, which has been associated with salt stress tolerance, therefore its high abundance in shoots could contribute towards enhancing the salt tolerance response. Finally, ABA is a phytohormone that can play an important role in salinity tolerance by reducing transpiration [77]. The ABA receptor 9 was found to over-accumulate in salt-stressed shoots but with equal abundance in roots, suggesting that it indeed has a key role in stomatal closure in the aerial parts of the plant.

Other proteins that could be involved in sensing stress signals (hyperosmotic stress and elevated Na^+ concentration) that were detected during the experiment were those involved in vesicular trafficking in cells (clathrin, coatomer, Sar1, secA) and the nucleus (transportin, importin), along with GDI (guanosine nucleotide diphosphate dissociation inhibitor) which is also implicated in vesicular transport [78–83]. These proteins had a higher abundance in the salinity treatment, being more abundant in roots compared to shoots. Similar to our findings, several authors have reported that salinity stress induces bulk-flow endocytosis in *A. thaliana* roots via clathrin-dependent and -independent pathways after only 10 minutes of exposure to NaCl [84–86]. Regarding nuclear vesicular trafficking, Luo et al. (2013) demonstrated that a gene encoding for importin β is required for the drought tolerance response, which is essentially the osmotic component of salinity stress, and this is in accordance with our observations of increased abundance of importin α in roots after 48 h of 100 mM NaCl [87]. Increased vesicular trafficking during salinity stress has been associated with removal of sodium transporters from the plasma membrane to limit the Na^+ uptake and internalizing of plasma membrane aquaporins to prevent water loss [82].

In our study, upregulation of proteins related to oxidative stress, protein protection and abiotic stress in general was observed in shoots and roots. Enzymes involved in ROS-scavenging such as APX, CAT, PDX, SOD and GME-1 (L-ascorbic acid biosynthesis) were upregulated but with different patterns, which suggests differential activation. PDX, contained in the *Saltol* QTL, was substantially upregulated in shoots at 48 h under 100 mM NaCl, whereas in roots its upregulation was observed at 6 and 24 h, suggesting different roles in both tissues. Several studies have found increased abundances of proteins involved in ROS-scavenging mechanisms, such as SOD in roots and shoots as observed by Abdollah Hosseini et al. (2015) for FL478. Li et al. (2011) and Dooki et al. (2006) reported upregulation of APX and

AP in the roots of the rice cultivar 93-11 and the panicles of the IR651 cultivar, respectively [68,88]. Rice mutants for the allene oxide cyclase, a protein downregulated in shoots and roots in the current work, have been reported as having increased salt tolerance [89]. An interesting finding is that proteins involved in protein protection (e.g. HSPs, peptidylprolyl isomerase and chaperonins) were only markedly upregulated in shoots, suggesting that roots cope better with excess salt because they withstand the stress without activating these proteins. Contrarily to our results, Li et al. (2011) found an over-abundance of two chaperonins in roots in a cultivar sensitive to salinity, which could explain the upregulation to cope with the stress [90]. As reported by us, Lakra et al. (2017) also found upregulation of a chaperonin for shoot proteins in rice plants harboring *Saltol* (Pokkali).

The osmotic component of the salt stress imposes changes in shoot and root cells, which could provoke plasmolysis and generate additional stress [8]. We found intriguing results regarding cytoskeleton-related proteins, as abundances between treatments varied greatly in both shoots and roots. Roots displayed generally lower abundances in the salinity treatment, whereas the shoot abundances were higher for some of the proteins. These results should be further studied to provide more conclusive results on the involvement of cytoskeleton-related proteins in tolerance to salinity stress, as the literature highlights a key role of the cytoskeleton in the response to high salinity due to its highly dynamic changes and complex regulatory networks [91]. In addition, related to the stress that the osmotic component could cause in the cells, several compatible solutes (osmoprotectants) have been studied in rice [10]. In our study, we only found upregulation of enzymes involved in the synthesis of sucrose (δP5CS) and glycine betaine (BADH2) in roots during the salinity stress, whereas in shoots BADH2 expression was lower during salinity stress, again suggesting a stronger specialization of roots for saline conditions. Lakra et al. (2017) evidenced a high

proline content in IR64 (salt sensitive variety) and Pokkali (salt-tolerant variety) under salinity stress, which suggests that this mechanism is not specific for salt-tolerant varieties but is widespread across rice varieties.

High salinity causes growth reduction in terms of length and fresh weight. It has been proposed that growth reductions can be triggered by two processes: one responsive to accumulation of salt in the shoot (days to weeks) and the other independent of salt accumulation (minutes to few days) [19]. Both can be experimentally distinguished because the latter explains effects triggered upon addition of salt before there has been time for salt to accumulate in the shoot. In fact, our results do confirm this because the Na^+/K^+ ratio in salt stressed shoots at 24 and 48 h in our work is half that recorded in other studies suggesting a lower Na^+ accumulation in this tissue [4,92]. Abdollah Hosseini et al. (2015) observed in FL478 that even after 14 days of salinity treatment the shoot Na^+/K^+ ratio was maintained at 0.5, similar to our values at 48 h, and this suggests that mechanisms are set in motion to prevent accumulation of Na^+ in this tissue. In contrast, the root Na^+/K^+ ratio increases continuously during salinity stress, as at 48 h we recorded values of around 2.5 and after 14 d of salinity in FL478 values of 3.4 have been observed [15]. Salinity treatment only reduced the shoot length because roots continued their natural development, probably in an effort to find less saline areas in the growing medium. Growth reduction in terms of fresh weight was observed for both tissues, which suggests a lower water availability in roots during salinity stress. Nevertheless, because the root water content was similar between treatments, the difference could be due to the development of thinner roots in the salinity treatment in order to create a greater surface area to cope with the salinity in the medium. In accordance with our results, Abdollah Hosseini et al. (2015) (14 d of salinity treatment) and Lakra et al. (2017) (15’ to 72 h of salinity treatment) evidenced reduction in fresh weight and length, respectively,

during salinity treatments in rice varieties possessing *Saltol*. Likewise, Khan and Panda (2008) reported reductions in the root fresh weight of a salt-tolerant rice variety (*O. sativa* L. cv. *Lunishree*) under salinity stress after 24 h of treatment [92]. Moreover, the overall growth reduction (length and weight) can be attributed to a low rate of CO_2 assimilation, which could be related to the over-abundance of ABAr9 (which enhances stomatal closure) as well as the downregulation of the majority of proteins related to photosynthesis and the reduction in relative chlorophyll content values.

Finally, in this study, as well as others that have evaluated salinity tolerance in *Saltol* varieties, the *OsHKT1;5* protein was not detected even when using a highly efficient and precise method such as shotgun proteomics, and it therefore still remains in doubt [4,15,41]. Nevertheless, we did detect SalT and PDX proteins, which are present in the *Saltol* QTL region. Furthermore, we provide a huge database for future studies of the salinity-stress response in rice varieties harboring the *Saltol* region, as we detected more than 2000 proteins during the early stages of salinity stress in the shoots and roots of the FL478 rice line. In addition, protein-protein interaction networks allow identification of key novel candidates that should have a major role in the tolerance to salinity stress and further detail about these will broaden our knowledge of this stress in rice. Moreover, this study highlights the importance of examining both the shoots and roots because their salinity tolerance traits are different and respond to different requirements of the rice plant. Finally, the information presented here expands our knowledge on adaptive processes under high salinity in rice plants and notably, in rice roots.

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The authors have declared no conflict of interest.

REFERENCES - CHAPTER 1

[1] Shrivastava, P., Kumar, R., Soil salinity: A serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. *Saudi J. Biol. Sci.* 2015, 22, 123–131.

[2] Mickelbart, M. V., Hasegawa, P.M., Bailey-Serres, J., Genetic mechanisms of abiotic stress tolerance that translate to crop yield stability. *Nat. Rev. Genet.* 2015, 16, 237–251.

[3] Kumar Dubey, S., Pandey, A., Singh Sangwan, R., *Current developments in biotechnology and bioengineering: crop modification, nutrition, and food production*, Elsevier, Amsterdam, Netherlands 2017.

[4] Lakra, N., Kaur, C., Anwar, K., Singla-Pareek, S.L., Pareek, A., Proteomics of contrasting rice genotypes: Identification of potential targets for raising crops for saline environment. *Plant Cell Environ.* 2017, 1.

[5] Genua-Olmedo, A., Alcaraz, C., Caiola, N., Ibáñez, C., Sea level rise impacts on rice production: The Ebro Delta as an example. *Sci. Total Environ.* 2016, 571, 1200–1210.

[6] Muthayya, S., Sugimoto, J.D., Montgomery, S., Maberly, G.F., An overview of global rice production, supply, trade, and consumption. *Ann. N. Y. Acad. Sci.* 2014, 1324, 7–14.

[7] FAO, *Rice Market Monitor*, vol. XX, 2017.

[8] Sahi, C., Singh, A., Kumar, K., Blumwald, E., Grover, A., Salt stress response in rice: genetics, molecular biology, and comparative genomics. *Plant Mol. Biol.* 2006, 6, 263–284.

[9] Sposito, G., in:, *Chem. Soils*, Oxford University Press, New York 2008, p. 330.

[10] Reddy, I.N.B.L., Kim, B.K., Yoon, I.S., Kim, K.H., Kwon, T.R., Salt tolerance in rice: focus on mechanisms and approaches. *Rice Sci.* 2017, 24, 123–144.

[11] Lutts, S., Kinet, J.M., Bouharmont, J., Changes in plant response to NaCl during development of rice (*Oryza sativa* L.) varieties differing in salinity resistance. *J. Exp. Bot.* 1995, 46, 1843–1852.

[12] Kumar, K., Kumar, M., Kim, S.-R., Ryu, H., Cho, Y.-G., Insights into genomics of salt stress response in rice. *Rice* 2013, 6, 27.

[13] Heenan, D., Lewin, L., McCaffery, D., Salinity tolerance in rice varieties at different growth stages. *Aust. J. Exp. Agric.* 1988, 28, 343–349.

[14] Munns, R., Tester, M., Mechanisms of salinity tolerance. *Annu Rev Plant Biol* 2008, 59, 651–681.

[15] Abdollah Hosseini, S., Gharechahi, J., Heidari, M., Koobaz, P., et al., Comparative proteomic and physiological characterisation of two closely related rice genotypes with contrasting responses to salt stress. *Funct. Plant Biol.* 2015, 42, 527–542.

[16] Negrão, S., Almadanim, M.C., Pires, I.S., Abreu, I.A., et al., New allelic variants found in key rice salt-tolerance genes: An association study. *Plant Biotechnol. J.* 2013, 11, 87–100.

[17] Gupta, B., Huang, B., Mechanism of salinity tolerance in plants: Physiological, biochemical, and molecular characterization. *Int. J. Genomics* 2014, 2014.

[18] Das, P., Nutan, K.K., Singla-Pareek, S.L., Pareek, A., Understanding salinity responses and adopting “omics-based” approaches to generate salinity tolerant cultivars of rice. *Front. Plant Sci.* 2015, 6, 712.

[19] Roy, S.J., Negrão, S., Tester, M., Salt resistant crop plants. *Curr. Opin. Biotechnol.* 2014, 26, 115–124.

[20] Djanaguiraman, M., Ramadass, R., Devi, D.D., Effect of salt stress on germination and

seedling growth in rice genotypes. *Madras Agric. J.* 2003, 90, 50–53.

[21] Platten, J.D., Egdane, J.A., Ismail, A.M., Salinity tolerance, Na^+ exclusion and allele mining of *HKT1;5* in *Oryza sativa* and *O. glaberrima*: many sources, many genes, one mechanism? *BMC Plant Biol.* 2013, 13, 32.

[22] Lin, H.X., Zhu, M.Z., Yano, M., Gao, J.P., et al., QTLs for Na^+ and K^+ uptake of the shoots and roots controlling rice salt tolerance. *Theor. Appl. Genet.* 2004, 108, 253–260.

[23] Kumar, V., Singh, A., Mithra, S.V.A., Krishnamurthy, S.L., et al., Genome-wide association mapping of salinity tolerance in rice (*Oryza sativa*). *DNA Res.* 2015, 22, 133–145.

[24] Singh, R.K., Gregorio, G.B., Jain, R.K., QTL Mapping for salinity tolerance in rice. *Physiol. Mol. Biol. Plants* 2007, 13, 87–99.

[25] Eckardt, N.A., Sequencing the Rice Genome. *Plant Cell Online* 2000, 12, 2011–2017.

[26] Zhang, G.Y., Guo, Y., Chen, S.L., Chen, S.Y., RFLP tagging of a salt tolerance gene in rice. *Plant Sci.* 1995, 110, 227–234.

[27] Gong, J., He, P., Qian, Q., Shen, L., et al., Identification of salt-tolerance QTL in rice (*Oryza sativa* L.). *Chinese Sci. Bull.* 1999, 44, 68–70.

[28] Prasad, S.R., Prashanth, G.B., Hittalmani, S., Shashidhar, H.E., Molecular mapping of quantitative trait loci associated with seedling tolerance to salt stress in rice (*Oryza sativa* L.). *Curr. Sci.* 2000, 78, 3.

[29] Koyama, M.L., Levesley, a, Koebner, R.M., Flowers, T.J., Yeo, a R., Quantitative trait loci for component physiological traits determining salt tolerance in rice. *Plant Physiol.* 2001, 125, 406–22.

[30] Gregorio, G.B., Senadhira, D., Mendoza, R.D., Manigbas, N.L., et al., Progress in breeding for salinity tolerance and associated abiotic stresses in rice. *F. Crop. Res.* 2002, 76, 91–101.

[31] Cotsaftis, O., Plett, D., Johnson, A.A., Walia, H., et al., Root-specific transcript profiling of contrasting rice genotypes in response to salinity stress. *Mol Plant* 2011, 4, 25–41.

[32] Bonilla, P., Dvorak, J., Mackill, D., Deal, K., Gregorio, G., RFLP and SSLP mapping of salinity tolerance genes in chromosome 1 of rice (*Oryza sativa* L.) using recombinante inbred lines. *Philipp. Agric. Sci.* 2002, 85, 9.

[33] Thomson, M.J., de Ocampo, M., Egdane, J., Rahman, M.A., et al., Characterizing the *Saltol* quantitative trait locus for salinity tolerance in rice. *Rice* 2010, 3, 148–160.

[34] Nutan, K.K., Kushwaha, H.R., Singla-Pareek, S.L., Pareek, A., Transcription dynamics of *Salto*l QTL localized genes encoding transcription factors, reveals their differential regulation in contrasting genotypes of rice. *Funct. Integr. Genomics* 2017, 17, 69–83.

[35] Waziri, A., Kumar, Purty, R., *Saltol* QTL and their role in salinity tolerance in rice. *Austin J Biotechnol Bioeng. Austin J Biotechnol Bioeng* 2016, 3, 1067–3.

[36] Krishnamurthy, S.L., Sharma, S.K., Kumar, V., Tiwari, S., Singh, N.K., Analysis of genomic region spanning *Saltol* using SSR markers in rice genotypes showing differential seedlings stage salt tolerance. *J. Plant Biochem. Biotechnol.* 2016, 25, 331–336.

[37] Samal, R., Roy, P.S., Dash, A.K., Rao, G.J.N., et al., Genetic diversity in the rice landraces (*Oryza sativa* L.) of coastal Sundarbans (India) and their adaptation to the local saline condition investigated both at molecular and physiological level. *Acta Physiol. Plant.* 2016, 38, 1–10.

[38] Kim, S.-H., Bhat, P., Cui, X., Walia, H., et al., Detection and validation of single feature polymorphisms using RNA expression data from a rice genome array. *BMC Plant Biol.* 2009, 9, 1–10.

[39] Platten, J.D., Cotsaftis, O., Berthomieu, P., Bohnert, H., et al., Nomenclature for HKT transporters, key determinants of plant salinity tolerance. *Trends Plant Sci.* 2006, 11, 372–374.

[40] Garciadeblás, B., Senn, M.E., Bañuelos, M.A., Rodríguez-Navarro, A., Sodium transport and HKT transporters: The rice model. *Plant J.* 2003, 34, 788–801.

[41] Singh, R., Jwa, N.S., Understanding the responses of rice to environmental stress using proteomics. *J. Proteome Res.* 2013, 12, 4652–4669.

[42] Ren, Z.H., Gao, J.P., Li, L.G., Cai, X.L., et al., A rice quantitative trait locus for salt tolerance encodes a sodium transporter. *Nat. Genet.* 2005, 37, 1141–1146.

[43] Golldack, D., Su, H., Quigley, F., Kamasani, U.R., et al., Characterization of a HKT-type transporter in rice as a general alkali cation transporter. *Plant J.* 2002, 31, 529–542.

[44] Walia, H., Wilson, C., Condamine, P., Liu, X., et al., Comparative transcriptional profiling of two contrasting rice genotypes under salinity stress during the vegetative growth stage. *Plant Physiol* 2005, 139, 822–835.

[45] Kobayashi, N.I., Yamaji, N., Yamamoto, H., Okubo, K., et al., *OsHKT1;5* mediates Na^+ exclusion in the vasculature to protect leaf blades and reproductive tissues from salt toxicity in rice. *Plant J.* 2017, 91, 657–670.

[46] Babu, N.N., Krishnan, S.G., Vinod, K.K., Krishnamurthy, S.L., et al., Marker aided incorporation of *Saltol*, a major QTL associated with seedling stage salt tolerance, into *Oryza sativa* 'Pusa Basmati 1121.' *Front. Plant Sci.* 2017, 8, 1–14.

[47] Gupta, R., Wang, Y., Agrawal, G.K., Rakwal, R., et al., Time to dig deep into the plant proteome: a hunt for low-abundance proteins. *Front. Plant Sci.* 2015, 6, 1–3.

[48] Mirzaei, M., Soltani, N., Sarhadi, E., Pascovici, D., et al., Shotgun proteomic analysis of long-distance drought signaling in rice roots. *J. Proteome Res.* 2012, 11, 348–358.

[49] Yoshida, S., Forno, D.A., Cock, J.H., Kwanchai, A.G., *Laboratory manual for physiological studies of rice*, International Rice Research Institute (IRRI), Los Baños, Laguna, Philippines 1976.

[50] Kim, Y.J., Lee, H.M., Wang, Y., Wu, J., et al., Depletion of abundant plant RuBisCO protein using the protamine sulfate precipitation method. *Proteomics* 2013, 13, 2176–2179.

[51] Gupta, R., Kim, S.T., in:, Posch A (Ed.), *Proteomic Profiling Methods Protoc.*, vol. 1295, Springer Science & Business Media, New York 2015, p. 9.

[52] Suzuki, Y., Nakabayashi, K., Yoshizawa, R., Mae, T., Makino, A., Differences in expression of the RBCS multigene family and rubisco protein content in various rice plant tissues at different growth stages. *Plant Cell Physiol.* 2009, 50, 1851–1855.

[53] Callister, S.J., Barry, R.C., Adkins, J.N., Johnson, E.T., et al., Normalization approaches for removing systematic biases associated with mass spectrometry and label-free proteomics. *J. Proteome Res.* 2006, 5, 277–286.

[54] Ogata, H., Goto, S., Sato, K., Fujibuchi, W., et al., KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* 2000, 27, 4.

[55] Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., Tanabe, M., KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res.* 2016, 44, 6.

[56] Oveland, E., Muth, T., Rapp, E., Martens, L., et al., Viewing the proteome: how to visualize proteomics data? *Proteomics* 2015, 15, 1341–1355.

[57] Halligan, B.D., Mirza, S.P., Pellitteri-Hahn, M.C., Olivier, M., Greene, A.S., in:, *Proc. Int. Conf. Inf. Vis.*, 2007, p. 8.

[58] Baehrecke, E.H., Dang, N., Babaria, K., Shneiderman, B., Visualization and analysis of microarray and gene ontology data with treemaps. *BMC Bioinformatics* 2004, 5, 1–12.

[59] Otto, A., Bernhardt, J., Meyer, H., Schaffer, M., et al., Systems-wide temporal proteomic profiling in glucose-starved *Bacillus subtilis*. *Nat. Commun.* 2010, 1.

[60] Cline, S.M., Smoot, M., Cerami, E., Kuchinsky, A., et al., Integration of biological networks and gene expression data using Cytoscape. *Nat. Protoc.* 2007, 2, 2366–2382.

[61] Key, M., A tutorial in displaying mass spectrometry-based proteomic data using heat maps. *BMC Bioinformatics* 2012, 13, S10.

[62] Benjamini, Y., Hochberg, Y., Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc.* 1995, 57, 289–300.

[63] Thomson, M., de Ocampo, M., Egdane, J., Rahman, M.A., et al., Characterizing the *Saltol* Quantitative Trait Locus for salinity tolerance in rice. *Rice* 2010, 3, 148–160.

[64] Huyen, L.T.N., Cuc, L.M., Ismail, A.M., Ham, L.H., Introgression the salinity tolerance QTLs Saltol into AS996, the elite rice variety of Vietnam. *Am. J. Plant Sci.* 2012, 3, 981–987.

[65] Krishnamurthy, S.L., Sharma, S.K., Kumar, V., Tiwari, S., et al., Assessment of genetic diversity in rice genotypes for salinity tolerance using *Saltol* markers of chromosome 1. *Indian J. Genet. Plant Breed.* 2014, 74, 243–247.

[66] Kim, M., Kim, H., Lee, W., Lee, Y., et al., Quantitative shotgun proteomics analysis of rice anther proteins after exposure to high temperature 2015, 2015.

[67] Zhang, Y., Fonslow, B.R., Shan, B., Baek, M.-C., Yates III, J.R., Protein analysis by shotgun/bottom-up proteomics. *Chem. Rev.* 2015, 113, 213–223.

[68] Li, X.J., Yang, M.F., Zhu, Y., Liang, Y., Shen, S.H., Proteomic Analysis of Salt Stress Responses in Rice Shoot. *J. Plant Biol.* 2011, 54, 384–395.

[69] Yan, S., Tang, Z., Su, W., Sun, W., Proteomic analysis of salt stress-responsive proteins in rice root. *Proteomics* 2005, 5, 235–244.

[70] Deinlein, U., Stephan, A.B., Horie, T., Luo, W., et al., Plant salt-tolerance mechanisms. *Trends Plant Sci* 2014, 19, 371–379.

[71] Moradi, F., Ismail, A.M., Responses of photosynthesis, chlorophyll fluorescence and ROS-scavenging systems to salt stress during seedling and reproductive stages in rice. *Ann. Bot.* 2007, 99, 1161–1173.

[72] Maathuis, F.J.M., Sodium in plants: Perception, signalling, and regulation of sodium fluxes. *J. Exp. Bot.* 2014, 65, 849–858.

[73] Kim, M.C., Chung, W.S., Yun, D.-J., Cho, M.J., Calcium and calmodulin-mediated regulation of gene expression in plants. *Mol. Plant* 2009, 2, 13–21.

[74] Mahajan, S., Pandey, G.K., Tuteja, N., Calcium- and salt-stress signaling in plants: shedding light on SOS pathway. *Arch. Biochem. Biophys.* 2008, 471, 146–158.

[75] Jami, S.K., Clark, G.B., Ayele, B.T., Roux, S.J., Kirti, P.B., Identification and characterization of annexin gene family in rice. *Plant Cell Rep.* 2012, 31, 813–825.

[76] Qiao, B., Zhang, Q., Liu, D., Wang, H., et al., A calcium-binding protein, rice annexin *OsANN1*, enhances heat stress tolerance by modulating the production of H_2O_2. *J. Exp. Bot.* 2015, 66, 5853–5866.

[77] Park, Y.C., Chapagain, S., Jang, C.S., A negative regulator in response to salinity in rice: *Oryza sativa* salt-, ABA- and drought-induced RING Finger Protein 1 (*OsSADR1*). *Plant Cell Physiol.* 2018, 59, 575–589.

[78] Karim, S., Aronsson, H., The puzzle of chloroplast vesicle transport - involvement of GTPases. *Front. Plant Sci.* 2014, 5, 1–12.
[79] Alezzawi, M., Vesicle transport in chloroplasts. University of Gothenburg, 2014.
[80] Lee, C., Goldberg, J., Structure of coatomer cage proteins and the relationship among COPI, COPII, and clathrin vesicle coats. *Cell* 2010, 142, 123–132.
[81] Memon, A.R., The role of ADP-ribosylation factor and SAR1 in vesicular trafficking in plants. *Biochim. Biophys. Acta - Biomembr.* 2004, 1664, 9–30.
[82] Baral, A., Shruthi, K.S., Mathew, M.K., Vesicular trafficking and salinity responses in plants. *IUBMB Life* 2015, 67, 677–686.
[83] Yang, Y., Wang, W., Chu, Z., Zhu, J.-K., Zhang, H., Roles of nuclear pores and nucleo-cytoplasmic trafficking in plant stress responses. *Front. Plant Sci.* 2017, 08, 1–8.
[84] Leshem, Y., Seri, L., Levine, A., Induction of phosphatidylinositol 3-kinase-mediated endocytosis by salt stress leads to intracellular production of reactive oxygen species and salt tolerance. *Plant J.* 2007, 51, 185–197.
[85] Zwiewka, M., Nodzyński, T., Robert, S., Vanneste, S., Friml, J., Osmotic stress modulates the balance between exocytosis and clathrin-mediated endocytosis in *Arabidopsis thaliana*. *Mol. Plant* 2015, 8, 1175–1187.
[86] Baral, A., Irani, N.G., Fujimoto, M., Nakano, A., et al., Salt-Induced remodeling of spatially restricted clathrin-independent endocytic pathways in *Arabidopsis* root. *Plant Cell* 2015, 27, 1297–1315.
[87] Luo, Y., Wang, Z., Ji, H., Fang, H., et al., An *Arabidopsis* homolog of importin β1 is required for ABA response and drought tolerance. *Plant J.* 2013, 75, 377–389.
[88] Dooki, A.D., Mayer-Posner, F.J., Askari, H., Zaiee, A.A., Salekdeh, G.H., Proteomic responses of rice young panicles to salinity. *Proteomics* 2006, 6, 6498–6507.
[89] Hazman, M., Hause, B., Eiche, E., Nick, P., Riemann, M., Increased tolerance to salt stress in OPDA-deficient rice ALLENE OXIDE CYCLASE mutants is linked to an increased ROS-scavenging activity. *J. Exp. Bot.* 2015, 66, 3339–3352.
[90] Wang, S., Cao, M., Ma, X., Chen, W., et al., Integrated RNA Sequencing and QTL mapping to identify candidate genes from *Oryza rufipogon* associated with salt tolerance at the seedling stage. *Front. Plant Sci.* 2017, 8, 1–11.
[91] Wang, C., Zhang, L.-J., Huang, R.-D., Cytoskeleton and plant salt stress tolerance. *Plant Signal. Behav.* 2011, 6, 29–31.
[92] Khan, M.H., Panda, S.K., Alterations in root lipid peroxidation and antioxidative responses in two rice cultivars under NaCl-salinity stress. *Acta Physiol. Plant.* 2008, 30, 81–89.

CHAPTER 2 - Phytohormone profiling method for rice: effects of *GA20ox* mutation on the gibberellin content of Mediterranean *japonica* rice varieties

Camilo López-Cristoffanini[1], Xavier Serrat[1], Olga Jáuregui[2], Salvador Nogués[1], Marta López-Carbonell[1]

[1]Departament de Biologia Evolutiva, Ecologia i Ciencies Ambientals, Secció de Fisiologia Vegetal, Universitat de Barcelona, 08028 Barcelona, España.

[2]Scientific & Technological Centers, Universitat de Barcelona (CCiTUB), c/Baldiri i, Reixach 10–12, 08028 Barcelona, España.

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ABSTRACT

Gibberellins (GAs) are an important group of phytohormones involved in seed germination, vegetative growth, flowering, and fruit development, being only active 4 of the 136 known: GA1, GA3, GA4 and GA7. Mutations in the OsGA20ox-2 gene generate rice (*Oryza sativa*) dwarf varieties, which were one of the main green revolution pillars. In this work two objectives were proposed: (i) develop a rapid and broad phytohormone profiling method and (ii) study the effects on the GA content of the GA20ox-2 mutation in rice developmental stages using three varieties (tall variety, elite variety, mutated variety). A phytohormone extraction that uses an SPE step and HPLC-MS/MS detection (QqQ instrument) was developed which allows to identify 13 GAs. This method has limits of detection (LOD) and limits of quantification (LOQ) for GAs between 0.1-0.7 and 0.3-2.3 pg·g-1 (f.w.) of sample respectively; and relative standard deviations (RSD) of 0.3-0.9% for a 40 ng·mL-1 standard. Overall, this method has high sensitivity and good reproducibility. Notoriously, GA1 was absent in the coleoptile and GA4 had the highest content in the majority of developmental stages. A significant increase of the four bioactive GAs in the internode of the flag leaf of the mutated variety was observed, allowing it to reach the elite variety's height. We provide a rapid and broad phytohormonal profiling that allows, to our knowledge, to report such a high number of gibberellins in rice (Oryza sativa ssp. japonica). Moreover, we provide evidence that the GA20ox-2 mutation is not the only factor generating dwarf varieties.

KEYWORDS: *GA20ox* gene, gibberellins (GA), Mediterranean *japonica* rice, phytohormone analysis, dwarfism, HPLC-MS/MS, solid phase extraction.

INTRODUCTION

Plants rely on plant hormones, also called phytohormones, for several processes throughout their life including growth, development and responses to stress. These small molecules are naturally occurring substances that act at very low concentrations and have signaling functions (Davies, 2010; Kudo et al., 2013). Nowadays, there is large knowledge regarding phytohormone biosynthesis, regulation and their specific role in signaling (Peleg and Blumwald, 2011). Gibberellins, a large hormone category, are a large group of tetracyclic diterpenoid carboxylic acids, which were first identified as secondary metabolites of the fungus Gibberella fujikuroi (Hedden and Thomas, 2012). Nowadays, more than 136 different gibberellin structures have been found, but four of them are highly bioactive: GA1, GA3, GA4 and GA7 (Hedden and Phillips, 2000; Macías et al., 2014; Yamaguchi, 2008).

Gibberellin biosynthesis, in plants, begins in plastids where trans-geranylgeranyl diphosphate (GGPP) is converted in two steps to ent-kaurene. Then, this molecule goes to the endoplasmic reticulum where it is converted into gibberellin GA12 where it can follow two pathways: (i) the GA12- or non-hydroxylated gibberellins pathway or (ii) be synthesized to GA53 through the GA 13-oxidase (*GA13ox*) to follow the GA53- or hydroxylated gibberellin pathway (Urbanová et al., 2013; Yamaguchi, 2008). Interestingly, both pathways have the same enzymes where GA 20-oxidase (GA20ox) produces GA9 and GA20 for GA12- and GA53- pathways respectively (Yamaguchi, 2008). Then, by the action of GA 3-oxidase (*GA3ox*) the bioactive gibberellins are produced: GA1 and GA3 (GA53-pathway) and GA4 and GA7 (GA12-pathway) (Hedden and Phillips, 2000). In addition, the GA 2-oxidases (GA2oxs) are enzymes that deactivate gibberellins through a change of the -OH position (Hedden, 2001). All these findings have revealed that there are several steps for GAs

biosynthesis regulation including genes for activation/deactivation and phytohormones interaction at several levels of the biosynthesis pathways (Wang et al., 2017).

Gibberellins have been largely viewed as phytohormones involved in processes such as seed germination, vegetative growth, flowering, and fruit development (Olszewski et al., 2002); but their main focus since 1960 (during the green revolution) has been their involvement in dwarfism traits of plants (Hedden, 2003). This reduction in height allowed to obtain high-yielding varieties which had a significant change in GA biosynthesis and signaling pathway (Hedden, 2003; Wang et al., 2017). Nevertheless, some semi-dwarf or dwarf mutants defective in hormone biosynthesis or signaling have undesirable secondary effects such as altered tillering, small grains, semi-sterility, malformed panicles and lower plant establishment (Liu et al., 2018a). In rice, four different mutations in the GA20 oxidase 2 gene (*GA20ox-2*, which has three exons) have been found to provoke a disruption in GA biosynthesis which generates plants with dwarfism traits, named sd-1 mutants (Sasaki et al., 2002). For indica varieties, the mutation is generally a 383-bp deletion (Dee-geo-woo-gen, between exon 1 and 2), whereas for japonica varieties the mutations are point mutations (Jikkoku in exon 1, Calrose in exon 2 and Reimein in exon 3) that result in single amino acid substitutions (Hedden, 2003; Sasaki et al., 2002). Independently of the allele that provokes height reduction, the gibberellins production pattern is disrupted as sd-1 mutated plants show GA53 accumulation and a lower content of the gibberellins that are produced by *GA20ox-2* (Ashikari et al., 2002; Sasaki et al., 2002; Spielmeyer et al., 2002a). Moreover, these mutants are only defective in GA biosynthesis, and not in GA perception, as external GA applications allow to recover normal height (Hedden, 2003). In addition to their crucial role in regulating plant height, they have also been shown to be involved in tolerance to abiotic stress such as salinity which can severely affect yield (Iqbal et al., 2012; Reddy et al., 2017; Sahoo et al., 2014).

Furthermore, taller plants have higher tolerance to salt stress than their shorter counterpart, because they can dilute the Na^+ content in the cells to reduce the damage (Das et al., 2015). Moreover, Sahoo et al. (2014) and Tuteja et al (2013) demonstrated that OsSUV3 over-expressing transgenic rice has higher endogenous levels of GA3 and higher yield under salinity than wild-type plants, suggesting a role of gibberellins in salinity tolerance.

Gibberellins are present in plants at very low concentrations that can range between 0.9 to 16.8 $ng \cdot g^{-1}$ of fresh weight, hence GAs in samples should be enriched prior to detection (Chen et al., 2012). Crucial points during gibberellin analysis are extraction and cleaning steps, which should ensure high presence of GAs and low presence of other molecules (Urbanová et al., 2013). The majority of current GAs extraction methods use the classic liquid-liquid extraction and solid phase extraction (SPE) with reverse phase C-18 cartridge for sample concentration and clean up (Macías et al., 2014). Nowadays, HPLC-MS/MS is the standard and routine technique for GAs separation and detection (Macías et al., 2014; Urbanová et al., 2013) mainly using triple quadrupole instruments for their quantification at trace levels. The biggest problem in plants, including rice, is the difficulty to detect a high number of gibberellins in one run (Chen et al., 2012; Kojima et al., 2009; Urbanová et al., 2013).

Due to the crucial role of gibberellins in regulating process such as growth, development and abiotic stress, and thanks to the current advances in HPLC-MS/MS techniques there is increased interest in studying whole hormonal profiles. Therefore, we report, for the first time, the application of a rapid and broad phytohormone profiling method, with high specific and accuracy, that can detect in one single run a total of 16 phytohormones (including 13 different gibberellins) on rice (*Oryza sativa*), using an SPE step and HPLC-MS/MS detection. This method was used to study the effect of a *GA20ox* mutation in three Mediterranean japonica rice varieties with differential heights.

MATERIALS AND METHODS

PLANT MATERIAL AND SAMPLING

Three Mediterranean japonica rice varieties were used in this study: NRVC980385 (N), Bomba (B) and *dwarf*-Bomba (dB). The dwarfism trait of dwarf-Bomba was verified by PCR and posterior Sanger sequencing. For this, DNA of the three varieties was extracted according to Doyle and Doyle (1987) with slightly modifications. Exon 2 of the *GA20ox-2* gene was amplified using the primers designed by Spielmeyer (2002b) following the PAQ5000 (Agilent, California, USA) manufacturer instructions. Afterwards, the PCR product was sequenced using Sanger method by the Genomic platform of the CCiT-UB (Barcelone, Spain).

Plants were germinated in Petri dishes with a humid autoclaved paper and in addition grown in greenhouse conditions at the Experimental Fields Service at the University of Barcelona (Barcelone, Spain) on four-liter plastic containers filled with rice substrate as described in Serrat et al (2014). For greenhouse grown plants, height was measured after one week of sowing and then on a two-week basis using a total of eight replicates for each variety.

Several growth stages were collected in triplicate from plants grown in greenhouse according to the rice development system proposed by Counce et al (2000) as detailed in Table 1. Collected samples were immediately frozen in N_2(l) and stored at -80ºC. Petri dish germinated plants were used to collect the coleoptile at S3 which occurred approximately after one week of germination. For tissue sampling, tissue was collected at five-leaves plant stages (V5) and at flag leaf/panicle exertion (R2 and R3-R4) (Table 3.2.1). Finally, we collected the panicles at 50% of booting which occurs between R3 and R4 reproductive growth stages.

Table 3.2.1. Growth stages, according to the rice development system proposed by Counce et al (2000), used for sample collection of the plants grown in the greenhouse.

Growth stage	Tissue	Code
S3 (emergence of prophyll from coleoptile)	Coleoptile	COL
V5 (collar formation on leaf 5 on main stem)	4th node	4N
	Internode between 4th and 5th node	4N5
	5th node	5N
	Basal part of the 5th leaf	B5L
	Apical part of the 5th leaf	A5L
R2 (flag leaf collar formation)	Node previous to the flag leaf node	pN
	Internode between flag leaf and previous leaf	pNF
	Flag leaf node	FN
	Basal part of the flag leaf	BFL
	Apical part of the flag leaf	AFL
R3-R4 (50 % heading)	Panicle and florets	50H

CHEMICALS AND MATERIAL

All reagents were obtained from LabBox (Vilassar de Dalt, Spain). All phytohormones, unlabeled (GA_1, GA_3, GA_4, GA_7, GA_8, GA_{12}, GA_{15}, GA_{19}, GA_{20}, GA_{29}, GA_{44}, GA_{51}, GA_{53}, ABA, JA, IAA) and deuterium-labeled (d_2-GA_1, d_2-GA_3, d_2-GA_4, d_2-GA_7, d_2-GA_8, d_2-GA_{12}, d_2-GA_{15}, d_2-GA_{19}, d_2-GA_{20}, d_2-GA_{29}, d_2-GA_{44}, d_2-GA_{51}, d_2-GA_{53}, d_6-ABA, d_6-JA, d_5-IAA) standards, were purchased from OlChemIm (Olomouc, Czech Republic). SPE columns and OASIS® HLB 1cc and OASIS® PRIME HLB 1cc were purchased from Waters (Milford, MA, USA). Fixed insert vials and pre-slit PTFE screw cap were purchased from Teknokroma (Sant Cugat del Vallès, Spain). The HPLC column Kinetex® 2.6 μm XB-C18 100 Å (30*2.1 mm) was purchased from Phenomenex (California, USA), and the HPLC column Mediterranea Sea 18 column (10*0.2 cm, 2.2 µm) was purchased from Teknokroma (Sant Cugat del Vallès, Spain).

HPLC-MS analysis

First of all, gibberellins were identified in rice samples using an HPLC-HRMS method, named HPLC-1 which is described in

Table 3.2.2. Positive identification of phytohormones was based on the accurate mass measurement with an error of two mDa using high-resolution LTQ Orbitrap Velos mass spectrometer. An inventory of 16 phytohormones (13 gibberellins, ABA, JA and IAA) was defined. Their theoretical exact masses were determined using a spectrum simulation tool of Xcalibur. Then, a list of possible candidates fitting the specific exact mass was generated using formula determination tools (elemental composition search) of Thermo Fischer Scientific Xcalibur softwares. The elemental number for phytohormones was restricted to include C, H and O. The formula constraints for gibberellins were 19≤C≥20, 22≤H≥28H, 4≤O≥7, whereas for ABA, JA and IAA the restriction was the exact formula for each compound. The search was based on single mass analysis and only considered the *m/z* value of the monoisotopic peak. Considering tentative identified phytohormones, we proceed to buy them and to inject in the HPLC-1 system. In this way, we confirmed the presence of 13 gibberellins as well as ABA, JA and IAA in rice samples.

Once phytohormones were identified and the SPE protocol optimized, phytohormones from the rice samples were extracted in triplicate with the method C' (explained below) and quantified in an HPLC-QqQ instrument with a method called HPLC-2 described in

Table 3.2.2. Multiple reaction monitoring (MRM) mode was used to identify and quantify analytes. MS/MS parameters for working in MRM mode were optimized by direct infusion of each individual standard at a concentration of 0.1 $mg \cdot L^{-1}$ in MeOH:H_2O (20:80, v/v) with 0.05% of HAc into the mass spectrometer using a syringe pump (Harvard Apparatus, Holliston, MA) at a constant flow rate of 10 $\mu L \cdot min^{-1}$. The scheduled MRM mode was

employed instead of conventional MRM, which allows the simultaneous monitoring of multiple transitions by using retention time windows. To establish these windows, individual standard solutions were injected into the HPLC-MS/MS system to find their retention times, and RT windows were then estimated based on peak widths. Analyst 1.6.2 Software was used for data acquisition and MultiQuant 3.0.1 for data processing both from ABSciex (Framingham, MA, USA).

Table 3.2.2. HPLC and MS conditions and parameters for HPLC-1 and HPLC-2 methods.

Conditions	**HPLC-1**	**HPLC-2**
HPLC		
LC system	Agilent 1290 Infinity LC System (Santa Clara, California, USA)	Accela chromatograph (Thermo Scientific, Hemel Hempstead, UK)
Column	Kinetex® 2.6 µm XB-C18 100 Å (30*2.1 mm) (Phenomenex, California, USA)	Mediterranea Sea 18 column (10*0.21 cm, 2.2 µm) (Teknokroma, Sant Cugat del Vallès, Spain)
Column T	30 ºC	30 ºC
Injection volume	10 µL	10 µL
Flow rate	400 µL min-1	600 µL min-1
Mobile phase	A: 0.05% of HAc; B: methanol	A: 0.05% of HAc; B: methanol
Gradient elution (t, %B)	0, 20 ➔ 5, 40 ➔ 20, 90 ➔ 30, 90 ➔ 32, 20 ➔ 40, 20	0, 20 ➔ 2, 50 ➔ 10, 90 ➔ 13, 90 ➔ 13.10, 20 ➔ 15, 20
MS		
MS System	LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK)	6500 QTRAP® MS/MS System (AB Sciex, Framingham, MA, USA)
Ionization mode	ESI (-)	IonDrive (-)
Resolution	30,000 at *m/z* 400	Unit
Acquisition	FTMS: *m/z* 100 to 1000	Scheduled MRM (Multiple Reaction Monitoring)
Operation parameters*	Source voltage: -3.5 kV, sheath gas: 50 au, auxiliary gas: 20 au, sweep gas: 20 au, capillary temperature: 375ºC	Ionspray voltage: - 4.5 kV, nebulizer gas: 50 au, auxiliary gas: 40 au, curtain gas: 30 au, collision gas: high (au), focusing potential: -200 V, entrance potential: -10V, declustering potential (DeP) and coliision energy (CE) can be revised in Table 3.2.3.

*au: arbitrary units

PHYTOHORMONE SPE (SOLID PHASE EXTRACTION) PROTOCOLS TEST

A total of five methods were tested that consisted in combinations of extraction solutions, extraction methods and SPE columns. On one hand, the three different extraction mediums (ACN: acetonitrile, HAc, acetic acid, MeOH: methanol) used were as following: (i)

ACN:H2O:HAc (99:0.9:0.1, v/v/v), (ii) ACN:H2O:HAc (90:9:1, v/v/v), and (iii) MeOH:H2O:HAc (90:9:1, v/v/v) (Figure 3.2.1). On the other hand, the two different extraction methods were: (i) pass-through and (ii) classic (Figure 3.2.1). For the classical method, we evaporated the sample in an Eppendorf concentrator 5301 (Hamburg, Germany) during 20 min for complete evaporation of the solvent, reconstituted in 200 µL of ACN:H2O (10:90, v/v) with 0.05% of HAc and then loaded on its corresponding column. For the pass-through approach we tested two methods: A and B using OASIS® PRIME HLB columns, whereas for classical approach three methods were tested: C, D and E we used OASIS® PRIME HLB and OASIS® HLB. Each method's flow chart can be observed in Figure 3.2.1.

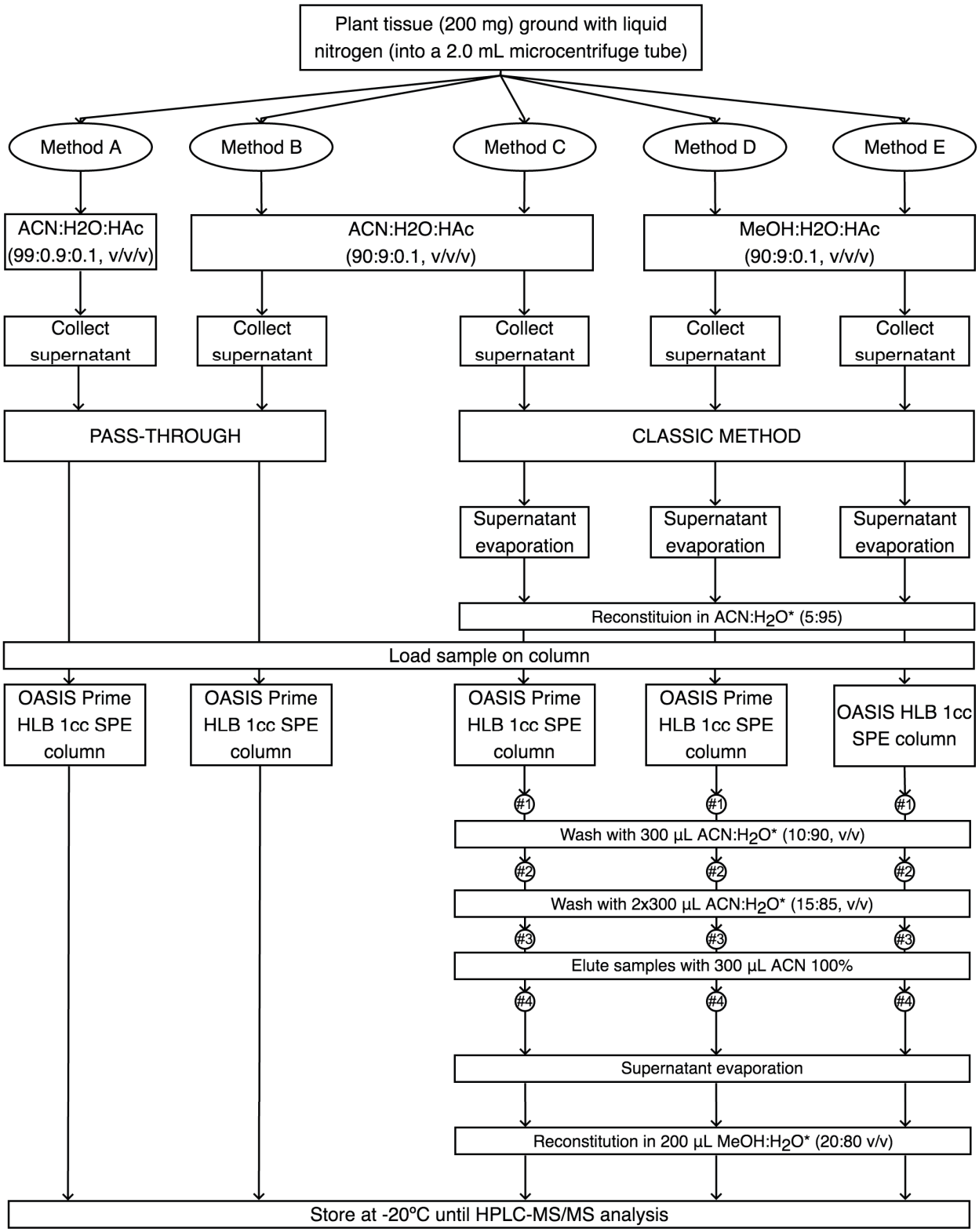

Figure 3.2.1. Flow chart of the phytohormone extraction protocols. In each numbered bullet, the fraction (#1, #2, #3) and eluate (#4) were collected to detect for possible phytohormone leakage. ACN: acetonitrile, HAc: acetic acid, MeOH: methanol, SPE: solid phase extraction, *: supplemented with 0.05% HAc.

For the five methods, two replicates of 200 mg of frozen Montsianell leaves were grounded to a fine powder in N_2(l) using a pistil and a mortar. The ground tissue was mixed in a relation 1:4 with its corresponding extraction medium for each method (see Figure 3.2.1). To each sample, a pool of standard containing five GAs (GA1, GA3, GA4, GA12, GA53) and d2-GA3 at 5 µg·mL-1 of each one was added to the mortar. The resulting solution is transferred to a microcentrifuge tube and centrifuged during 12 min at 14000 g. The resulting supernatant is transferred to a new microcentrifuge tube and the remaining pellet is re-extracted with a ¼ of the extraction medium volume added previously. This pellet is centrifuged during 12 min at 14000 g and the supernatant is transferred to the microcentrifuge tube containing the first supernatant.

For methods A and B, sample was directly loaded in the Oasis Prime HLB 1cc SPE columns using the pass-through approach and the eluate was collected on a fixed insert vial with a screw cap and stored at -20ºC until analysis. For methods C, D and E, samples were evaporated and reconstituted in ACN:H2O (10:90, v/v) with 0.05% of HAc. In method D, the Oasis HLB 1cc SPE column was conditioned with MeOH and water, whereas for methods C and E sample was directly loaded onto the Oasis Prime HLB 1cc SPE columns. For methods C, D and E, all washing fractions (#1, #2 and #3) and the eluate (#4) from the column were collected to determine if phytohormones were lost in any step (Figure 3.2.1).

All samples obtained using the five methods (A, B, C, D and E) were analyzed by the HPLC-1 method explained above (

Table 3.2.2). To determine the effectiveness of each protocol tested, the deuterated gibberellin d2-GA3 at 5 µg·mL–1 each one was run with each sample. Afterwards, peak area of d2-GA3 was compared with a standard directly loaded on on a vial with a screw cap that was injected in the same way as the samples to calculate the recovery percentage of d2-

GA3. After comparing the five protocols, a slightly modified method C was established and tested using ten Montsianell leaf samples. In this new method, named C', the evaporated samples were reconstituted in 600 µL ACN:H2O (5:95, v/v) with 0.05% of HAc and loaded in the Oasis Prime HLB 1cc SPE column. Sample was washed twice with 300 µL ACN:H2O (5:95, v/v) with 0.05% of HAc and eluted three times with 300 µL ACN 100% which was collected in a microcentrifuge tube. This solution was evaporated and the sample reconstituted in 200 µL MeOH:H2O (20:80, v/v) with 0.05% of HAc, and then transferred on a fixed insert vial with a screw cap and stored at -20ºC until analysis.

STATISTICAL ANALYSIS

Height data in the three varieties was verified for normality and homoscedasticity for each week of measurement. It was observed that all data showed normal distribution except for weeks 17, 19 and 21 when using the Shapiro-Wilkinson test and an $\alpha=0.05$. Each week data was heteroscedastic using the Levene's test for homoscedasticity except for data of the first week (W1). For homoscedastic data, a one-way ANOVA test, which is very robust and accept transgressions to normality, followed by a Tukey *post-hoc* test were used. On the other hand, for heteroscedastic data, a Kruskal-Wallis test for non-parametric data followed by a Conover-Iman post-hoc test were used. For all tests, differences were considered to be significant at a probability of 5% ($p < 0.05$). For the five extraction protocols tested (A, B, C, D and E) and method C', a one-way ANOVA followed by a Tukey post-hoc test was performed on the fraction with the highest recovery percentages, after checking that the data was homoscedastic and normal using Levene's and Shapiro Wilkinson tests respectively.

Phytohormone content (reported as $ng \cdot g^{-1}$ of fresh weight) was normalized for life cycle stages S3 and V5, and stages R2 and R3-R4 separately using the formula: $x' = (x_i - x_{min})/(x_{max} - x_{min})$. For representing all the data, heatmaps were used using the normalized data for each

couple of life cycle stages. In addition, for each phytohormone, normality and homoscedasticity were checked for the three varieties in each tissue (COL, 4N, 4N5, 5N, B5L, A5L, pN, pNF, FN, BFL, AFL, 50H) using Shapiro Wilkinson and Levene's tests, respectively. Normal and homoscedastic data was analyzed using a one-way ANOVA followed by a Tukey post-hoc test, normal and heteroscedastic data was analyzed using Kruskal-Wallis followed by a Conover-Iman multiple non-parametric pairwise test, and not normal and heteroscedastic data was analyzed using Welch's ANOVA followed by a Games-Howell post-hoc test.

Table SM.2.1 shows the statistical *p*-values and F values for each of the analysis performed on each tissue and phytohormone.

RESULTS

MEDITERRANEAN JAPONICA RICE VARIETIES CHARACTERIZATION

The mutation present in dwarf-Bomba was verified with Sanger sequencing. Figure 3.2.2 shows the substitution of G by A in the position 1006 of the second exon, which corresponds to the Calrose mutation. The development of the three Mediterranean japonica rice varieties (Montsianell, Bomba and dwarf-Bomba) was registered through height measurement (Figure 3.2.3). It can be seen that Bomba is significantly taller than dwarf-Bomba and Montsianell starting from week three and seven, respectively. The trend of Bomba being taller than the other two varieties is constant throughout the measurement period. Moreover, it is worth noting that between weeks eleven and thirteen the height of dwarf-Bomba increases rapidly, making this variety significantly taller than Montsianell from week 17 although having the GA20ox gene mutated (Figure 3.2.3).

```
GA20ox-2       768  GAGGGTGTACCAGAAGTACTGCGAGGAGATGAAGGAGCTGTCGCTGACGATCATGGAACT
NRCV980385     768  GAGGGTGTACCAGAAGTACTGCGAGGAGATGAAGGAGCTGTCGCTGACGATCATGGAACT
Bomba          768  GAGGGTGTACCAGAAGTACTGCGAGGAGATGAAGGAGCTGTCGCTGACGATCATGGAACT
dwarf-Bomba    768  GAGGGTGTACCAGAAGTACTGCGAGGAGATGAAGGAGCTGTCGCTGACGATCATGGAACT

GA20ox-2       828  CCTGGAGCTGAGCCTGGGCGTGGAGCGAGGCTACTACAGGGAGTTCTTCGCGGACAGCAG
NRCV980385     828  CCTGGAGCTGAGCCTGGGCGTGGAGCGAGGCTACTACAGGGAGTTCTTCGCGGACAGCAG
Bomba          828  CCTGGAGCTGAGCCTGGGCGTGGAGCGAGGCTACTACAGGGAGTTCTTCGCGGACAGCAG
dwarf-Bomba    828  CCTGGAGCTGAGCCTGGGCGTGGAGCGAGGCTACTACAGGGAGTTCTTCGCGGACAGCAG

GA20ox-2       888  CTCAATCATGCGGTGCAACTACTACCCGCCATGCCCGGAGCCGGAGCGGACGCTCGGCAC
NRCV980385     888  CTCAATCATGCGGTGCAACTACTACCCGCCATGCCCGGAGCCGGAGCGGACGCTCGGCAC
Bomba          888  CTCAATCATGCGGTGCAACTACTACCCGCCATGCCCGGAGCCGGAGCGGACGCTCGGCAC
dwarf-Bomba    888  CTCAATCATGCGGTGCAACTACTACCCGCCATGCCCGGAGCCGGAGCGGACGCTCGGCAC

GA20ox-2       948  GGGCCCGCACTGCGACCCCACCGCCCTCACCATCCTCCTCCAGGACGACGTCGGCGGCCT
NRCV980385     948  GGGCCCGCACTGCGACCCCACCGCCCTCACCATCCTCCTCCAGGACGACGTCGGCGGCCT
Bomba          948  GGGCCCGCACTGCGACCCCACCGCCCTCACCATCCTCCTCCAGGACGACGTCGGCGGCCT
dwarf-Bomba    948  GGGCCCGCACTGCGACCCCACCGCCCTCACCATCCTCCTCCAGGACGACGTCGGCGGCTT

GA20ox-2       1008 CGAGGTCCTCGTCGACGGCGAATGGCGCCCCGTCAGCCCCGTCCCCGGCGCCATGGTCAT
NRCV980385     1008 CGAGGTCCTCGTCGACGGCGAATGGCGCCCCGTCAGCCCCGTCCCCGGCGCCATGGTCAT
Bomba          1008 CGAGGTCCTCGTCGACGGCGAATGGCGCCCCGTCAGCCCCGTCCCCGGCGCCATGGTCAT
dwarf-Bomba    1008 CGAGGTCCTCGTCGACGGCGAATGGCGCCCCGTCAGCCCCGTCCCCGGCGCCATGGTCAT

GA20ox-2       1068 CAACATCGGCGACACCTTCATG
NRCV980385     1068 CAACATCGGCGACACCTTCATG
Bomba          1068 CAACATCGGCGACACCTTCATG
dwarf-Bomba    1068 CAACATCGGCGACACCTTCATG
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Figure 3.2.2. Alignment of the second exon of GA20ox-2, with the corresponding base numbers. The Calrose mutation present in dwarf-Bomba is blackened.

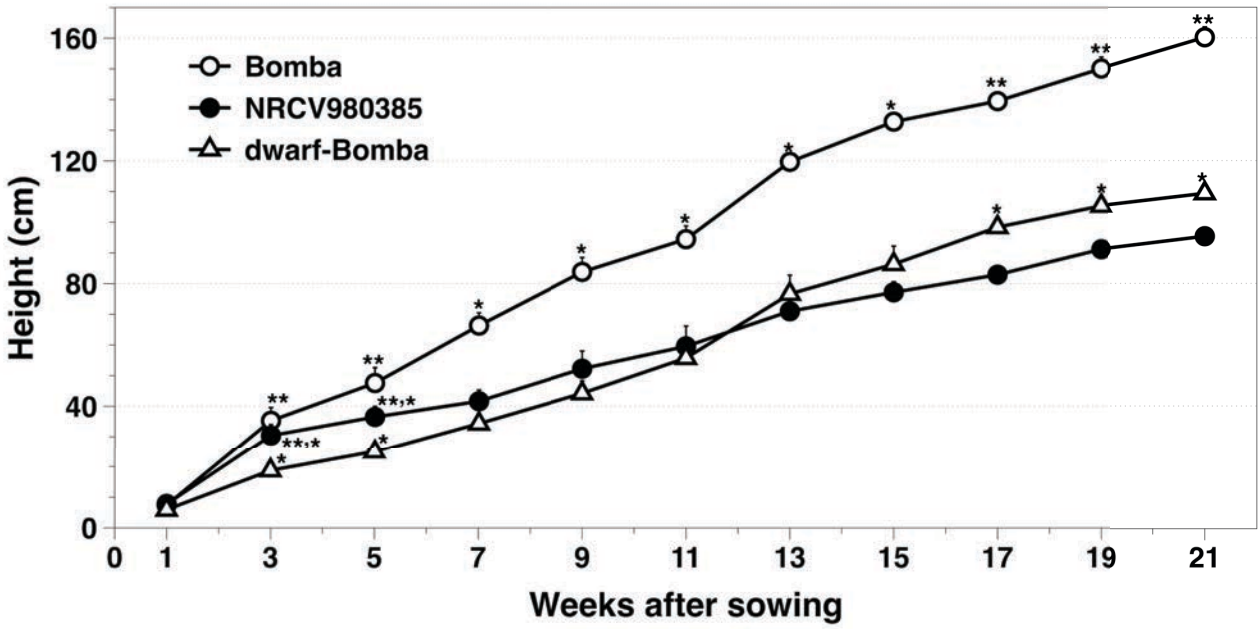


Figure 3.2.3. Monitoring of the rice heights of the three Mediterranean japonica rice varieties used. Values correspond to Asterisks indicate significant differences between rice varieties in each measurement week.

PHYTOHORMONE EXTRACTION PROTOCOL AND HPLC–MS/MS OPTIMIZATION

Five methods for extracting phytohormones (i.e. methods A, B, C, D and E) were tested in this study (Figure 3.2.1). All methods display a good peak of the hormone d2-GA3 in the trace chromatogram (Figure SM.2.1). The washing fractions as well as the eluate of all the methods were analyzed to determine the best solution to clean the columns before elution (Figure 3.2.4). When comparing the highest recovery percentage of each method (including C'), significant differences were observed between them (ANOVA: $F=13.96$; p-value<0.0001). Methods E displayed the significantly lower recovery percentage in any of the elutes, followed closely by method A (Figure 3.2.4). It can be observed for methods C and D that the eluate (#4) is low compared to the fraction #3 elute, because the second wash [ACN:H2O (15:85) with 0.05% of HAc] dragged the majority of d2-GA3 out of the column. In fact, the recovery percentage for those two methods is very good in the fraction #3 (67.2±12.0 and 41.0±6.4, mean ± SE respectively). On the other hand, method B although faster than C and D has lower recovery percentage if all fractions (#1, #2, and #3) and eluate (#4) of method C and D are respectively summed together. The low recovery percentages in fractions is probably due to the fact that sample contains an excessive percentage of water. For method C, the recovery percentage is 85.1±7.1 (mean ± SE) for the sum of all fractions and eluate. Therefore, method C was chosen but some adjustments were performed in order to not lose phytohormones during the washes, and this method was established as C'. Washes in method C' are performed with a ACN:H2O (5:95) solution with 0.05% of HAc, which corresponds to the fraction #1 that displayed a phytohormone recovery of 0.4%, and the elution was performed with 100% ACN. The recovery percentage was 76.4±5.0 (mean ± SE) corroborating the validity of this new method C' (Figure 3.2.4).

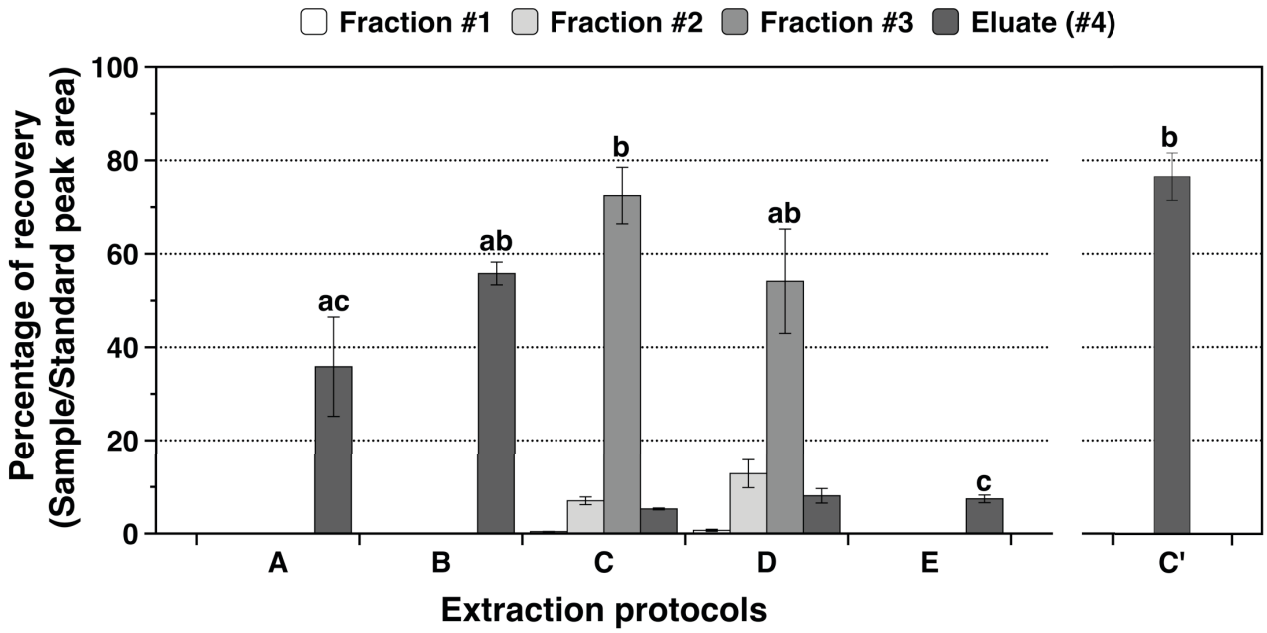


Figure 3.2.4. Recovery percentages for all the fractions (#1, #2 and #3) and eluates (#4) of the five extractions protocols (A, B, C, D and E; for details see Figure 3.2.1 in page 160) and new method (C') on NRVC980385 leaves. Columns correspond to mean ± SE of 4 replicates for methods A to E whereas mean ± SE of 6 replicates for method C', letters above bars indicate significant differences between varieties for each tissue analyzed (Tukey tests at $p<0.05$).

Furthermore, method HPLC-1 was further optimized into method HPLC-2 in which a full phytohormone can be carried out in 20 min of chromatography instead of in 40 min as observed in Figure SM.2.2. It can be observed that the retention time (RT) of d2-GA8 is similar for both methods, and in contrast the RT of d2-GA12 is almost half the time for HPLC-2 compared to HPLC-1 (Figure SM.2.2). Moreover, detecting the gibberellins in the QTRAP6500 (QqQ; HPLC-2) instead of the LTQ Orbitrap Velos (HRMS; HPLC-1) allows to have higher sensibility. In fact, the signal to noise ratio (S/N) was 5 and 1.5 times higher in HPLC-2 than in HPLC-1 for d2-GA8 and d2-GA12, respectively (Figure SM.2.2).

The declustering potential and collision energy parameters of the MS and MS/MS were optimized to generate the highest signal intensities for each phytohormone (Table 3.2.3). A

scheduled MRM method was established with an MRM detection window of 60 s and a target scan time of 0.7 s. In Figure SM.2.3, trace chromatograms of the 13 detected gibberellins in a sample (a replicate of dwarf-Bomba FN) can be observed showing a clear peak for each phytohormone. Quantification of gibberellins was done by the isotopic dilution method. Calibration curve was constructed with standard solutions between 0.2-200 ng·mL-1 diluted in MeOH:H2O (20:80 v/v) with 0.05% of HAc. The linear range for each hormone is presented in table S1 and for the majority it displays a broad linear range that goes between 0.2-200 ng·mL-1. Linear regression was adjusted (1/x or 1/x2) in order to have accuracies between 80-120% for all the standards. Moreover, limit of detection (LOD) and limit of quantification (LOQ) was calculated for each phytohormone (gibberellins, ABA, JA and IAA) as the concentration of phytohormone in rice sample that gives a S/N = 3 for LOD and S/N = 10 for LOQ (Table 3.2.3). LODs are very low for the majority of phytohormones, ranging from 0.1 to 1.6 pg·g-1 (f.w.) Good reproducibility was observed, as the relative standard deviations (RSDs) for a standard pool varied between 0.3 and 1.6%. High RSD was found for IAA and GA44 (20.0 and 9.3% respectively) when standards of 0.4 ppb were analyzed, as this concentration is near the LOD values for those phytohormones.

Table 3.2.3. MRM transitions, retention time (RT), declustering potential (DP), collision energy (CE), limit of detection (LOD) and limit of quantification (LOQ) for the phytohormones analyzed in method HPLC-2 (6500QTRAP).

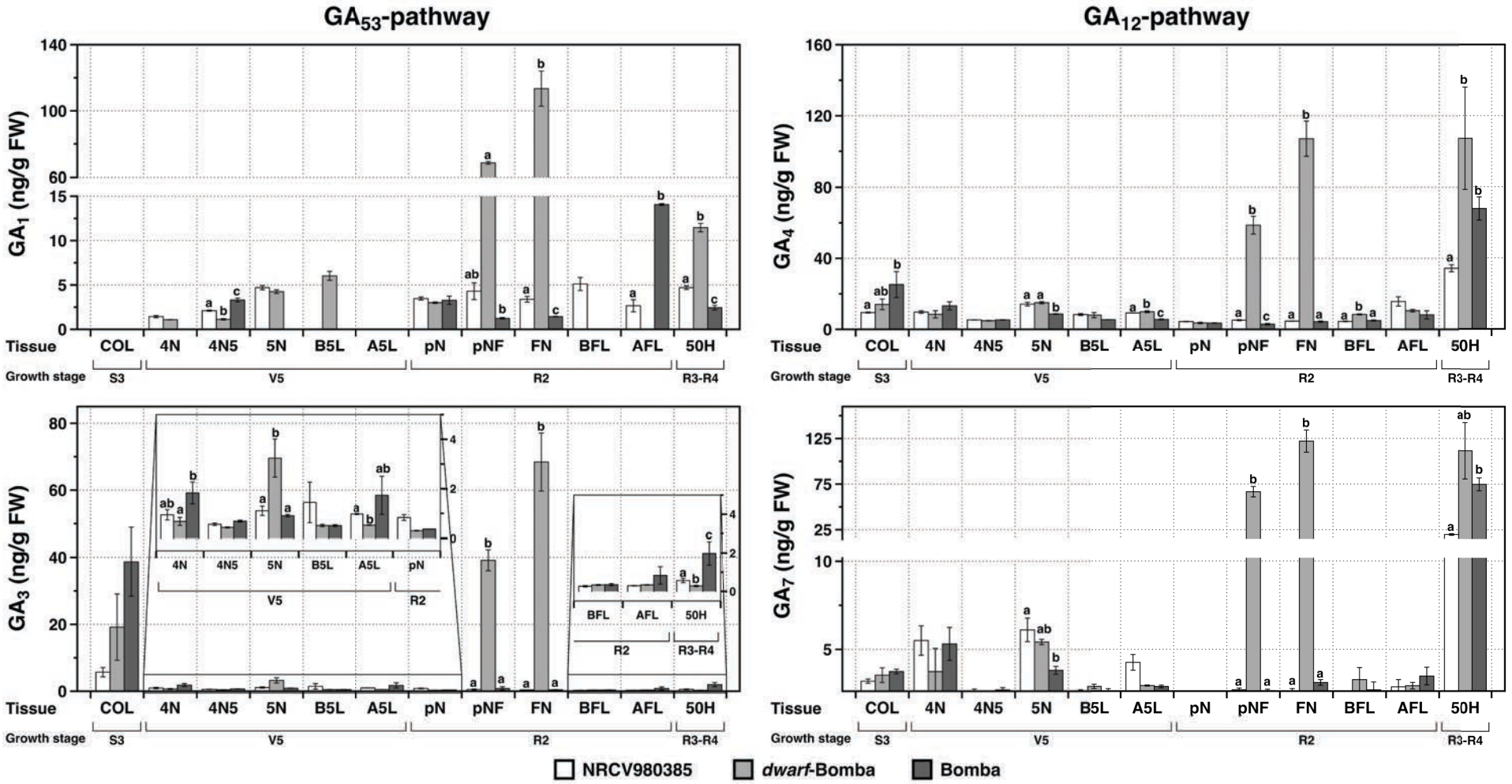
Compound	**MRM transition**	**RT** (min)	**DeP** (V)	**CE** (V)	**LOD**[a] ($pg \cdot g^{-1}$)	**LOQ**[a] ($pg \cdot g^{-1}$)	**Linear range** ($ng \cdot mL^{-1}$)	**RSD** (%, 0.4 $ng \cdot mL^{-1}$)[b]	**RSD** (%, 40 $ng \cdot mL^{-1}$)[b]
GA_1	347.0 / 259.0	2.6	-65	-26	0.4	1.2	0.2-200	1.9	0.9
d_2-GA_1	349.0 / 261.2	2.6	-140	-26	-	-	-	-	-
GA_3	345.0 / 239.1	2.5	-95	-20	0.2	0.5	0.2-175	1.3	0.5
d_2-GA_3	347.0 / 241.1	2.5	-105	-20	-	-	-	-	-
GA_4	331.0 / 243.2	5.6	-105	-26	0.7	2.2	0.2-200	5.4	0.7
d_2-GA_4	333.0 / 259.0	5.6	-120	-32	-	-	-	-	-
GA_7	329.0 / 223.1	5.3	-80	-26	0.3	0.8	0.2-175	1	0.4
d_2-GA_7	331.0 / 225.1	5.3	-115	-24	-	-	-	-	-
GA_8	363.0 / 275.2	1.7	-105	-24	0.7	2.3	0.2-90	1.5	0.4
d_2-GA_8	364.9 / 277.0	1.7	-125	-24	-	-	-	-	-
GA_{12}	331.0 / 313.1	8.4	-20	-38	0.4	1.2	0.2-175	2.5	0.3
d_2-GA_{12}	333.0 / 315.2	8.4	-145	-36	-	-	-	-	-
GA_{15}	329.0 / 257.0	6.7	-20	-34	0.1	0.3	0.2-90	0.5	0.3
d_2-GA_{15}	331.0 / 259.1	6.7	-35	-34	-	-	-	-	-
GA_{19}	361.1 / 273.0	4.6	-60	-34	0.1	0.4	0.2-200	2.5	0.7
d_2-GA_{19}	362.9 / 275.0	4.6	-115	-36	-	-	-	-	-
GA_{20}	331.1 / 225.2	3.9	-105	-34	0.5	1.8	0.2-200	2.4	0.7
d_2-GA_{20}	332.9 / 227.1	3.9	-130	-34	-	-	-	-	-
GA_{29}	347.1 / 259.1	1.9	-120	-24	0.4	1.2	0.2-90	0.9	0.9
d_2-GA_{29}	348.9 / 261.2	1.9	-135	-22	-	-	-	-	-
GA_{44}	345.1 / 272.9	4.3	-130	-34	0.2	0.6	0.2-175	9.3	0.9
d_2-GA_{44}	347.0 / 275.0	4.3	-120	-36	-	-	-	-	-
GA_{51}	331.1 / 243.1	4.7	-105	-22	0.5	1.6	0.2-200	2.1	0.4
d_2-GA_{51}	332.9 / 245.0	4.7	-105	-24	-	-	-	-	-
GA_{53}	347.1 / 189.0	6.0	-125	-46	0.2	0.5	0.2-200	7.1	0.3
d_2-GA_{53}	349.0 / 188.9	6.0	-120	-48	-	-	-	-	-
ABA	263.1 / 153.1	3.4	-55	-16	0.1	0.3	0.2-200	1.1	0.8
d_6-ABA	269.0 / 159.0	3.4	-95	-16	-	-	-	-	-
JA	209.1 / 58.9	4.1	-70	-16	0.1	0.4	0.2-200	1.2	0.9
d_6-JA	215.0 / 62.2	4.1	-60	-18	-	-	-	-	-
IAA	174.0 / 129.9	2.7	-95	-16	1.6	5.2	0.2-200	20.0	1.6
d_5-IAA	178.9 / 135.0	2.7	-35	-16	-	-	-	-	-

[a]Limits of detection (LOD) and quantification (LOQ) were determined in rice samples and are expressed as $pg \cdot g^{-1}$ of fresh weight.
[b]Values are average of ten replicates.

GIBBERELLLIN PROFILING OF THREE MEDITERRANEAN JAPONICA RICE VARIETIES

Regarding the bioactive gibberellins, it is worth noting that their contents in the different tissues is different being GA4 in average the one displaying the highest values (Figure 3.2.5) for the three varieties. In addition, GA1 is not detected in the coleoptile (COL) whereas the other three bioactive gibberellins are present, and have a similar pattern in which Bomba is the one displaying the highest values followed by dwarf-bomba and then Montsianell. Moreover, GA1 in contrast with GA3, GA4 and GA7 was not detected in several analyzed tissues. Strikingly, the content of these 4 gibberellins is significantly highly increased in dwarf-bomba for the internode between flag leaf and previous leaf (pNF) and the flag leaf node (FN) compared to the other two varieties (Figure 3.2.5 and Table SM.2.1). Similarly, the content of the bioactive gibberellins in the panicle and florets (50H) is higher in dwarf-Bomba compared to Bomba (although concentrations are lower), and significantly higher for both when compared to Montsianell (Figure 3.2.5 and Table SM.2.1).

Figure 3.2.5 (page 171). Content of the bioactive gibberellins (GA_1, GA_3, GA_4 and GA_7) in the different tissues (see Table 3.2.1, page 155) for the three analyzed varieties: NRVC980385, *dwarf*-bomba and Bomba. COL: coleoptile; 4N: 4th node; 4N5: internode between 4th and 5th node; 5N: 5th node; B5L: basal part of the 5th leaf; A5L: apical part of the 5th leaf; pN: node previous to the flag leaf node; pNF: internode between flag leaf and previous leaf; FN: flag leaf node; BFL: basal part of the flag leaf; AFL: apical part of the flag leaf. Columns correspond to mean ± SE of 3 replicates, and letters above bars indicate significant differences between varieties for each tissue analyzed.


GA53-pathway
GA12-pathway
GA1 (ng/g FW)
GA4 (ng/g FW)
GA3 (ng/g FW)
GA7 (ng/g FW)
Tissue
COL
4N
4N5
5N
B5L
A5L
pN
pNF
FN
BFL
AFL
50H
Growth stage
S3
V5
R2
R3-R4
NRCV980385
dwarf-Bomba
Bomba

It seems that phytohormones GA19 is crucial to the normal development of rice plants, because their concentration is very high in all varieties for Stages S3 and V5 (Figure 3.2.6 and Figure SM.2.4). More in detail, the first phytohormone profiling was performed for the life cycle stage S3 (emergence of prophyll from coleoptile) where it is noteworthy that GA29 and GA1 were not detected in neither of the three rice varieties (Figure 3.2.6 and Figure SM.2.5). In this same pathway, only GA3 showed high values which were higher in Bomba. Concomitantly, GA4 of the GA12-pathway also showed high values for Bomba as well as other phytohormones which contrasts to the tendency observed for the gibberellins of the GA53-pathway. When focusing on the five-leaves plants (Stage V5), it was observed that GA1 was not detected on the coleoptile, and barely detected in other tissues. Similarly, GA29 was only detected for Montsianell in the 4th node (4N; Figure 3.2.6 and Figure SM.2.5). On one hand, the content of GA44 and GA20 for the majority of tissues analyzed in this growth stage for the three varieties were increased when compared to the coleoptile (COL). On the other hand, content levels of GA12, GA15 and GA51 were not very different to those obtained in COL, being GA12 even not detected in several tissues (Figure 3.2.6 and Figure SM.2.6). Finally, it is worth noting that GA15 contents are very similar between all varieties and tissues. Regarding growth stage R2 and R3-R4 growth stages, GA19 is also crucial as its contents is the highest of all the analyzed (Figure 3.2.7 and Figure SM.2.4). As in stages S3 and V5, GA29 was almost undetected in all tissues and GA1 was not detected in three tissues (Figure 3.2.5, Figure 3.2.7 and Figure SM.2.5). Interestingly, all the GA53-pathway gibberellins contents are significantly increased in the internode between flag leaf and previous leaf (pNF) and flag leaf node (FN) for dwarf-Bomba (Figure SM.2.4 and Table SM.2.1). This trend also appears when looking at R3-R4 for the same variety, where Panicle and florets (50H) show a higher content of the GAs (Figure 3.2.7). In addition, as for the GA53-pathway, gibberellins

contents for pNF, FN and 50 are also increased in dwarf-Bomba when analyzing the GA12-pathway. In a similar way as in S3 and V5, GA15 contents were all similar between tissues and varieties for R2 and R3-34 (Figure 3.2.7 and Figure SM.2.6).

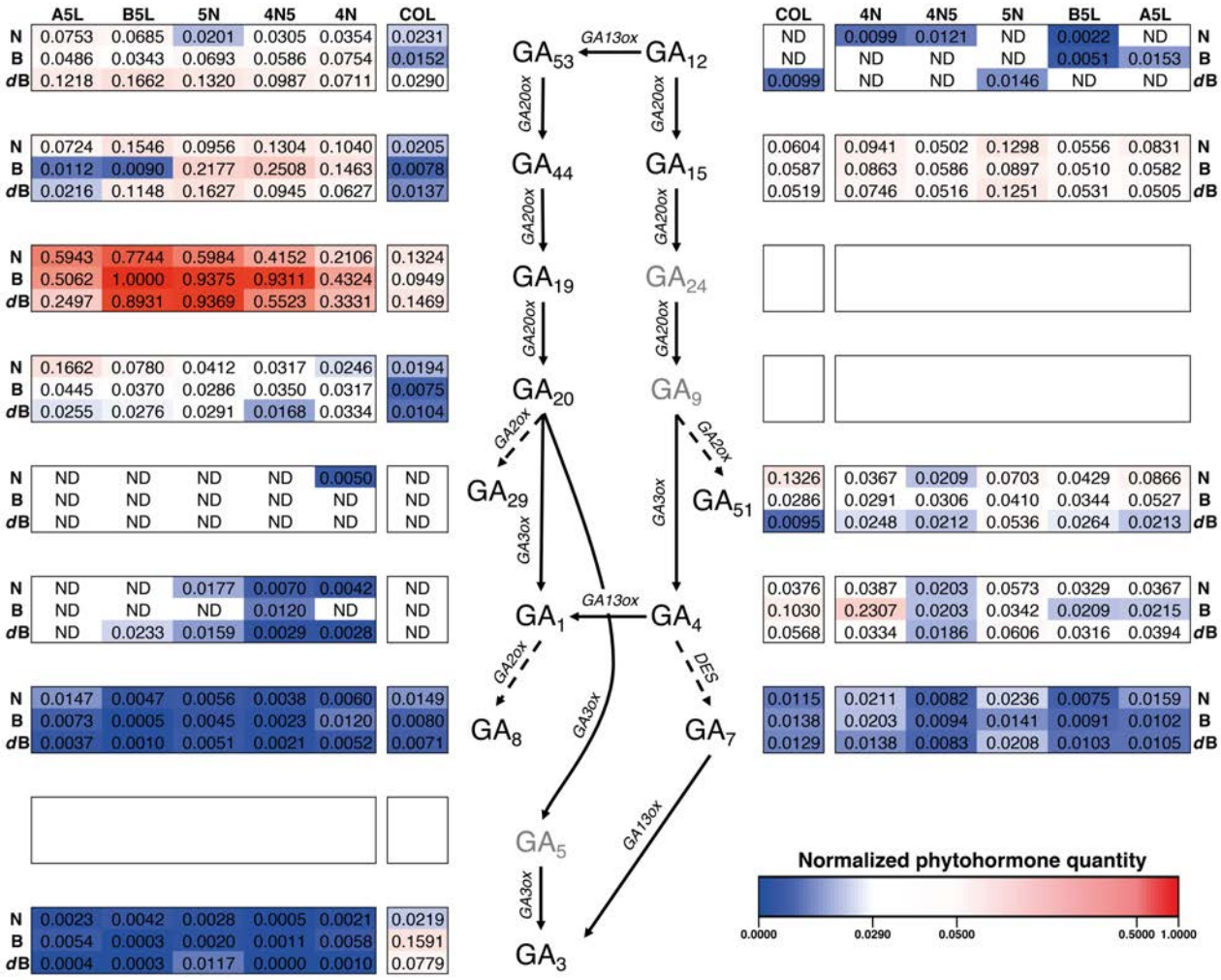


Figure 3.2.6. Heatmap of the hormone profiling of the three Mediterranean japonica rice varieties in the stages S3 and V5 (see Table 3.2.1, page 155). Normalized phytohormone quantity scale is shown at the lower right of the image and corresponds to the mean of 3 replicates. Three phytohormones (GA_5, GA_9 and GA_{24}, in grey) were not analyzed, ND: not determined. M: NRVC980385; B: Bomba; *d*B: *dwarf*-bomba; COL: coleoptile; 4N: 4th node; 4N5: internode between 4th and 5th node; 5N: 5th node; B5L: basal part of the 5th leaf; A5L: apical part of the 5th leaf.

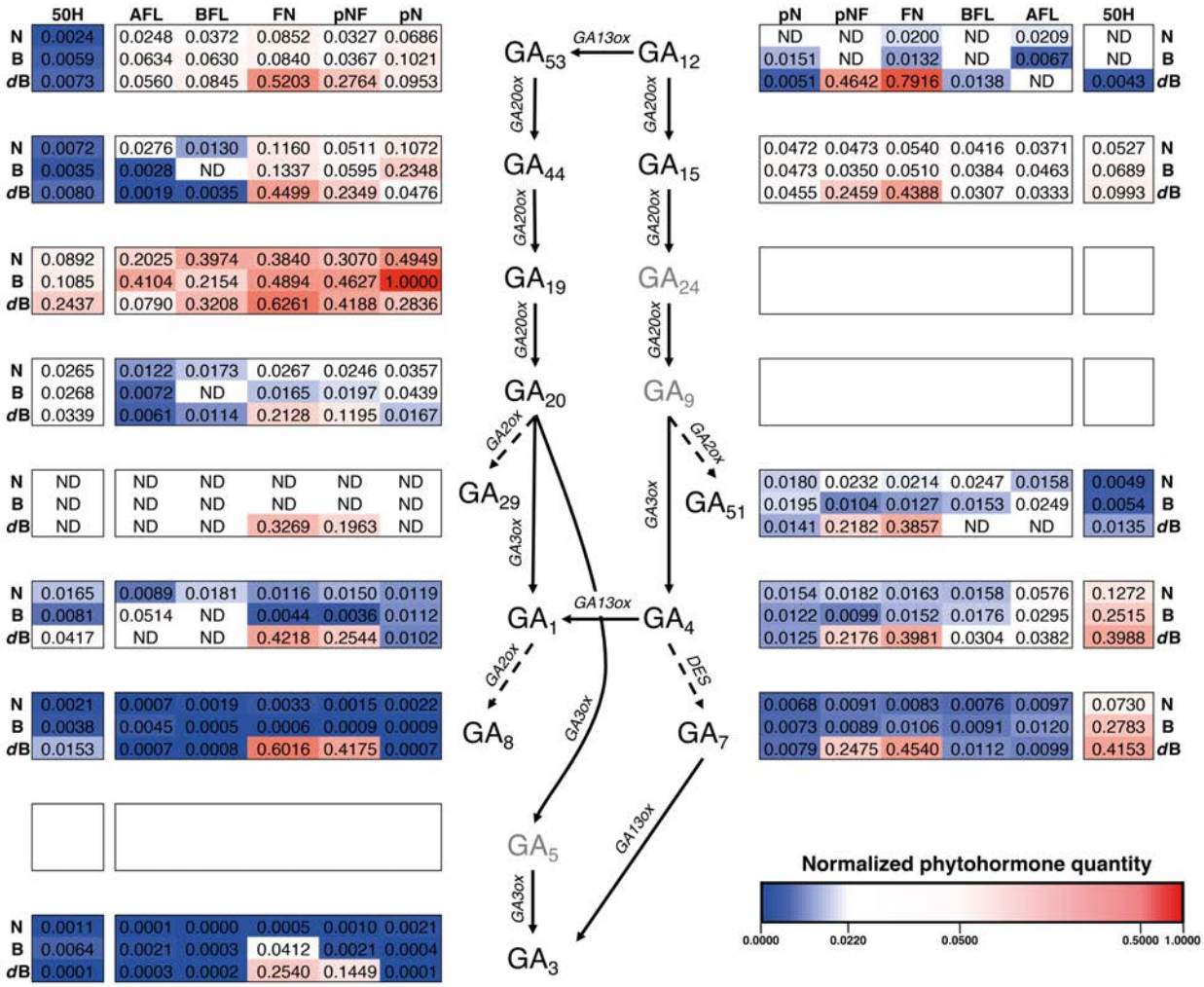


Figure 3.2.7. Heatmap of the hormone profiling of the three Mediterranean japonica rice varieties in the stages R2 and R3-R4 (see Table 3.2.1, page 155). Normalized phytohormone quantity scale is shown at the lower right of the image and corresponds to the mean of 3 replicates. Three phytohormones (GA_5, GA_9 and GA_{24}, in grey) were not analyzed, ND: not determined phytohormone. M: NRVC980385; B: Bomba; *d*B: *dwarf*-bomba; pN: node previous to the flag leaf node; pNF: internode between flag leaf and previous leaf; FN: flag leaf node; BFL: basal part of the flag leaf; AFL: apical part of the flag leaf.

ABA, JA AND IAA PROFILING OF THREE MEDITERRANEAN JAPONICA RICE VARIETIES

In addition to the 13 gibberellins detected and quantified in several tissues and growth stages of rice tissues, our method allows to analyze in addition the phytohormones abscisic acid (ABA) and jasmonic acid (JA) in all tissues and varieties; indole-3-acetic acid (IAA; Figure SM.2.7). ABA concentrations were in general very similar among the three varieties, except for the coleoptile (COL) where dwarf-Bomba has the higher values and both parts of the leaf in the V5 growth stage where Bomba has the higher values. JA levels were very similar in all tissues and varieties displaying significant differences in only two tissues (pNF in R2 and 50H in R3-R4; Table SM.2.1). In addition, it is also noteworthy that IAA in the flag leaf (stage R2) was absent in almost all the varieties. Finally, in the heading stage (50H) no IAA was detected in Bomba whereas Montsianaell and dwarf-Bomba displayed very high values (Figure SM.2.7).

DISCUSSION

It is well established that studying phytohormones in plants is crucial for understanding several developmental and physiological processes, including tolerance to different stresses. We have established a protocol for analyzing and quantifying more than 15 phytohormones, including a total of 13 different gibberellins, in different rice tissues with detection at trace levels. To our knowledge, this is the first study that has reported such a high number of analyzed gibberellins at the same time in rice (*Oryza sativa* ssp. japonica). We have also established that bioactive gibberellins content in rice tissues not only depends on the presence of a wild-type GA20 oxidase 2 gene (*GA20ox-2*), but it must also depend on whether or not other bioactive GAs are present in the variety that contains the Calrose mutation. Interestingly, we found that (i) dwarf plants do not have a drastically lower gibberellin content in comparison to their non-dwarf counterpart and (ii) their growth is primarily halted only in the early stages as its growth is faster in the later phenological stages. In this work, a reliable and broad phytohormone extraction protocol for rice was developed. Acetonitrile was a better organic solvent than methanol as the recovery percentages were highest in methods A, B and C. This is corroborated by other studies (Cui et al., 2015; Flores et al., 2011; Urbanová et al., 2013). Urbanová et al (2013) also reported that acetonitrile extract fewer interfering pigments than methanol. It is crucial to achieve high recovery percentages, because a loss of phytohormones during extraction could lead to wrong detection and, therefore, to results misinterpretation (Chen et al., 2012; Cui et al., 2015). The best acetonitrile method was C, and it was further improved into method C', and tested in leaf samples which yielded recovery percentages similar to other broad profiling protocols (Chen et al., 2012; Urbanová et al., 2013). Moreover, our method is simpler than others, because only one SPE columns is used, whereas normally others authors employ two or even three

columns for sample purification (Chen et al., 2012; Kojima et al., 2009; Urbanová et al., 2013). In addition, the relative standard deviation we found in our samples is very low when compared to the study made by Chen et al (2012), suggesting that our method is very precise. The only exceptions were IAA and GA_{44} when analyzed at a 0.4 ppb concentration, but this is due to the fact that this concentration is within the LOD values for those phytohormones. Finally, our method shows good detectability (e.g. 0.1 $pg \cdot g^{-1}$ (f.w.) for 4 phytohormones), good reproducibility (no more than 1.6 at 40 ppb for stanndards) and good separation of all the studied gibberellins as it does not have interferences between isobaric species (e.g. between GA_4 and GA_{51} or d_2-GA_7 and GA_{20}).

In this work a broad phytohormone profiling was performed, which allows to analyze changes during growth development. In fact, changes between different developmental stages and even tissues within a developmental stage were observed in the three varieties. The first notorious finding was that independently of the variety, GA_1 was absent in the coleoptile which disagrees with an article published by L. Liu et al (2018). Nevertheless, in that study GA content was measured after four days of germination whereas in our study measurement was done after seven days, therefore this particular GA may not be needed for coleoptile elongation. In this same tissue, it was evident that GA_4 is the most important bioactive gibberellin followed by GA_3 and GA_7, due to their concentration differences. In agreement with this, it has been shown that low levels of GA_4 in *Arabidopsis thaliana* are related to no germination of seeds, proving that GA_4 is a crucial bioactive gibberellin in the coleoptile (Yamaguchi, 2008). Moreover, Kaneko et al (2003) have shown that the embryo has differential gibberellin genes expression patterns which suggests that the genes for GA_3 and GA_4 could be under- and over-expressed respectively. In fact, the low availability of GA_1 and GA_3, final active products of the GA_{53} pathway, could be explained by the high quantities

detected in their precursor (GA_{19}). Regarding gibberellins production in different tissues, as also reported by Kojima et al (2009), we evidenced higher GA levels in the nodes compared to the internodes in both V5 and R2 growth stages. These findings are supported by Kaneko et al (2003), that showed higher activity of *OsGA3ox2* and *OsGA20ox2* in the node of elongating stems. In the later phenological stages, such as heading, GA contents have been shown to be high which is also in correlation with our findings (Yang et al., 2000). GA_7 ang GA_3 levels throughout the development of the three varieties had low concentrations, suggesting that GA_4 is the key active gibberellin in this species. This is in concordance with Binenbaum et al (2018) that claim that GA_7 and GA_3 are biologically active but present at minor levels. The exception was *dwarf*-Bomba which showed high levels of GA_1, GA_3, GA_4 and GA_7 at pNF and FN, which could explain its faster growth in later phenological stages.

Regarding the other three phytohormones also studied in this work, it is worth noting that both JA and ABA are present throughout the plant development. The high levels observed in Bomba for B5L and A5L compared to the other two varieties are expected since this phytohormone has a crucial role in stomata movements (López-Carbonell et al., 2009). In contrast to JA and ABA, IAA is almost absolutely absent in Bomba at the R2 and R3-R4 stages, but has elevated levels in the heading stage (R3-R4) for NRVC980385 and *dwarf*-Bomba. Since IAA has been shown to be increased during heading (Yang et al., 2000), it is surprising that Bomba levels are so low compared to those of the three varieties have high GA levels. These results for JA, ABA and IAA are not conclusive but give insights in phytohormone mechanisms in different tissues, therefore more studies are needed to fully understand hormone patterns during life cycles.

Sanger sequencing results confirmed that only *dwarf*-Bomba had a mutation in the *GA20ox-2* gene that corresponded to a deletion between exon 1 and exon 2. This deletion has been

reported by other authors and is one of the four *GA20ox-2* mutations that lead to the semi-dwarf varieties (Ashikari et al., 2002; Hedden, 2003; Monna et al., 2002; Sasaki et al., 2002; Wolfgang Spielmeyer et al., 2002). The deletion clearly explains the semi-dwarfism traits observed by other authors the 21 weeks of plant development: Bomba (traditional variety) was significantly higher than its mutated counterpart, *dwarf*-Bomba. Strangely, NRVC980385 was also significantly shorter than Bomba throughout the monitoring. The height values observed for NRVC980385 are in accordance with those of the literature [e.g. NRVC980385 in Serrat et al (2014)], and those reported for Bomba are not surprising since traditional varieties are known to have higher heights (Franquet Bernis and Borràs Pàmies, 2004; Okuno et al., 2014)]. Therefore, heights differences observed are not exclusively caused by the well-studied *sd-1* mutation. Interestingly, NRVC980385 during the first half of rice development was higher than *dwarf*-Bomba even though its *GA20ox-2* gene is not mutated. It is already well known that other genes involved in GAs biosynthesis and signaling pathways as well as other phytohormones are also contributing to height in rice (F. Liu et al., 2018). JA and IAA levels were very similar in tissues of the V5 growth stage between NRVC980385 and *dwarf*-Bomba, so their difference in height might respond to changes in other GA genes or phytohormones such as brassinosteroids (BRs) or strigolactones (SLs; F. Liu et al., 2018). Surprisingly, during week 11 and 13, *dwarf*-Bomba height surpasses that of NRVC980385 which is in correlation with the elevated contents of bioactive gibberellins of both the GA_{53}-(GA_1 and GA_3) and the GA_{12}-pathway (GA_4 and GA_7) reported in the internode between flag leaf and previous leaf (pNF) and the flag leaf node (FN). This increment in GAs levels are most likely caused by either other one or all of the other three *GA20ox* genes [i.e. *GA20ox-1*, *GA20ox-3* and *GA20ox-4*, Sakamoto (2004)]. As gibberellins are crucial for internode elongation, this is the phenotypical characteristic that should explain the height increment in

dwarf-Bomba during flag leaf collar formation stage (Ayano et al., 2014; Counce et al., 2000; Wang et al., 2017).

In conclusion, we have shown that GA_1 is not a crucial gibberellin in the rice coleoptiles neither in more advanced phenological stages, because its levels are in general low. Moreover, GA_{19} seems to have a crucial role in gibberellin availability in rice as its levels were much higher than all the other gibberellins in all tissues. In addition, it has been demonstrated that the *GA20ox-2* mutation is not the only factor affecting height in rice, as a mutated variety had an increased growth during flag leaf collar formation stage (R2). It was corroborated that GA_3 and GA_7 are present at low levels in the majority of rice tissues. Finally, all these findings were possible due to the establishment, for the first time, of a simple and broad phytohormone extraction and detection protocol that allows to identify 13 gibberellins and ABA, JA and IAA in several tissues at different phenological stages.

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REFERENCES - CHAPTER 2

Ashikari, M., Sasaki, A., Ueguchi-Tanaka, M., Itoh, H., Nishimura, A., Datta, S., et al. (2002) Loss-of-function of a rice gibberellin biosynthetic gene, GA20 oxidase (GA20ox-2), led to the rice 'Green Revolution'. Breed. Sci., 52, 143–150.

Ayano, M., Kani, T., Kojima, M., Sakakibara, H., Kitaoka, T., Kuroha, T., et al. (2014) Gibberellin biosynthesis and signal transduction is essential for internode elongation in deepwater rice. Plant, Cell Environ., 37, 2313–2324.

Binenbaum, J., Weinstain, R., and Shani, E. (2018) Gibberellin localization and transport in plants. Trends Plant Sci., 23, 410–421.

Chen, M.L., Fu, X.M., Liu, J.Q., Ye, T.T., Hou, S.Y., Huang, Y.Q., et al. (2012) Highly sensitive and quantitative profiling of acidic phytohormones using derivatization approach coupled with nano-LC-ESI-Q-TOF-MS analysis. J. Chromatogr. B Anal. Technol. Biomed. Life Sci., 905, 67–74.

Counce, P.A., Keisling, T.C., and Mitchell, A.J. (2000) A uniform, objective, and adaptive system for expressing rice development. Crop Sci., 40, 436–443.

Cui, K., Lin, Y., Zhou, X., Li, S., Liu, H., Zeng, F., et al. (2015) Comparison of sample pretreatment methods for the determination of multiple phytohormones in plant samples by liquid chromatography-electrospray ionization-tandem mass spectrometry. Microchem. J., 121, 25–31.

Das, P., Nutan, K.K., Singla-Pareek, S.L., and Pareek, A. (2015) Understanding salinity responses and adopting 'omics-based' approaches to generate salinity tolerant cultivars of rice. Front. Plant Sci., 6, 712.

Davies, P.J. (2010) Plant hormones: biosynthesis, signal transduction, action!, 3rd edn, (Davies,P.J., ed). London, New York: Springer.

Doyle and Doyle, J. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull., 19.

Flores, M.I.A., Romero-González, R., Frenich, A.G., and Vidal, J.L.M. (2011) QuEChERS-based extraction procedure for multifamily analysis of phytohormones in vegetables by UHPLC-MS/MS. J. Sep. Sci., 34, 1517–1524.

Franquet Bernis, J.M. and Borràs Pàmies, C. (2004) Variedades y mejora del arroz (Oryza sativa L.), 1st editio. Tortosa, Spain.

Hedden, P. (2001) Gibberellin metabolism and its regulation. J. Plant Growth Regul., 20, 317–318.

Hedden, P. (2003) The genes of the Green Revolution. Trends Genet, 19, 5–9.

Hedden, P. and Phillips, A.L. (2000) Gibberellin metabolism: New insights revealed by the genes. Trends Plant Sci., 5, 523–530.

Hedden, P. and Thomas, S.G. (2012) Gibberellin biosynthesis and its regulation. Biochem. J., 444, 11–25.

Iqbal, N., Masood, A., and Khan, N.A. (2012) Phytohormones in sailinity tolerance: ethylene and gibberellins cross talk. In: Phytohormones and abiotic stress tolerance in plants (Khan,N.A., Nazar,R., Iqbal,N., and Anjum,N.A., eds) , pp. 77–98. London New York: Springer.

Kaneko, M., Itoh, H., Inukai, Y., Sakamoto, T., Ueguchi-Tanaka, M., Ashikari, M., and Matsuoka, M. (2003) Where do gibberellin biosynthesis and gibberellin signaling occur in rice plants? Plant J., 35, 104–115.

Kojima, M., Kamada-Nobusada, T., Komatsu, H., Takei, K., Kuroha, T., Mizutani, M., et al. (2009) Highly sensitive and high-throughput analysis of plant hormones using ms-probe modification

and liquid chromatographytandem mass spectrometry: An application for hormone profiling in Oryza sativa. Plant Cell Physiol., 50, 1201–1214.

Kudo, T., Akiyama, K., Kojima, M., Makita, N., Sakurai, T., and Sakakibara, H. (2013) UniVIO: A multiple omics database with hormonome and transcriptome data from rice. Plant Cell Physiol., 54, 1–12.

Liu, F., Wang, P., Zhang, X., Li, X., Yan, X., Fu, D., and Wu, G. (2018) The genetic and molecular basis of crop height based on a rice model. Planta, 247, 1–26.

Liu, L., Xia, W., Li, H., Zeng, H., Wei, B., Han, S., and Yin, C. (2018) Salinity Inhibits Rice Seed Germination by Reducing α-Amylase Activity via Decreased Bioactive Gibberellin Content. Front. Plant Sci., 9, 1–9.

López-Carbonell, M., Gabasa, M., and Jáuregui, O. (2009) Enhanced determination of abscisic acid (ABA) and abscisic acid glucose ester (ABA-GE) in Cistus albidus plants by liquid chromatography-mass spectrometry in tandem mode. Plant Physiol. Biochem., 47, 256–261.

Macías, J.M., Pournavab, R.F., Reyes-Valdés, M.H., and Benavides-Mendoza, A. (2014) Development of a rapid and efficient liquid chromatography method for determination of gibberellin A4 in plant tissue, with solid phase extraction for purification and quantification. Am. J. Plant Sci., 2014, 573–583.

Monna, L., Kitazawa, N., Yoshino, R., Suzuki, J., Masuda, H., Maehara, Y., et al. (2002) Positional cloning of rice semidwarfing gene, sd-1: rice 'Green Revolution Gene' encodes a mutant enzyme involved in gibberellin synthesis. DNA Res., 9, 11–17.

Okuno, A., Hirano, K., Asano, K., Takase, W., Masuda, R., Morinaka, Y., et al. (2014) New approach to increasing rice lodging resistance and biomass yield through the use of high gibberellin producing varieties. PLoS One, 9.

Olszewski, N., Sun, T.-P., and Gubler, F. (2002) Gibberellin signaling: biosynthesis, catabolism, and response pathways. Plant Cell, 14 Suppl, S61–S80.

Peleg, Z. and Blumwald, E. (2011) Hormone balance and abiotic stress tolerance in crop plants. Curr. Opin. Plant Biol., 14, 290–295.

Reddy, I.N.B.L., Kim, B.K., Yoon, I.S., Kim, K.H., and Kwon, T.R. (2017) Salt tolerance in rice: focus on mechanisms and approaches. Rice Sci., 24, 123–144.

Sahoo, R.K., Ansari, M.W., Tuteja, R., and Tuteja, N. (2014) OsSUV3 transgenic rice maintains higher endogenous levels of plant hormones that mitigates adverse effects of salinity and sustains crop productivity. Rice, 7, 1–3.

Sakamoto, T. (2004) An overview of gibberellin metabolism enzyme genes and their related mutants in rice. Plant Physiol., 134, 1642–1653.

Sasaki, A., Ashikari, M., Ueguchi-Tanaka, M., Itoh, H., Nishimura, A., Swapan, D., et al. (2002) Green revolution: a mutant gibberellin-synthesis gene in rice – new insight into the rice variant that helped to avert famine over thirty years ago. Nature, 416, 701–702.

Serrat, X., Cardona, M., Gil, J., Brito, A.M., Moysset, L., Nogués, S., and Lalanne, E. (2014) A Mediterranean japonica rice (Oryza sativa) cultivar improvement through anther culture. Euphytica, 195, 31–44.

Spielmeyer, W., Ellis, M.H., and Chandler, P.M. (2002) Semidwarf (sd-1), 'green revolution' rice, contains a defective gibberellin 20-oxidase gene. Proc Natl Acad Sci U S A, 99, 9043–9048.

Spielmeyer, W., Ellis, M.H., and Chandler, P.M. (2002) Semidwarf (sd-1), 'green revolution' rice, contains a defective gibberellin 20-oxidase gene. Proc Natl Acad Sci U S A, 99, 9043–9048.

Tuteja, N., Sahoo, R.K., Garg, B., and Tuteja, R. (2013) OsSUV3 dual helicase functions in salinity stress tolerance by maintaining photosynthesis and antioxidant machinery in rice (Oryza sativa L. cv. IR64). Plant J., 76, 115–127.

Urbanová, T., Tarkowská, D., Novák, O., Hedden, P., and Strnad, M. (2013) Analysis of gibberellins as free acids by ultra performance liquid chromatography-tandem mass spectrometry. Talanta, 112, 85–94.

Wang, Y., Zhao, J., Lu, W., and Deng, D. (2017) Gibberellin in plant height control: old player, new story. Plant Cell Rep., 36, 391–398.

Yamaguchi, S. (2008) Gibberellin metabolism and its regulation. Annu. Rev. Plant Biol., 59, 225–251.

Yang, J., Peng, S., Visperas, R.M., Sanico, A.L., Zhu, Q., and Gu, S. (2000) Grain filling pattern and cytokinin content in the grains and roots of rice plants. Plant Growth Regul., 30, 261–270.

CHAPTER 3 – *In vitro* anther culture studies

CHAPTER 3.1 - An improved anther culture procedure for obtaining new commercial Mediterranean temperate japonica rice (Oryza sativa) genotypes

Camilo López-Cristoffanini[1], Xavier Serrat[1], Eduardo Ramos-Fuentes[1,2], Isidre Hooghvorst[1,3], Roser Llaó[2], Marta López-Carbonell[1], Salvador Nogués[1]

[1]Departament de Biologia Evolutiva, Ecologia i Ciencies Ambientals, Secció de Fisiologia Vegetal, Universitat de Barcelona, 08028 Barcelona, España.

[2]Càmara Arrossera del Montsià i Secció de Crèdit SCCL, 43870 Amposta, Tarragona, España.

[3]ROCALBA S.A., c/Barcelona 15 PO BOX 156, 17001 Girona, España.

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ABSTRACT

Rice is one of the greatest calorie supply for the world population, especially since its production is almost entirely destined to direct human consumption and its demand will increase along with the world population. There are efforts worldwide to increase rice yields by obtaining new improved and stabilized rice lines. The rice anther culture, a fast and cheap technique, allows to obtain double haploid lines in less than one year. We report its application with an improved protocol in four Mediterranean *japonica* rice genotypes at F_2 generation. We performed a screening test for cold-pretreatment at 5.0±0.1ºC and concluded that the optimum duration was 9 days as it produced the higher rate of anther-derived *callus* induction. This revised protocol was successfully applied to the four genotypes, obtaining good results in all the procedure's steps. At the end, more than 100 of double haploid green plants were generated. Moreover, 9 lines obtained from the anther culture procedure showed good qualities for the Spanish market at the growing, farming and grain production level during the field assays. Therefore, we report an improved anther culture procedure for obtaining double haploid lines from temperate *japonica* rice genotypes showing high commercialization expectance.

KEYWORDS: *Oryza sativa*, Mediterranean rice, anther culture, field assays, cold-pretreatment.

Rice is a highly important cereal cultivar in the world, with a total of 490.9 million tonnes (milled equivalent) produced in 2015/16 of which more than 80% was destined to direct human consumption (FAO Trade and Market Division 2017). In addition, it has been proposed that rice will be one of the main calorie supplies in the forthcoming years (FAO Rice Market Monitor 2016). Thus, there are efforts worldwide to accelerate the development of new rice varieties either to attain higher yielding rates and/or to obtain higher quality grains (Moon et al. 2003; Khush 2005; Peng et al. 2008; Guimaraes 2009; Zeng et al. 2017). Despite the efforts made, rice breeders' seed suffer recurrent deteriorations due to successive annual cultivation (IRRI 1988; Serrat et al. 2014). Programs for ensuring rice breeders' seeds stability are laborious and time-consuming (Briggs and Knowles 1967; Jennings et al. 1979; Serrat et al. 2014). In addition, selecting and stabilizing new rice lines from an F_1 cross is a long process that usually takes about 8 years minimum (Martínez et al. 1996; Serrat et al. 2014). The anther culture technique, first developed in rice by Niizeki and Oono (1968), allows to obtain stabilized double haploid (DH) plants bypassing the inbreeding process. Moreover, it is the fastest method to obtain DH rice plants as can be performed in less than one year (Miah et al. 1985; Agache et al. 1989). Roughly, this technique is a two-step process from the initial development of *calli* to the subsequent regeneration of green plants from embryogenic *calli* (Mishra and Rao 2016). This technique has been used to obtain pure parental lines and to speed up descendant's selection after an artificial cross (Courtois 1993; Mishra and Rao 2016). Over the years, it has been shown that it is much easier to apply this technique on tropical *japonica* varieties, since they are more responsive at the *callus* formation and plant regeneration stages than Mediterranean *japonica* or *indica* varieties (Hu 1985; Miah et al. 1985; Yan et al. 1996; Herath et al. 2007; Mishra and Rao 2016; Chen et al. 1986; Serrat et al. 2014). Despite of that, we have previously reported an anther culture

technique adaptation for a Mediterranean temperate *japonica* rice (*Oryza sativa*) cultivar (NRVC980385) to produce a new commercial cultivar (NRVC20110077; Serrat el al. (2014)), which however showed a very poor anther-derived *callus* induction.

Therefore, the main aim of this study is to test for the first time and improved anther culture procedure on F_2 rice genotypes coming from self-pollination of four crosses between different temperate Mediterranean *japonica* rice varieties. In addition, a secondary aim was to test the effect of a colder cold-pretreatment performed at different days of exposure for increasing the anther induction rate. This will allow to establish a standard and fast technique for obtaining commercial DH plants, with a high anther induction efficiency, from any temperate Mediterranean *japonica* rice line in development.

For testing this improved protocol, four different F_2 rice genotypes that resulted from self-pollination of an F_1 generation generated by crosses between Mediterranean temperate *japonica* rice cultivars were used (Table 3.3.1.1; germplasm rice genotypes were coded according to La Càmara cooperative seed producer simplified coding system). We employed the F_2 generation as characters segregation is maximum and plants will therefore provide high variability when obtaining the double haploid green plants (Guimaraes 2009). Plants were grown in greenhouse conditions at the Experimental Fields Service at the University of Barcelona (Barcelona, Spain) on four litre plastic containers filled with rice substrate as described in Serrat et al. (2014).

Table 3.3.1.1. Parental cultivar (P1 and P2) and F_2 rice genotypes produced by P1*P2 cross are listed using the simplified code system according to La Càmara seed producer. Anther culture *in-vitro* results are shown: number of plated anthers, number of anthers producing *calli*, number of *calli* generated, *callus* induction percentage (CI,%), *callus* production ratio (CPratio) and number of green double haploid plants regenerated in the four rice genotypes assayed. The error for CI corresponds to the confidence interval.

Parental cultivar 1 (P1)[a]	**Parental cultivar 2 (P2)[a]**	**F_2 rice genotype (P1xP2→F_1→F_2)**	**No of plated anthers**	**No of anther producing *calli***	**No of produced *calli***	***Callus* induction (CI,%)**	**CP_{ratio}**	**No of green double haploid plants regenerated**
rG3	NRVC980385 (rG0)	**F_2-30**	20185	27	160	0.133±0.050	5.93	30
rG4	NRVC980385 (rG0)	**F_2-40**	21301	99	547	0.465±0.091	5.53	70
rG4	rG2	**F_2-42**	18880	37	152	0.196±0.063	4.11	7
rG4	rG5	**F_2-45**	17456	72	360	0.412±0.095	5	17
-	-	**NRVC980385[b]**	42660	4	66	0.009±0.009	16.5	29

[a]rG: Rice genotype.
[b]Data for NRVC980385 was obtained from supplementary material in Serrat et al. (2014).

The anther culture procedure was performed similar to Serrat et al. (2014). The cold-pretreament was modified in order to enhance the anther-derived *callus* induction stage according to (Chen et al. 1986; Trejo-Tapia et al. 2002a, 2002b). We performed a screening test at 5.0 ± 0.1ºC during 8 to 12 days to select the best cold-pretreatment duration for using it for the anther protocol. Haploid *calli* spontaneously double their ploidy during the plantlet regeneration step, and thus develop into double haploid (DH) plants but could also develop into haploid, triploid or polyploid plants (Alemanno and Guiderdoni 1994). Further, the ploidy level was analyzed with the aim of reducing greenhouse space and costs since haploid plants are sterile. The ploidy determination was performed by flow cytometry following the protocol described in Serrat et al. (2014). Dihaploid plants were cultured in greenhouse until seed-set, and seeds were harvested for the subsequent field assays.

For comparing the suitability of the improved anther protocol, several parameters were analyzed in the four F_2 rice genotypes tested and NRVC980385 cultivar used in Serrat et al. (2014). These were: *callus* induction percentage (**CI%**) = number of anthers producing *calli*/number of plated anthers *100; *callus* production ratio (**CP_{ratio}**) = number of produced *calli*/number of anthers producing *calli*; green plant percentage (**GR%**) = number of green plant regenerated/number of transferred *calli* *100; green double haploid plant percentage (**GRDH%**) = number of green DH plants regenerated/number of transferred *calli* *100. For comparing data among rice genotypes, we used two approximations: (i) visually, we calculated the confidence intervals (ConInt) using the following formula $CI = \% \pm 1.96 \times \sqrt[2]{\frac{\%\times(1-\%)}{\text{number of observations}}}$ and used them as a mean of standard error; and (ii) statistically, we performed a chi-squared test with Yates correction (Zar 2010). No visual nor statistical approximations were used for CP_{ratio}, since due to its nature neither CI nor Chi-squared test with Yates correction were possible to calculate. Please note that due to the

experimental procedure of the anther culture, we did not use replicates, thus total values of several parameters for each genotype assayed were used instead.

Finally, for testing rice genotypes with commercial interest, we performed a general field assay on 70 double haploid in order to screen overall diseases resistance and production estimates. Selected genotypes were assayed in small scale field assays in La Càmara experimental fields (Amposta, Tarragona, Spain). For this, two designs were used: (i) plant agronomical trait evaluation: 25 plants per genotype assayed were planted in row as to have 20 cm between each plant and 50 cm between rows; (ii) plant production evaluation: 80 plants per genotype assayed were planted in row as to have 20 cm between each plant and 25 cm between rows. Plant agronomical traits such as plant height (i.e. from the base of the plant to the top of the panicle), susceptibility to rice stem borers and resistance to blast and brown spot (Mew and Gonzales 2002), number of spikes per plant and inter-homogeneity (homogeneity between plants of the same genotype) were recorded 120 days after sowing. For plant production traits, the parameters evaluated were humidity (%) at the time of data recollection, 1,000-grains weight, percentage of whole grains (unshattered milled grains/total milled grains * 100) and yield (kg of grains per hectare, kg/ha). NRVC980385 was used as a control to monitor field behavior as it is a parental cultivar for F_2-30 and F_2-40, and is also common variety cultivated in the region (Serrat et al. 2014; Català et al. 2007).

Results of the cold-pretreatment duration test at 5.0±0.1ºC as well as that performed at 7ºC by Serrat et al. 2014 is shown in Figure 3.3.1.1. It was observed that for the *callus* induction (CI,%), there were significant differences between the duration in days of the cold-pretreament ($\chi^2_{Yates}(4) = 94.0699$, $p < 0.0001$; Figure 3.3.1.1), being 9 days the optimum for anther-derived *callus* induction. Moreover, it was also observed that a cold-pretreament at 5ºC during 9 days instead of 7ºC during 7-12 days had a higher CI% in all the days tested,

being 0.254±0.072 in average and 0.009±0.009 respectively. Our results differ to those of Kaushal et al. (2014b) where the optimum is for 5 days at 12ºC. These contrasting results can be explained by the fact that Kaushal et al. (2014b) used *indica* varieties, whereas the ones used in our experiment are Mediterranean temperate *japonica* genotypes. Trejo-Tapia et al. (2002a, 2002b) also studied the effect of cold-pretreament in anther-derived *callus* induction, but at 4ºC. In their first study, 14 days was the best for the majority of the cultivars (tropical *japonica* sub-species; Trejo-Tapia et al. 2002a), whereas in their second study 7 days was the best for H2500 cultivar (tropical *japonica* sub-species; Trejo-Tapia et al. 2002b). Our cold-pretreament duration is situated between both works, suggesting that 9 days is an ideal time for this stage of the anther culture procedure to enhance the *callus* production in Mediterranean temperate rice *japonica* varieties. Moreover, the cold-pretreament at 5ºC during 9 days radically increases the CI%, as the lowest CI% value reported in this study is almost 15 times higher than the one reported with NRVC980385 (Serrat et al. 2014).

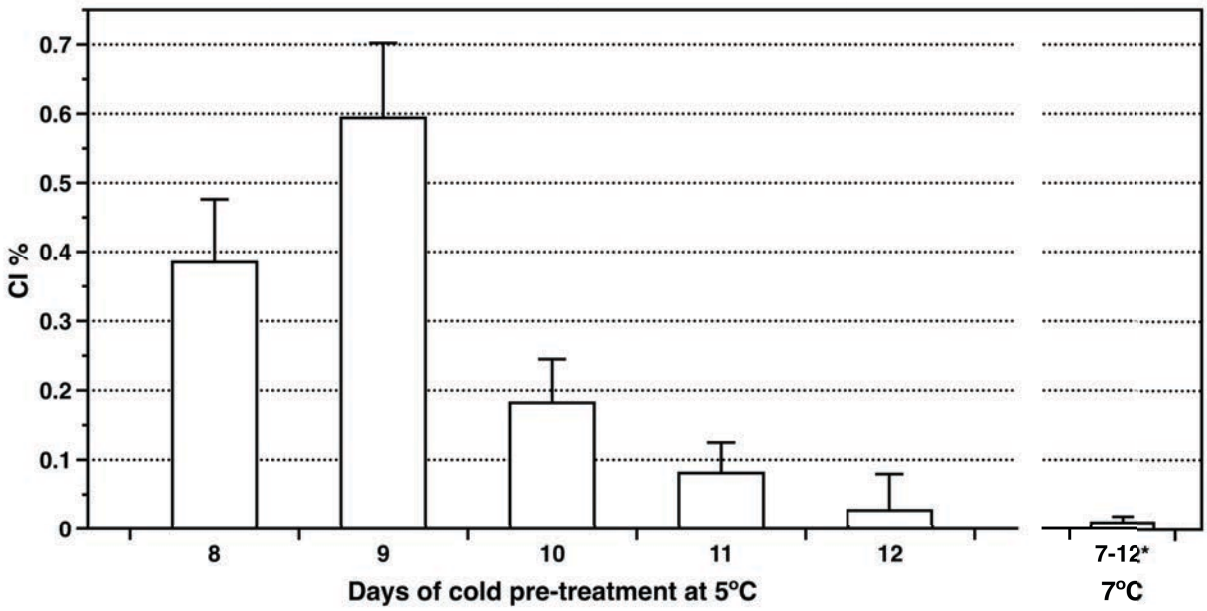

Figure 3.3.1.1. Effect of the cold pre-treatment on the *callus* induction percentage (CI,%) in the four rice lines assayed. The star (*) data on the right side of the graph corresponds to NRVC980385 retrieved from supplementary material in Serrat et al. (2014), in which the cold pre-treatment was performed at 7ºC during 7-12 days. Error bars correspond to the confidence intervals.

Regarding the anther culture procedure for obtaining new rice line, an average of approximately 19,500±1,600 anthers was plated for each of the four F_2 rice genotypes used in this experiment (details for each genotype in Table 3.3.1.1). *Calli* were produced from all of the four assessed genotypes differing in the CI%, but in average was higher than the one reported by Serrat et al. (2014). More in detail, it was observed that the genotype F_2-30, the one with the lowest CI%, had an anther-derived *callus* induction 12 times greater than NRVC980385 (Table 3.3.1.1). On the other hand, genotypes F_2-40 and F_2-45 displayed a CI% 51.7 and 45.8 times higher, respectively, when compared to NRVC980385. It is worth noting that the three of the F_2 genotypes in which the cultivar 4 was one of the used parental, yielded the highest CI%, being the higher the genotype F_2-40, cross between cultivar 4 and NRVC980385, with 0.465%. Statistical analysis showed significant differences between all the five rice genotypes (four F_2 rice genotypes and NRVC980385 cultivar) ($\chi^2_{Yates}(4) = 193.9229$, $p < 0.0001$). In the literature, CI% vary between as low as 0.2% to up to 77.9%, being the genotype the most important factor that determines these percentages (Bishnoi et al. 2000; Shahnewaz et al. 2004; Herath et al. 2007; Kaushal et al. 2014a, 2014b). Our CI% is situated in the lower ones, and it is probably due to the genotype of our F_2 rice genotypes, which do not favour *callus* formation as also observed in the study performed by Serrat *et al* 2014. Several studies support this affirmation, since most of the differences can be explained by the genotype factor (Kaushal et al. 2014a; Herath et al. 2007; Shahnewaz et al. 2004; Khanna and Raina 1998; Yan et al. 1996). Despite the low CI% reported in this study when compared to the majority of the literature, the number of *callus* obtained was higher than that reported by other authors (Shahnewaz et al. 2003, 2004).

The higher anther-derived *callus* induction translated in a higher number of *calli*, 1,219 in total for the four F_2 genotypes assayed (Table 3.3.1.1). Moreover, the number of *calli* produced

was much higher in this study compared to NRV980385. The *callus* production ratio (CP_{ratio}) was similar in the four rice genotypes, being F_2-30 and F_2-40 the ones that showed higher values of 5.9 and 5.5, respectively. In average, the CP_{ratio} only corresponded to 31.2% of the displayed by NRVC980385 (Table 3.3.1.1) though we expected, as seen for CI%, that this parameter would be higher. This can be explained by considering the following: (i) average of anthers producing *callus* for our experiment was 59±17 whereas for Serrat et al. (2014) only four anthers were used for *callus* production; (ii) average of *callus* produced were 305±94 and 66 in our experiment and in Serrat et al. (2014), respectively. Thus, although having in average a low CP_{ratio} in our study, we expect to have a higher chromosomal variability of the rice genotypes since an elevated number of *calli* coming from a larger number of anthers was obtained. It is worth nothing that not much data is available in the literature regarding CP_{ratio} which in turn does not allow for much comparison. Nevertheless, it is of high importance since it gives information if regenerated plants come from several or few *calli*, thus we propose that this ratio should be regularly given.

The green plant percentage (GR,%) was in average 89.6±5.9% among the four rice genotypes tested as seen in Figure 3.3.1.2, which is similar to our prior results using NRVC980385. But, it is noteworthy that the GR% was considerably higher than in other articles (ranging from 2 to even 16 times more; Trejo-Tapia et al. 2002a, 2002b; Shahnewaz et al. 2003, 2004; Herath et al. 2007; Kaushal et al. 2014a, 2014b). Statistical analysis also showed that there are significant differences between the 5 rice genotypes ($\chi^2_{Yates}(4) = 6.3447$, $p = 0.1478$; Figure 3.3.1.2). The green double haploid plant percentage (GRDH,%) reported in this study ranges from 11.5 to 47.1% of that reported with NRVC980385, being the genotypes F_2-30 and F_2-40 the ones displaying the higher GRDH% among our four F_2 rice genotypes (20.7 and 15.5%, respectively). In this study, more than 75 plants for each

genotype were analyzed, whereas in Serrat et al. (2014) only 43 in total were analyzed. The GRDH% was significantly different among the 5 varieties ($\chi^2_{Yates}(4) = 67.6942$, $p < 0.0001$; Figure 3.3.1.2). This parameter is of the uttermost importance since those plants are viable and suited for field assay evaluations, which is the final purpose of this procedure. No data for this is available in the literature thus we cannot further compare. The last stage of the process was to acclimatize *in-vitro* plants to greenhouse conditions in rice substrate. We transplanted a total of 547 green double haploid plants produced from the four F_2 genotypes, of which in average 89±4% were successfully grown to maturity (data not shown), which is greater than the 67±8% in average that is reported by Herath et al. (2007). Similarly, only 12±8% of the total of the transplanted plants showed more than 5% of sterility (data not shown), which is a better success rate than 24±31%, value observed in the work by Herath et al. (2007).

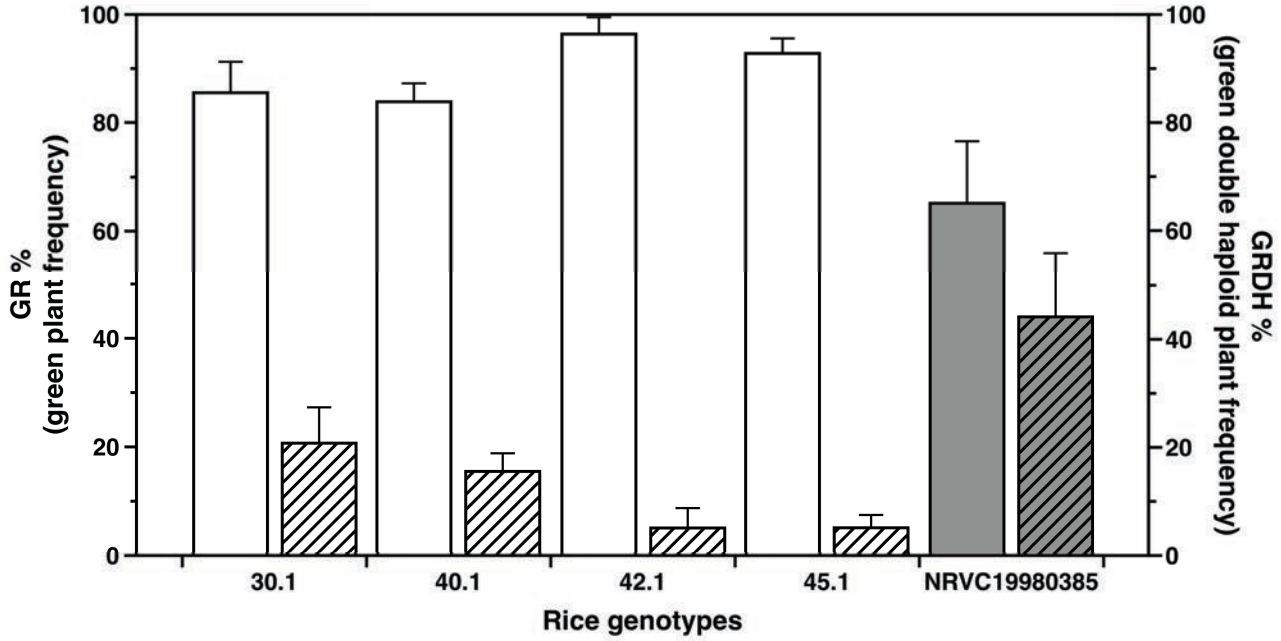

Figure 3.3.1.2. Total percentage of green regenerated plants (GR%) and total percentage of green double haploid regenerated plants (GRDH%) in the four rice lines assayed (white bars). Data for NRVC980385 was retrieved from supplementary material in Serrat et al. (2014) (grey bars). Solid bars correspond to GR% and hatched bars to GRDH%. Error bars correspond to the confidence intervals.

During the general field assay of 70 double haploid (DH) lines, it was observed that all DH plants coming from F_2-42 and F_2-45 along with some DH plants of F_2-30 and F_2-40 lacked agronomic and commercial interest (data not shown). Therefore, a total of 9 lines were selected for agronomical and production traits, which showed high inter-homogeneity and a high tillering activity (more than 40 tillers per plant; data not shown). The average height of the lines was 70.7±2.2 cm, which is in the range of those cultivated in the Ebro Delta. Moreover, 7 of them were shorter than Gleva, the shortest cultivated variety in the region (Pla et al. 2017). Overall, all evaluated lines showed a high resistance to fungal diseases and medium resistance to rice stem borers (Table 3.3.1.2), except for F_2-40.D266 plants which was promising in terms of production during the general field assay. Regarding blast

resistance, it was in general higher in comparison to the varieties regularly cultivated in the region of the Ebro Delta (Pla et al. 2017; Català et al. 2009). No literature was available for comparing tolerance to brown spot disease and the rice stem borers in local field conditions. Despite this, resistance to brown spot was similar to that of the control variety (NRCC980385), and rice stem borers resistance was higher than NRVC980835 for several lines. The 1,000-grain weight (determined by grain length, width and thickness) of the lines was in average 31.6 g, comparable among them since the humidity range was 13.6-14.2%. Of the assayed lines, all of them with the exception of F_2-30.C1, as seen in Table 3.3.1.2, showed a 1,000-grain weight higher than NRVC980385 and other *indica* and *japonica* varieties (Koutroubas and Ntanos 2003; Fan et al. 2006). The whole grains percentage was variable among the 9 lines assayed but ranged between 60-70%, similar to NRVC980385 and several other Spanish varieties values (Català et al. 2009), thus these lines are suitable for large scale production. In terms of yield, lines of the F_2-30 genotype were below those reported for Gleva (most cultivated variety in the region) and NRVC980835 cultivar (Pla et al. 2017), and half of the values reported for F_2-40 genotype lines (Table 3.3.1.2). On the other hand, lines of the F_2-40 genotype displayed higher yields than Gleva (Pla et al. 2017). Furthermore, the observation that F_2-40.D266 was promising in terms of production was certain as its yield was the highest among the lines tested and higher than the Spanish rice varieties including the NRVC980835 cultivar (Pla et al. 2017). Nevertheless, to fully characterize and evaluate the assayed lines, direct seeded field assays should be performed in a medium (and maybe even large scale) in order to better assess for pathogens resistance, plant height and yield.

In conclusion, we have shown that the improved anther culture protocol can be successfully applied in different F_2 rice genotypes between temperate *japonica* rice genotypes to obtain

green double haploid plants. Moreover, we have observed that genotype is one of the main factors that affects the anther culture protocol success. Despite this, we have determined that the cold-pretreatment improvement, 9 days at 5.0±0.1ºC, greatly increases the anther-derived *callus* induction in temperate *japonica* Mediterranean rice crossed genotypes at the F_2 generation, since the number of green double haploid plants obtained at the end of the anther culture procedure was high. Furthermore, 7 of the 9 lines evaluated in the field showed good qualities at the agricultural and production level. Therefore, these varieties are suited to be submitted to direct seeded medium scale assays before registry for their subsequent commercialization. Thus, in conclusion, our proposed method for Mediterranean *japonica* rice is highly applicable to rice genotypes at the F_2 generation of different *japonica* rice cultivars for producing new lines that could be registered and commercialized as new varieties.

Table 3.3.1.2. Agronomical and production traits evaluation for the lines assayed in the field assays.

Anther-derived rice line	Height (cm)[a]	Plant agronomical traits evaluation			Plant production traits evaluation[d]			
		Fungal disease resistance[b]		Rice stem borers resistance[b,c]	Humidity (%)	1,000-grain weight (g)	Whole grains (%)	Yield (kg/ha)
		Blast[c]	Brown spot[c]					
F_2-30.C1	68.5 ± 1.9	+++	+++	++	13.8	27.5	67.3	5740
F_2-30.C2	70.5 ± 0.6	+++	+++	++	14	28.8	66.7	5828
F_2-40.C15	66.3 ± 4.1	+++	+++	++	13.9	32.5	67.8	11393
F_2-40.D37	68.5 ± 1.3	+++	+++	+	13.8	35	63.3	11598
F_2-40.D39	62.8 ± 2.1	+++	+++	+++	13.6	35	61.4	10338
F_2-40.D118	65.3 ± 0.5	+++	+++	+	13.8	35	66.9	11328
F_2-40.D173	59.0 ± 2.7	+++	+++	++	13.9	30	63	11670
F_2-40.D174	61.5 ± 1.3	+++	+++	++	13.9	32.5	65.9	11760
F_2-40.D266	90.3 ± 4.4	+++	+	-	14.1	35	63.9	13553
NRVC980385	94.0 ± 2.7	+++	+++	+	14.2	27.5	65.3	11325

[a]The value shown correspond to the mean of 25 plants and the SD.
[b]Resistance scale is the following: -: sensible; +: low resistance; ++: medium resistance; +++: high resistance.
[c]Blast and brown spot diseases are caused by *Magnaporthe oryzae* and *Helminthosporium sp*. respectively, and rice stem borers *Chillo suppresalils*.
[d]Production traits evaluation data for NRVC980385 was recorded at the same time of the F2 rice lines assayed.

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The authors declare that they have no conflict of interest.

REFERENCES - CHAPTER 3.1

Agache S, Bachelier B, de Buyser J, Henry Y, Snape J (1989) Genetic analysis of anther culture response in wheat using aneuploid, chromosome substitution and translocation lines. *Theoretical and Applied Genetics* 77: 7-11

Alemanno L, Guiderdoni E (1994) Increased doubled haploid plant-regeneration from rice (*Oryza sativa* L.) anthers cultured on colchicine-supplemented media. *Plant Cell Reports* 13: 432-436

Bishnoi U, Jain RK, Rohilla JS, Chowdhury VK, Gupta KR, Chowdhury JB (2000) Anther culture of recalcitrant *indica* × Basmati rice hybrids. *Euphytica* 114: 93-101

Briggs FN, Knowles PF (1967) Introduction to plant breeding. Reinhold Publishing Corporation, New York

Català MM, Jardí M, Pla E (2007) Evolución de las variedades de arroz cultivadas en el Delta del Ebro durante los últimos 20 años. *Agrícola Vergel* 303: 132-135

Català MM, Tomàs N, Martinez M, Pla E (2009) Valoración agronómica de nuevas variedades de arroz. Ensayos en el Delta del Ebro: 2004-2008. *Agrícola Vergel* 327: 161-166

Chen C-C, Tsay H-S, Huang C-R (1986) Rice (*Oryza sativa* L.): factors affecting androgenesis. In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry, vol 2., Springer Berlin Heidelberg, Heidelberg, Germany, pp 123-128

Courtois B (1993) Comparison of single seed descent and anther culture-derived lines of three single crosses of rice. *Theoretical and Applied Genetics* 85: 625-631

Fan C, Xing Y, Mao H, Lu T, Han B, Xu C, Li X, Zhang Q (2006) *GS3*, a major QTL for grain length and weight and minor QTL for grain width and thickness in rice, encodes a putative transmembrane protein. *Theoretical and Applied Genetics* 112: 1164-1171

FAO Rice Market Monitor (2016) Rice Market Monitor. Trade and Markets Division. Food and Agriculture Organization of the United Nations.

FAO Trade and Markets Division (2016) Food Outlook: Biannual report on global food markets, November 2017. Food Outlook, Food and Agriculture Organization of the United Nations.

Guimaraes EP (2009) Rice Breeding. In: Carena MJ (ed) Cereals, The Banks and the Italian Economy. Springer-Verlag New York, New York, pp 99-126

Herath HMI, Bandara DC, Samarajeewa PK (2007) Effect of culture media for anther culture of *indica* rice varieties and hybrids of *Indica* and *Japonica*. *Tropical Agricultural Research & Extension* 10: 17-22

Hu H (1985) Use of haploids for crop improvement in China. *Genetic Manipulation in Crops Newsletter* 1: 11-23

IRRI (1988) Rice seed health. Proceedings of the international Workshop on Rice Seed Health. International Rice Research Institute, Manila

Jennings PR, Coffman WR, Kauffman HE (1979) Rice improvement. International Rice Research Institute (IRRI), Los Baños, Laguna, Philippines

Kaushal L, Balachandram SM, Ulaganathan K, Shenoy V (2014a) Effect of culture media on improving anther culture response of rice (*Oryza sativa* L.). *International Journal of Agriculture Innovations and Research* 3: 218-224

Kaushal L, Sharma R, Balachandram SM, Ulaganathan K, Shenoy V (2014b) Effect of cold pretreatment on improving anther culture response of rice (*Oryza sativa* L.). *Journal of Experimental Biology and Agricultural Sciences* 2: 233-242

Khanna HK, Raina SK (1998) Genotype x culture media interaction effects on regeneration response of three *indica* rice cultivars. *Plant Cell, Tissue and Organ Culture* 52: 145-153

Khush GS (2005) What it will take to feed 5.0 billion rice consumers in 2030. *Plant Molecular Biology* 59: 1-6

Koutroubas SD, Ntanos DA (2003) Genotypic differences for grain yield and nitrogen utilization in *Indica* and *Japonica* rice under Mediterranean conditions. *Field Crops Research* 83: 251-260

Martínez CP, Correa Victoria F, Amézquita MC, Tulande E, Lema G, Zeigler RS (1996) Comparison of rice lines derived through anther culture and the pedigree method in relation to blast (*Pyricularia grisea* Sacc.) resistance. *Theoretical and Applied Genetics* 92: 583-590

Mew TW, Gonzales P (2002) A handbook of rice seedborne fungi. IRRI, Los Baños, Philippines

Miah MAA, Earle ED, Khush GS (1985) Inheritance of *callus* formation ability in anther cultures of rice, *Oryza sativa* L. *Theoretical and Applied Genetics* 70: 113-116

Mishra R, Rao GJN (2016) *In-vitro* Androgenesis in rice: advantages, constraints and future prospects. *Rice Science* 23: 57-68

Moon HP, Kang KH, Choi IS, Jeong OY, Hong HC, Choi SH, Choi HC (2003) Comparing agronomic performance of breeding populations derived from anther culture and single-seed descent in rice. In: Advances in Rice Genetics. Los Baños, Laguna, Philippines, pp 3-5

Niizeki H, Oono K (1968) Induction of haploid rice plant from anther culture. *Proceedings of the Japan Academy* 44: 554-557

Peng S, Khush GS, Virk P, Tang Q, Zou Y (2008) Progress in ideotype breeding to increase rice yield potential. *Field Crops Research* 108: 32-38

Pla E, Català MM, Tomàs N (2017) Variedades de arroz registradas en España entre los años 2013 y 2016. *Vida Rural* 424: 14-18

Serrat X, Cardona M, Gil J, Brito AM, Moysset L, Nogués S, Lalanne E (2014) A Mediterranean *japonica* rice (*Oryza sativa*) cultivar improvement through anther culture. *Euphytica* 195: 31-44

Shahnewaz S, Bari MA, Siddique NA, Khatun N, Rahman MH, Haque ME (2003) Induction of haploid rice plants through *in vitro* anther culture. *Pakistan Journal of Biological Sciences* 6: 1250-1252

Shahnewaz S, Bari MA, Siddique NA, Rahman MH (2004) Effects of genotype on induction of *callus* and plant regeneration potential *in vitro* anther culture of rice (*Oryza sativa* L.). *Pakistan Journal of Biological Sciences* 7: 235-237

Trejo-Tapia G, Maldonado Amaya U, Salcedo Morales G, De Jesús Sánchez A, Martínez Bonfil B, Rodríguez-Monroy M, Antonio J-A (2002a) The effects of cold-pretreatment, auxins and carbon source on anther culture of rice. *Plant Cell, Tissue and Organ Culture* 71: 41-46

Trejo-Tapia G, Maldonado-Amaya U, Jiménez-Aparicio A, Blanqueto-Illescas M, Salcedo-Morales G, Martínez-Bonfil BP, De Jesús-Sánchez A (2002b) Effect of time at low temperature treatment and growth regulators on regeneration of plants from anthers of rice *Oryza sativa* L. (Japoonica H2005 cultivar). *Agrociencia* 36: 441-449

Yan J, Xue Q, Zhu J (1996) Genetic studies of anther culture ability in rice (*Oryza sativa*). *Plant Cell, Tissue and Organ Culture* 45: 253-258

Zar JH (2010) Biostatistical Analysis. Statistics and Mathematics, 5th ed. Prentice Hall, Upper Saddle River, NJ, USA

Zeng D, Tian Z, Rao Y, Dong G, Yang Y, Huang L, Leng Y, Xu J, Sun C, Zhang G, Hu J, Zhu L, Gao Z, Hu X, Guo L, Xiong G, Wang Y, Li J, Qian Q (2017) Rational design of high-yield and superior-quality rice. *Nature Plants* 3: 17031.

CHAPTER 3.2 - Antimitotic and hormone effects on green double haploid plant production through anther culture of Mediterranean japonica rice

Isidre Hooghvorst[1,2], Eduardo Ramos-Fuentes[1,3], Camilo López-Cristoffanini[1], Mirari Ortega[1], Raimon Vidal[1], Xavier Serrat[1], Salvador Nogués[1]

[1]Departament de Biologia Evolutiva, Ecologia i Ciencies Ambientals, Secció de Fisiologia Vegetal, Universitat de Barcelona, 08028 Barcelona, España.

[2]ROCALBA S.A., c/Barcelona 15 PO BOX 156, 17001 Girona, España.

[3]Càmara Arrossera del Montsià i Secció de Crèdit SCCL, 43870 Amposta, Tarragona, España.

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ABSTRACT

Rice double haploid (DH) plants are produced mainly through anther culture. In order to improve the anther culture protocol, microspores of two japonica rice genotypes (NRVC980385 and H28) were subjected to three growth regulator combinations and four colchicine treatments on induction medium. In addition, a post anther culture procedure using colchicine or oryzalin was tested to induce double haploid plantlets from haploid plantlets. A cold pre-treatment of microspores for 9 days at 10ºC increased *callus* induction 50-fold in the NRVC980385 genotype. For both genotypes, 2 mg·L-1 2,4-D and 1 mg·L^{-1} kinetin on colchicine-free induction medium gave the best culture responses. The culturability of both genotypes changed on colchicine-supplemented induction media. A high genotype dependency was recorded for *callus* induction, *callus* regenerating green plantlets and regeneration of green double haploid plantlets. Colchicine at 300 mg·L^{-1} for 48 hours enhanced *callus* induction 100-fold in H28. Colchicine-supplemented media clearly improved green double haploid plantlet regeneration. We showed that the post anther culture treatment of haploid plantlets at 500 mg·L^{-1} of colchicine permitted fertile double haploid plantlets to be generated. Finally, an enhanced medium-throughput flow cytometry protocol for rice was tested to analyze all the plantlets from anther and post anther culture.

KEYWORDS: Mediterranean japonica rice, anther culture, hormones, colchicine, antimitotics, double haploid.

INTRODUCTION

Doubled haploid lines (DHs) are produced when spontaneous or induced chromosome duplication of haploid cells occurs. DH plants are complete homozygous individuals that can be produced within a year through anther or microspore culture. Therefore, the production of homozygous lines from heterozygous parents is feasible and shortens the time required to obtain them (Germanà 2011). Nowadays, anther culture is being used to produce DH plants in more than 250 species, including major cereals such as rice, wheat, maize, barley and also economically important trees, fruit crops and medicinal plants (Maluszynska 2003).

Rice DH plant production is mainly obtained through anther culture. Niizeki and Oono (1968) were the first to produce haploid rice plantlets through anther culture. Rice anther culture is a two-step process with initial *callus* development and subsequent green plantlet regeneration from embryogenic *callus* (Mishra 2016). Since the first report of anther culture, much research has aimed at optimizing the media used at each step in the process to enhance *callus* induction and *callus* regeneration (Herath et al. 2010; Pauk et al. 2009). This work has focused on overcoming limiting factors that reduce the efficiency of green DH plantlet production such as high genotypic dependency, low frequency of *callus* induction and plantlet regeneration, the low percentage of doubled haploids produced and the high ratio of albino plantlets (Lentini et al. 1997). The application of stress during the developmental period of pollen grains, osmotic stress applied to cells during culture, the composition of the culture media, and the addition of antimitotic agents, gelling agents or growth regulators amongst many exogenous factors may affect the success of anther culture in rice (Mishra and Rao 2016). Endogenous factors such as the rice variety and genotype also affect anther culture success. Indica rice varieties have a limited response to anther culture due to early necrosis,

poor *callus* proliferation and a high regeneration of albino plantlets (Chen et al. 1991), unlike japonica varieties where green DH plant production is more efficient (He et al. 2006).

Despite the improvements and progress achieved in every step of the anther culture procedure, there is still a need to optimize conditions for higher rates of green DH plant production while reducing the amount of work in each step. Colchicine is an antimitotic compound widely used in microspore culture and has been shown to improve results in terms of green double haploid plant production (Forster et al. 2007) in maize (Obert and Barnabás 2004), barley (Thompson et al. 1991), wheat (Barnabás et al. 1991), rapeseed (Weber et al. 2005), and other species. However, few authors have reported the use of colchicine in rice anther culture. Alemanno and Guiderdoni (1994) were the first to study a routine *in vitro* colchicine treatment to increase DH plant production in rice. In addition, post anther culture procedures have rarely been used in green haploid plantlets regenerated from anther culture. Finally, such a procedure can be undertaken either *in vivo* by treating tillers with antimitotic compounds such as colchicine in order to increase the DH recovery from haploid plantlets (Jensen 1974; Zapata-Arias 2003; Chen et al. 2002) or *in vitro* as explained in this work. Among the auxins, 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthaleneacetic acid (NAA) are the most used hormones for rice *callus* induction from anthers (Trejo-Tapia et al. 2002).

The aim of this study is to improve the anther culture efficiency in two japonica rice genotypes by assessing some factors that could improve the numbers of green double haploid plants. Thus, we tested (i) the effect of different growth regulators (2,4-D, NAA and Kinetin) in the anther culture induction medium, (ii) the effect of different colchicine doses in the anther culture induction medium and (iii) a post anther culture procedure to increase plant DH production from haploid plantlets through colchicine and oryzalin *in vitro* treatments. We showed that colchicine-supplemented media increase green plantlet double haploid

production and 500 mg·L^{-1} of colchicine in a post anther culture procedure enabled recovery of green double haploid plantlets from haploid plantlets to be maximized.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

The commercial temperate japonica rice variety NRVC980385 and a temperate japonica F2 hybrid called H28, provided by the *Càmara Arrossera del Montsià SCCL* cooperative, were used as plant material. Plants were grown in greenhouse conditions at the *Servei de Camps Experimentals* at the University of Barcelona (Barcelona, Spain) in 4 litre plastic containers filled with rice substrate as previously described (Serrat et al. 2014).

ANTHER CULTURE PROCEDURE

Tillers were selected at the booting stage, when the distance from the flag leaf to the auricle of the penultimate leaf was 5-12 cm. The time of collection was from 8:00 to 9:30 as recommended by Chen et al. (1991). Collected tillers were soaked in 70% ethanol for 1 min, rinsed twice with distilled water and were then cold pre-treated for 9 days at 10ºC in polystyrene bags, prior to being surface disinfected again as above. Tillers were dissected to obtain the panicles in a laminar flow cabinet. Panicles were soaked in 70% ethanol for 1 min, rinsed twice and soaked in 10% sodium hypochlorite solution with Tween 20 (30 drops L^{-1}) and 35% HCl (50 drops L^{-1}) for 3 min, and rinsed five times in sterile distilled water. Anthers were obtained from the panicles and plated into 90 mm petri dishes (Sterilin LTD, Cambridge). Basal induction medium consisted of Chu N6 modified as follows: N6 standard salts and vitamins fortified with a combination of growth regulators, 1 $g \cdot L^{-1}$ casein enzymatic hydrolysate, 250 $mg \cdot L^{-1}$ L-proline, 2 $mg \cdot L^{-1}$ 500 $mg \cdot L^{-1}$ 2-(N-morpholino) ethanesulfonic acid (MES), 30 $g \cdot L^{-1}$ sucrose and 3 $g \cdot L^{-1}$ Gelrite.

The anther culture procedure was carried out in parallel for all the combinations of different growth regulators and their concentrations, colchicine concentrations and colchicine exposure times (Table 3.3.2.1). Regarding growth regulators, three combinations were used:

(i) treatment D1, 1 mg·L^{-1} 2,4-D and 1 mg·L^{-1} kinetin; (ii) treatment D2, 2 mg·L^{-1} 2,4-D and 1 mg·L^{-1} kinetin, as used by Serrat et al. (2014) and Chen et al. (2002); and (iii) treatment NA, 2 mg·L^{-1} NAA and 0,5 mg·L^{-1} kinetin, as used by Alemanno and Guiderdoni (1994). Colchicine was assayed at 0 (control), 150 and 300 mg·L^{-1}, each for the two exposure times of 24 and 48 hours. Thus, five colchicine treatments were tested and named as concentration/exposure time: 0/0 (control), 150/24, 150/48, 300/24 and 300/48. Six petri dishes were sown with approximately 100 anthers for each condition. After 24 or 48 hours, anthers inoculated in colchicine-supplemented media were transferred to exactly the same medium but colchicine-free.

Table 3.3.2.1. Conditions for induction media assayed for both genotypes.

Growth regulator			**Colchicine treatment**		**Conditions**
2,4-D (mg·L^{-1})	**NAA (mg·L^{-1})**	**Kinetin (mg·L^{-1})**	**Concentration (mg·L^{-1})**	**Exposure Time (h)**	**Concentration/Time-Hormone**
1	0	1	0	0	0/0-D1
			150	24	150/24-D1
			150	48	150/48-D1
			300	24	300/24-D1
			300	48	300/48-D1
2	0	1	0	0	0/0-D2
			150	24	150/24-D2
			150	48	150/48-D2
			300	24	300/24-D2
			300	48	300/48-D2
0	2	0.5	0	0	0/0-NA
			150	24	150/24-NA
			150	48	150/48-NA
			300	24	300/24-NA
			300	48	300/48-NA

Anthers were kept in the dark at 24ºC and analyzed weekly for 8 weeks. Microspore-derived *calluses* of 1 to 2 mm diameter that emerged from anthers were transferred to *callus*

regeneration medium as described by Serrat et al. (2014). Anthers that induced *callus* were removed to ensure a count of one *callus* per anther, to avoid overestimation of callus induction and to match the number of calluses and induced *calli* for data analysis. Calluses were transferred to plantlet regeneration medium containing Chu N6 (Chu et al. 1975) standard salts and vitamins fortified with 1 $g \cdot L^{-1}$ casein hydrolysate, 250 $mg \cdot L^{-1}$ L-proline, 1 $mg \cdot L^{-1}$ naphthaleneacetic acid, 2 $mg \cdot L^{-1}$ kinetin, 500 $mg \cdot L^{-1}$ MES, 30 $g \cdot L^{-1}$ sucrose and 3 $g \cdot L^{-1}$ Gelrite. IWAKI 94 mm petri dishes (Asahi Techno Glass Corporation, Amagasaki) were filled with 25 mL of the medium. Calluses were transferred after 28 days onto fresh regeneration medium. Cultures were kept at 25 °C and illuminated with 50-70 $\mu mol \cdot m^{-2} \cdot s^{-1}$ fluorescent light under a 16/8 h day/night photoperiod until plantlet formation occurred.

The tiny but fully formed albino and green plantlets (0.5-3 cm length) were transferred into tubes with hormone-free MS (Murashige and Skoog 1962) medium as described by Serrat et al. (2014). Subsequently, clearly sprouting individual plantlets were propagated under conditions as described for regeneration above.

All components of the media were supplied by Duchefa Biochemie BV (The Netherlands). Media were prepared using distilled water and the pH was adjusted to 5.7 by adding 1M KOH (Sigma-Aldrich Co). All components including growth regulators were added before standard autoclave sterilization (121 °C for 20 min).

PLOIDY-LEVEL DETERMINATION

The ploidy of green and albino regenerated plantlets was determined by flow cytometry following the procedure of Cousin et al. (2009) with slight modifications. About 5 mg of young leaves were collected and put into ice-cold 2 mL microcentrifuge tubes each with a single steel bead (3 mm diameter). To each tube, 300 µL of cold lysis buffer (0.1 M citric acid and 0.5% Triton X-100 in distilled water) were added. Tubes were cooled at -20ºC for 10 min.

Samples were shaken at 25 Hz for a total of 48 s in a MM 400 tissue lyser (Retsch, Haan, Germany). The aliquot from each tube was filtered through a 22 µm nylon filter (Sefar Maissa, Blacktown, Australia), gently vacuumed and transferred to a flow cytometry sample tube (Beckman Coulter Inc., Pasadena, California, USA). Afterwards, 150 µL of propidium iodide (PI) stain solution [0.25mM Na_2HPO_4, 10 mL 10x stock (100 mM sodium citrate, 250 mM sodium sulfate) and 9 M PI made up to 100 mL with Milli-Q water] was added to each tube. Tubes were then sealed and kept on ice in the dark for 1 h before flow cytometry (FCM) analysis. The stained nuclei samples were analyzed using a Gallios™ Flow Cytometer (Beckman Coulter Inc., Pasadena, California, USA) with a 488-nm laser at the Cytometry Unit (Scientific and Technological Centres, University of Barcelona) and a 32-well carrousel. One diploid control (NRVC980385) sample was included every seven measurements. Samples analyzed with a clearly defined peak as the reference ploidy control were classified as DH, whereas those producing half the fluorescence were classified as haploids. Flow cytometry data was analyzed using Summit Software v4.3 (Beckman Coulter Inc., Pasadena, California, USA).

DIPLOIDIZATION OF HAPLOID GREEN PLANTLETS

Green haploid plantlets regenerated from anther culture were subjected to a post anther culture *in vitro* treatment with colchicine at 1000, 500 and 250 $mg \cdot L^{-1}$, or oryzalin at 5, 2.5 and 1.25 $mg \cdot L^{-1}$; both in a solution containing 1% DMSO and Tween 20 (4 $drops \cdot L^{-1}$) in sterilized distilled water. Prior to the antimitotic treatment, plantlet stems and roots were trimmed to 3 cm in length and were incubated in the antimitotic solution for 5 h on a shaker at 120 rpm at 25ºC and maintained under sterile conditions in a laminar flow cabinet. Thereafter, the plantlets were transferred to hormone-free MS medium as described before and by 3-4 weeks

of growth plantlets that had survived and reached 10-15 cm in size were collected to perform flow cytometry analysis as described before.

STATISTICS

All parameters were divided by the number of anthers sown for each treatment and multiplied by 100 in order to obtain percentages: *callus* induction (CI, %), number of calluses regenerating green plantlets (CGR,%), number of calluses regenerating albino plantlets (CAL,%), number of calluses regenerating green and albino plantlets (CGRAL,%), number of green plantlets regenerated (GPR), number of double haploid plantlets regenerated (DHPR) and number of green double haploid plantlets regenerated (GDHPR). The three concentrations of growth regulators in the colchicine-free media were compared with each other, and each colchicine treatment was also compared individually with the corresponding control medium (0/0) according to the growth regulator hormone concentration (D1, D2 and NA). Growth regulators and colchicine treatments were analyzed separately for both genotypes. To determine significant differences between the conditions assayed, a Chi-Square ($P<0.05$) test for homogeneity was used.

RESULTS

EFFECT OF THE COLD PRE-TREATMENT ON CALLUS INDUCTION

The percentages of *callus* induction (CI) and green double haploid plantlets regenerated (GDHPR) were compared according to the cold pre-treatment applied (Figure 3.3.2.1) Cold pre-treatment was adjusted to 9 days at 10ºC for NRVC980385 (data not shown) as suggested by Serrat et al. (2014). Following cold pre-treatment for 9 days at 10ºC the CI and GDHPR percentages were 51 times and 33 times higher, respectively, than the 7-12 day pre-treatments at 7ºC.

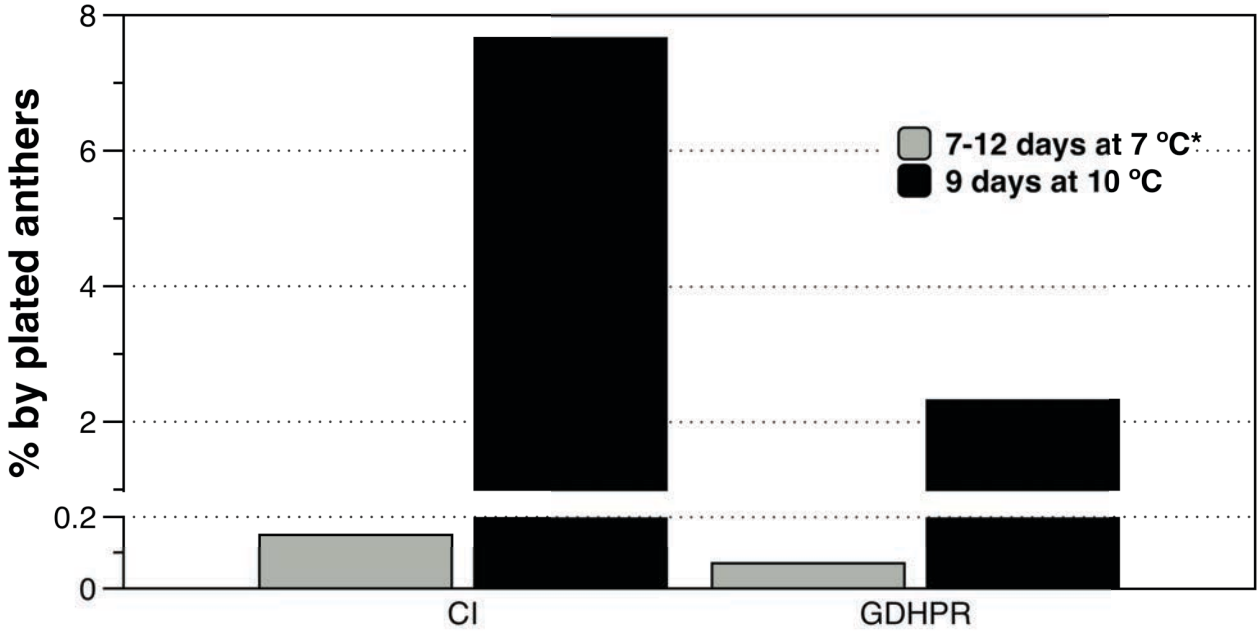

Figure 3.3.2.1. Induced *calluses* (IC,%) and green double haploid plantlets regenerated (GDHPR) for NRVC980385 in 0/0-D2 treatment with a cold pre-treatment of 7-12 days at 7ºC* and 9 days at 10ºC (this work). * Data retrieved from Serrat et al. (2014).

EFFECT OF DIFFERENT GROWTH REGULATORS ON RICE ANTHER CULTURE

The culturability of both genotypes with the different growth regulators in colchicine-free induction media is shown in Table 3.3.2.2. In D2 medium, the culturabilities of NRVC980385

and H28 were greater for all the parameters analyzed. For NRVC980385, there were no significant differences (P>0.05) between the three growth regulator treatments (Table 3.3.2.2) for any of the anther culture parameters. Nevertheless, there was a tendency for D2 conditions (2 mg·L^{-1} of 2,4-D and 1 mg·L^{-1} kinetin) to yield higher values than the other treatments for *callus* induction (CI), *callus* that regenerates green plantlets (CGR), regenerated green plantlets (GPR), regenerated double haploid plantlets (DHPR) and regenerated green double haploid plantlets (GDHPR). The values of the D1 and NA growth hormone regulator treatments were similar, but the D2 treatment had slightly higher values for the majority of the parameters.

Table 3.3.2.2. Culturability results for the temperate *japonica* rice variety, NRVC980385, and the temperate j*aponica* hybrid, H28, among the three growth regulator treatments assayed without colchicine. . D1: 1 mg·L^{-1} 2,4-D and 1 mg·L^{-1} kinetin, *D2* 2 mg·L^{-1} 2,4-D and 1 mg·L^{-1} kinetin; and treatment NA, 2 mg·L^{-1} NAA and 0.5 mg·L^{-1} kinetin.

Rice genotype	**Growth regulator**	**Anther culture parameters (in % by plated anthers)**				
		CI	**CGR**	**GPR**	**DHPR**	**GDHPR**
NRVC980385	D1	6.05	0.36	1.96	0.36	1.60
	D2	7.66	0.89	3.21	0.89	2.32
	NA	6.96	0.17	1.39	0.17	1.22
H28	D1	0.18*	0.00	0.00	0.00	0.00
	D2	4.18	0.70	1.57	0.70	0.87
	NA	5.10	0.17	0.51	0.17	0.34

The percentage of *callus* induction (CI) in H28 with the D1 treatment was significantly lower (P<0.05) than the D2 and NA treatments. Therefore, in D1 treatment, parameters that are dependent on CI (CGR, GPR, DHPRP and GDHPR) were zero, due to a low *callus* induction. No culture parameters between D2 and NA for H28 were statistically significantly (P<0.05).

Although D2 showed higher values than NA for *callus* that regenerates green plantlets (CGR), regenerated green plantlets (GPR), regenerated double haploid plantlets (DHPR) and regenerated green double haploid plantlets (GDHPR), these differences were not significant ($P<0.05$).

EFFECTS OF COLCHICINE TREATMENT ON CALLUS INDUCTION AND PLANTLET REGENERATION

Callus induction was observed in almost all conditions assayed for both genotypes with the colchicine-supplemented treatments (Table 3.3.2.3). The only exceptions were the 150/24-NA and 300/24-NA media for NRVC980385, with both media used as 24 h colchicine treatments. On one hand, IC in NRVC980385 in the colchicine treatments was significantly lower ($P<0.05$) than the control. On the other hand, IC for H28 seemed to increase with colchicine, showing significant differences ($P<0.05$) in both colchicine treatments over 48 h. Moreover, the values were higher in comparison to their respective controls (0/0). Finally, several 24 h colchicine treatments in H28, such as 150/24, 300/24 for D1 and 150/24 for NA, had significantly higher percentages of induced calluses ($P<0.05$) than their respective controls (0/0-D1 and 0/0-NA).

Table 3.3.2.3. *Callus* induction (CI,%) in all treatments assayed for the temperate *japonica* rice variety, NRVC980385, and the temperate *japonica* F2 hybrid, H28.

Colchicine	**NRVC980385**			**H28**		
(concentration[a]/time[b])	**D1**	**D2**	**NA**	**D1**	**D2**	**NA**
0/0	6.05	7.66	6.96	0.18	4.18	5.10
150/24	7.39	2.40*	0.00*	6.44*	2.73	8.67*
150/48	2.39*	3.51*	7.80	14.10*	17.73*	11.39*
300/24	5.23	3.44*	0.00*	10.55*	3.28	1.37*
300/48	5.81	5.12	8.56	18.75*	9.63*	12.54*

[a]$mg \cdot L^{-1}$, [b]hours

Regenerated plantlets were obtained from thirteen and fourteen out of fifteen different media for NRVC980385 and H28, respectively. The exceptions were the NA treatments supplemented with colchicine for 24 h (150/24-NA and 300/24-NA) in NRVC980385 and 0/0-D1 in H28. Plantlets regenerated from calluses were either albino or green, although some calluses were capable of regenerating both (Figure 3.3.2.2 and Figure 3.3.2.3 for NRVC980385 and H28, respectively). There was a tendency for higher numbers of albino plantlets to be present when there was a high rate of plantlet regeneration. Albino plantlets regenerating calluses were the most frequent, representing 77% of and 75% of NRVC980385 and H28 plantlets, respectively, when all media were grouped together.

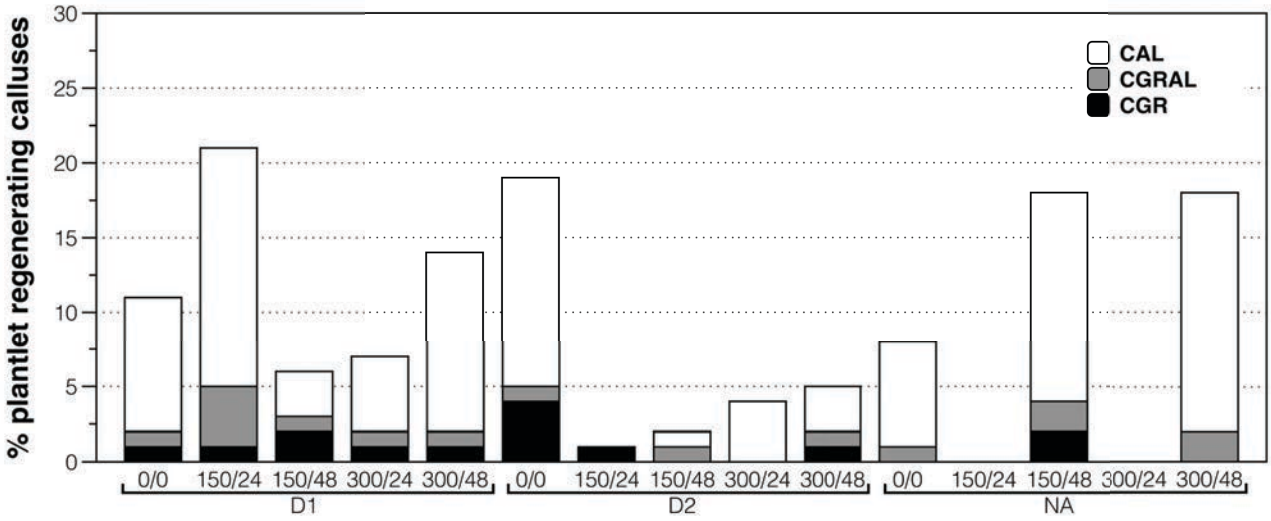

Figure 3.3.2.2. Percentage of plantlets regenerating calluses for each induction media assayed in NRVC980385. CGR: *callus* regenerating green plantlets, CGRAL: *callus* regenerating green and albino plantlets, CAL: *callus* regenerating albino plantlets.

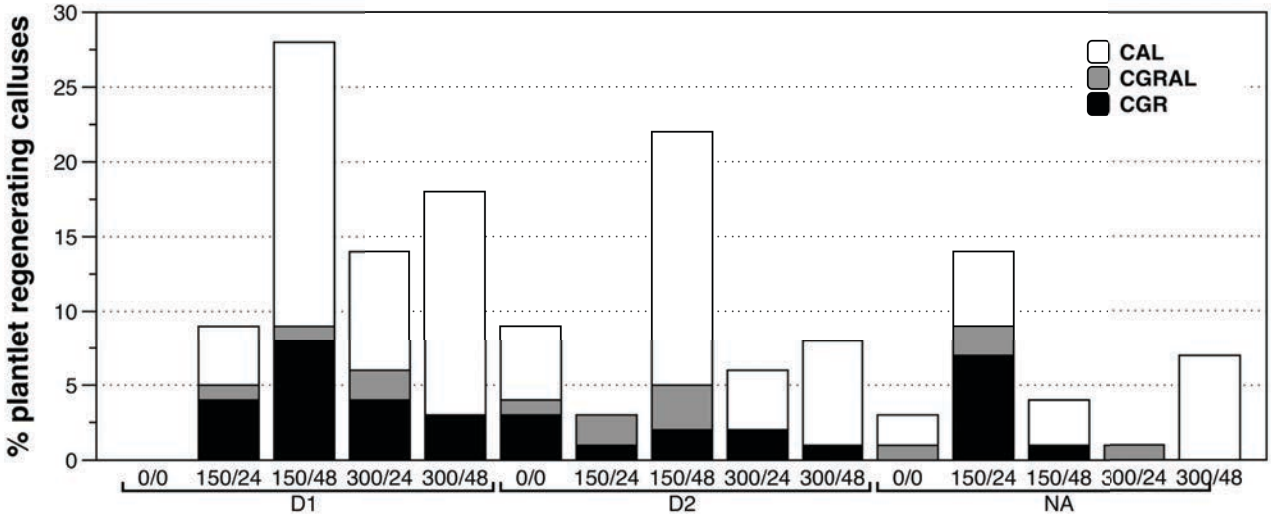

Figure 3.3.2.3. Percentage of plantlets regenerating calluses for each induction media assayed H28. CGR: *callus* regenerating green plantlets, CGRAL: *callus* regenerating green and albino plantlets, CAL: *callus* regenerating albino plantlets.

NRVC980385 regenerated green plantlets in all D1 hormone media (1 mg·L^{-1} of 2,4-D and 1 mg·L^{-1} kinetin). In contrast, under D2 hormone conditions (2 mg·L^{-1} of 2,4-D and 1 mg·L^{-1}

kinetin) the values for the total number of calluses regenerating plantlets were higher under colchicine-free conditions, with the number of green plantlets also being higher. Plantlets regenerated in the 150/24-D2 treatment were all green, unlike 300/24-D2 where all plantlets were albino. Finally, 0/0-NA and 300/48-NA conditions caused calluses to regenerate either albino plantlets or albino and green plantlets, but none of the calluses regenerated green plantlets alone.

The ability of H28 calluses to regenerate green plantlets was higher than NRVC980385 in almost all conditions (Figure 3.3.2.2 and Figure 3.3.2.3). Furthermore, colchicine treatments combined with D1 and NA hormone conditions displayed higher CGR than their control treatments (0/0). Meanwhile, D2 hormone treatment without colchicine regenerated a higher number of green plantlets compared to colchicine treatments with this hormone, a tendency also observed for NRVC980385. Finally, 0/0-NA, 300/24-NA and 300/48-NA conditions had no calluses that regenerated green plantlets exclusively.

EFFECTS OF COLCHICINE TREATMENT ON DHPR*, G/A AND* GDHPR

The hormone factor has been grouped for both genotypes with the aim of analysing the colchicine effect on regenerated double haploid plantlets (DHPR), the green/albino plantlet ratio (G/A) and regenerated green double haploid plantlets (GDHPR) (Table 3.3.2.4). Values of DHPR and GDHPR for NRVC980385 were greater than for H28, but on average the G/A ratio was lower in NRVC980385 (Table 3.3.2.4). In addition, DHPR values for H28 in the four colchicine treatments at 48 h were higher than in the control, with both treatments showing significant differences.

On the most part, the G/A ratio for NRVC980385 and H28 was not significantly affected by the colchicine treatments. The one exception was H28 in the 150/24 colchicine treatment, which had a doubled G/A ratio in comparison to the colchicine-free control.

Calluses from all colchicine treatments regenerated green double haploid plantlets and the values were similar. The values for regenerated green double haploid plantlets (GDHPR) were always higher on colchicine treatments than on the colchicine-free treatment for both genotypes, with NRVC980385 having the highest values. For NRVC980385, the 150/24 and 300/48 colchicine treatments were significantly different ($P<0.05$) from their 0/0 controls, with the number of regenerated green double haploid plantlets being 2.5 and 4 times higher respectively. For H28, the GDHPR values showed no significant differences when compared to its control due to the absence of regenerating green double haploid plantlets on 0/0. The best colchicine treatments for H28 were 150 $mg \cdot L^{-1}$ at 24 and 48 hours.

Table 3.3.2.4. Regenerating double haploid plantlets per 100 plated anthers (DHPR), green/albino plantlets ratio (G/A) and regenerating green double haploid plantlets per 100 plated anthers (GDHPR) by genotypes according to colchicine treatment.

Colchicine (concentration[a]/time[b])	DHPR		G/A		GDHPR	
	NRVC[c]	H28	NRVC	H28	NRVC	H28
0/0	3.89	0.23	0.19	0.67	0.18	0.00
150/24	2.38	0.54	0.14	1.33	0.49*	0.22
150/48	1.77*	2.26*	0.28	0.55	0.22	0.29
300/24	1.80*	0.46	0.09	0.65	0.31	0.12
300/48	4.22	1.34*	0.12	0.20	0.75*	0.17

[a]$mg \cdot L^{-1}$, [b]hours, [c]NRVC980385

HAPLOID DIPLOIDIZATION

Application of antimitotic agents to haploid plantlets obtained from the anther culture procedure hindered the rate of plantlet survival. After antimitotic treatment, most analyzed plantlets were haploids (Table 3.3.2.5). Plantlets showing only double haploid ploidy were observed at the lower antimitotic concentrations (250 $mg \cdot L^{-1}$ of colchicine and 1.25 $mg \cdot L^{-1}$ of oryzalin). Within the plantlets that changed his chromosome content, mixiploids, including

double haploid ploidy, were the majority. Moreover, when a higher antimitotic concentration was used the plantlet mortality rate increased, reaching 91.11% mortality with 1000 mg·L^{-1} of colchicine. In the case of oryzalin, the mid-range concentration treatment (2.5 mg·L^{-1}) showed the highest mortality, with a value of 34.78%. The percentage of plantlets that remained haploid after the treatment was higher when the antimitotic oryzalin was used (Table 3.3.2.5) at any concentration. The percentage of haploid plantlets and dead plantlets tended to increase with increases in the antimitotic concentrations.

Table 3.3.2.5. Post anther culture parameters of treated plants. H: haploid, DH: double haploid.

Antimitotic	**Concentration (mg·L^{-1})**	**Post anther culture parameters**					
		Total plants	**% H**	**% DH**	**% 2n and mixploid**	**% mixploid**	**% dead**
Control	0	20	100	0.00	0.00	0.00	0.00
Colchicine	250	47	38.30	2.13	29.79	2.13	27.66
	500	37	18.92	0.00	35.14	0.00	45.95
	1000	45	4.44	0.00	4.44	0.00	91.11
Oryzalin	1.25	31	77.42	6.45	3.23	0.00	12.90
	2.5	46	60.87	0.00	2.17	2.17	34.78
	5	47	70.21	0.00	6.38	0.00	23.40

DISCUSSION

Anther culture is a powerful technique to produce rice DH plants. Nevertheless, the genotype effect is the major limiting factor, causing a differential response in *callus* induction as well as plantlet regeneration, ploidy and pigmentation previously reported by a number of authors Mishra and Rao 2016; Herath et al. 2010; Khanna and Raina 1998; Raina and Zapata 1997; Moloney et al. 1989). Additionally, obtaining a high number of regenerating calluses is essential to increase the number of green double haploid plantlets displaying differential genotypes. Regenerated plantlets from the same *callus* or calluses from the same anther are more likely to be clones and therefore will have poor genetic variability. In addition, due to the fact that anther culture is a two-step process (i.e. initial development of calluses and subsequent regeneration of green plantlets from embryogenic calluses), researchers interested in obtaining new rice varieties from anther culture must avoid bottlenecks in the procedure. Low *callus* induction, low green plantlet regeneration and low double haploid regeneration can drastically limit the outcomes of anther culture. To minimize this, we proposed a workflow to study the response of the desired genotypes to anther culture over a six-month period using a range of induction media. Consequently, this study has focused on making preliminary assays to determine factors that could improve the yield of green double haploid plants. Additionally, the procedure has reported ways of reducing the amount of work to obtain DH plants in rice by: (i) reducing the time needed for the anther culture procedure, (ii) introducing a fast ploidy determination method, and (iii) a post anther culture diploidization protocol for rice.

We included in our study the NRVC980385 genotype and the growth regulator 2,4-D in the induction medium at 2 $mg \cdot L^{-1}$ in order to compare results with Serrat et al. (2014). In fact, the number of induced calluses was higher in our study than the previous one, which in turn

resulted in a greater number of green double haploid plantlets. The cold pre-treatment was the main difference between the studies, which was changed from 7ºC to 10ºC and adjusted from a variable 7-12 days to a fixed 9 days. Another factor that could have affected the results is that in this experiment NRVC980385 genotype was a stabilized commercial genotype rather than a heterozygous seed batch as used previously in Serrat et al. (2014). Many authors have confirmed that cold pre-treatment has a stimulatory effect on androgenic response in several genotypes Tian et al. 2015; Herath et al. 2010; Touraev et al. 2009). Moreover, a temperature of 10ºC is commonly used as a cold pre-treatment (Rukmini et al. 2013; Naik et al. 2017). Indeed, Naik et al. (2017) described that 7 days at 10ºC resulted in the best *callus* induction in a japonica cultivar. Therefore, the changes in cold pre-treatment enhanced the anther culture protocol with a higher rate of induced calluses.

Among the auxins, 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthaleneacetic acid (NAA) are the most commonly used hormones for rice *callus* induction from anthers (Trejo-Tapia et al. 2002). The colchicine-free D2 treatment (2 $mg \cdot L^{-1}$ of 2,4-D and 1 $mg \cdot L^{-1}$ of kinetin), induced the best culture conditions overall for both the NRVC980385 and H28 genotypes. These results agree with many authors who have determined that 2 $mg \cdot L^{-1}$ of 2,4-D results in the best culturability results for many genotypes (Chen et al. 2002; Herath et al. 2008). The effect of 2,4-D may be the promotion of rapid cell proliferation and formation of non-embryogenic *callus* as described for spring wheat (Ball et al. 1993). In addition, this auxin at this concentration is widely used in rice anther culture, although regularly combined with other auxins or other cytokines to obtain the best results (Serrat et al. 2014; Afza et al. 2000; Kaushal et al. 2014; Chen et al. 2002). In contrast, the effect of NAA, which is also commonly used in rice anther culture, may be to induce direct androgenesis (Yi et al. 2015; Alemanno and Guiderdoni 1994; Reiffers and Freire 1990). Finally, many authors use combinations of

2,4-D and NAA, to obtain better results (He et al. 2006; Xie et al. 1995). Nevertheless, our results do not show clear patterns of rice anther culturability between the two growth regulators.

A differential anther culture response was observed when adding colchicine to the induction medium, which depended on the genotype cultured. NRVC980385 *callus* induction was negatively affected by colchicine. On the other hand, H28 induced more *calluses* on colchicine-supplemented induction media. In maize, an increase in embryo frequency has been reported in the presence of colchicine (Barnabás et al. 1999; Obert and Barnabás 2004). In wheat, an absence of effect of colchicine on microspore embryogenesis has been reported by Barnabás et al. (1991), and a reduction was reported by Navarro-Alvarez et al. (1994). In the present study, 75% of the colchicine-supplemented induction media assayed for H28 had significant *callus* induction enhancement. Moreover, H28 *callus* induction increased in both colchicine concentrations at 48 h for all of the growth regulators assayed. This effect also agrees with Alemanno and Guiderdoni (1994), who described a significant (50%) increase in rice anther *callusing* with 500 $mg \cdot L^{-1}$ of colchicine at 24 and 48 h.

The ability to regenerate plantlets, and specifically green plantlets, was different between the genotypes and the media assayed. NRVC980385 in colchicine-supplemented induction media had no positive effects on green plantlet regeneration. Indeed, treatments with 2,4-D at 1 $mg \cdot L^{-1}$ regenerated as efficiently with colchicine-supplementation as the colchicine-free control. The ability of H28 to regenerate plantlets from *calluses* was greater than NRVC980385. At 1 $mg \cdot L^{-1}$ of 2,4-D, colchicine increased the percentage of regenerating *calluses*, which was defined as the ability to regenerate higher numbers of green plantlets. Alemanno and Guiderdoni (1994) doubled the number of green plantlet-regenerating *calluses* with 250 $mg \cdot L^{-1}$ of colchicine for 24 h relative to the colchicine-free treatment using the Miara

genotype. In contrast, colchicine in the regenerating medium at 30 mg·L^{-1} increased 7 times the number of green plantlets in comparison to the control in the Zao jing 26 genotype (Chen et al. 2002). Like these earlier reports, the number of calluses that regenerate green plantlets in H28 genotype, was increased. It is clear from the current work and previous reports that there is a strong effect of genotype on the outcome of anther culture.

In our study, the incidence of albinism was high for both genotypes. Albinism in plants is characterized by a lack of chlorophyll pigments and/or incomplete differentiation of chloroplast membranes in normally green tissues. Many studies have suggested that the use of colchicine in anther culture reduces the albinism ratio (Kumari et al. 2009; Barnabás et al. 1991; Ferrie et al. 2014). In our study, a reduction in albinism was only observed with 150 mg·L^{-1} of colchicine during the 24 h treatment of the H28 genotype. This observation agrees with other authors who have noted no increases in the proportions of green plantlets after colchicine treatment at the *callus* stage (Alemanno and Guiderdoni 1994; Hansen and Andersen 1998). Furthermore, the number of double haploid regenerated plantlets also seems to be unaffected by colchicine treatments, irrespective of the different concentrations and exposure times tested. These results are in contrast with reports of an increase in regenerating double haploid plantlets when using colchicine (Barnabás et al. 1991). In our work, the ploidy of green and albino plantlets was analyzed to obtain the parameter of regenerated double haploid plantlets (DHPR), whereas other authors have usually only considered the regenerated green plantlets. Nevertheless, in our study, colchicine-supplemented media increased the percentagen of regenerated green double haploid plantlets (GDHPR), which is in accordance with other studies (Alemanno and Guiderdoni 1994; Barnabás et al. 1991; Weber, Ünker et al. 2005). Finally, all colchicine treatments yielded higher proportions of regenerated green double haploid plantlets for both genotypes

in comparison to the colchicine-free induction media. In NRVC980385, the 150 $mg \cdot L^{-1}$ colchicine treatment for 24 h and the 300 $mg \cdot L^{-1}$ treatment for 48 h gave the best results ($P<0.05$) compared to 0/0 control, and the numbers of GDHPR s were 2.5 and 4 times greater than the control, respectively. The H28 genotype was not able to regenerate green double haploid plantlets in colchicine-free induction media, and because of that a statistical test was not possible. This lack of green double haploid plantlets from colchicine-free media may be due to a low endoreduplication or low ability for endomitosis in H28, which entails a spontaneous duplication of chromosomes from the haploid (Chen and Chen 1980).

In both genotypes assayed, colchicine seemed to affect the endomitosis rate in the treated microspores. Endomitosis is described as nuclear chromosome doubling due to a failure of the spindle during metaphase (Kasha 2005). C-mitosis is a form of endomitosis caused by colchicine, which has the ability to abort mitosis and inhibit tubulin polymerization in animal and plant cells (Pickett-Heaps 1967; Fltzgerald 1976; Kasha 2005), and this explains the high yield of green double haploid plantlets in rice. Our observed 2.5-fold increase in the proportion of *calluses* regenerating diploid green plantlets is in accordance with the work of Alemanno and Guiderdoni (1994). Chen et al. (2002) also observed an increase in regenerated green double haploid plantlets when using regeneration media fortified with 75 $mg \cdot L^{-1}$ colchicine, although higher concentrations caused harmful effects on *calluses* and regenerated plantlets. For this reason we assayed 300 $mg \cdot L^{-1}$ and 150 $mg \cdot L^{-1}$ concentrations to test lower concentrations than the 500 $mg \cdot L^{-1}$ used by Alemanno and Guiderdoni (1994) and delimit the best colchicine concentration while avoiding toxicity. Finally, colchicine is widely used to increase the number of green double haploid plantlets in anther culture of other species and has had positive results in wheat (Hansen and Andersen 1998; Soriano et al. 2007), maize (Saisingtong et al. 1996), oats (Ferrie et al. 2014) and rapeseed (Mollers et al. 1994; Weber

et al. 2005). Despite our results and those reported in the literature, we suggest that further studies should be performed in rice to investigate the ability of colchicine to increase the numbers of regenerated green double haploids above albino double haploids.

The main limitation of anther culture is the unknown interaction that occurs between media and genotypes. Our results describe completely different responses for *callus* induction, regenerating *calluses*, plant albinism and the ploidy of regenerated plantlets that are dependent on the growth regulator, their concentrations and the exposure time. Many authors have reported that the genotype affects the androgenic response (Lentini et al. 1997; He et al. 2006; Bagheri and Jelodar 2008) and that changes in medium composition can alter the response of different rice cultivars (Trejo-Tapia et al. 2002; Q. F. Chen et al. 2002; Herath et al. 2008; Herath et al. 2010). However, manipulation of colchicine in induction media has not been reported previously for rice.

A complementary way to obtain DH plants is to perform a post anther procedure treatment of green haploid plantlets with antimitotics. It has been widely reported that antimitotic treatments of plantlets may change their ploidy (Chen et al. 2002; Ascough et al. 2008; Gallone et al. 2014; Sarathum et al. 2010; Omidbaigi et al. 2012; de Carvalho et al. 2005). In this study, the *in vitro* production of double haploid plantlets from already formed haploid plantlets was achieved. Most of the plantlets that survived were mixiploid with levels of diploidy, and it was from these latter plants that we were able to obtain double haploid seed from those tillers that were double haploids. Colchicine at 500 $mg \cdot L^{-1}$ was the best *in vitro* treatment to double the ploidy (35.14% of plantlets treated). This concentration of colchicine was also used previously in an *in vivo* treatment of tillers with an effectiveness of 11.5% (Chen et al. 2002). Ascough et al. 2008 reported that when lower antimitotic concentrations were used the number of surviving plantlets was higher, but on the other hand the level of

diploidization was also lower. Omidbaigi et al. 2012 reported that high concentrations of oryzalin did not have much effect on the survival ratio.

Anther culture in rice has been studied in many genotypes to achieve the best method of maximizing green double haploid plantlet formation through different stresses. Taking this earlier work into account, we selected a range of stresses to formulate protocols for two Mediterranean japonica rice genotypes that will form the basis of an anther culture procedure for a wide range of genotypes. The genotypes trialled each had specific responses to the experimental conditions. We have demonstrated that cold pre-treatment at 10ºC for 9 days increases *callus* induction. Without colchicine in the induction medium, we recommend 2,4-D at 2 $mg \cdot L^{-1}$ and kinetin at 1 $mg \cdot L^{-1}$ to obtain the highest values for *callus* induction and green double haploid plantlet regeneration for Mediterranean japonica rice varieties. Colchicine-supplemented induction media may increase the level of *callus* induction, depending on the genotype. Colchicine on the induction medium increases the green double haploid plantlet production in all treatments of concentration and time assayed. We have shown that post anther culture colchicine treatment at 500 $mg \cdot L^{-1}$ increases the green double haploid production from green haploid plantlets. Our results highlight the importance of the genotype and media interactions effects on the anther culture efficiency in rice. This study stands out the necessity to continue studying the response of rice to anther culture to better understand the main mechanism of interaction between genotype and induction media.

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The authors declare that they have no conflict of interest.

REFERENCES - CHAPTER 3.2

Afza, R., Shen, M., Zapata-Arias, F. J., Xie, J., Fundi, H. K., Lee, K. S., ... & Kodym, A. (2000). Effect of spikelet position on rice anther culture efficiency. Plant Science, 153(2), 155-159.

Alemanno, L, and E Guiderdoni. 1994. “Increased Doubled Haploid Plant Regeneration from Rice (Oryza Sativa L.) Anthers Cultured on Colchicine-Supplemented Media.” *Plant Cell Reports* 13 (8): 432–36. doi:10.1007/BF00231961.

Ascough, G. D., Van Staden, J., and Erwin, J.E.. 2008. “Effectiveness of Colchicine and Oryzalin at Inducing Polyploidy in *Watsonia Lepida* NE Brown.” *HortScience* 43 (7). American Society for Horticultural Science: 2248–51.

Bagheri, N, and N B Jelodar. 2008. “Combining Ability and Heritability of Callus Induction and Green Plant Regeneration in Rice Anther Culture.” *Biotechnology* 7 (2): 287–92.

Ball, S. T., Zhou, H. and Konzak, C. F.. 1993. “Influence of 2, 4-D, IAA, and Duration of Callus Induction in Anther Cultures of Spring Wheat.” *Plant Science* 90 (2). Elsevier: 195–200.

Barnabás, B, B Obert, and G Kovács. 1999. “Colchicine, an Efficient Genome-Doubling Agent for Maize (*Zea Mays* L.) Microspores Cultured in Anthero.” *Plant Cell Reports* 18 (10). Springer: 858–62.

Barnabás, B, P L Pfahler, and G Kovacs. 1991. “Direct Effect of Colchicine on the Microspore Embryogenesis to Produce Dihaploid Plants in Wheat (*Triticum Aestivum* L.).” *TAG Theoretical and Applied Genetics* 81 (5). Springer: 675–78.

de Carvalho, J. F. R. P., de Carvalho, C. R. D. P., & Otoni, W. C. 2005. “In Vitro Induction of Polyploidy in Annatto (*Bixa Orellana*).” Plant Cell, Tissue and Organ Culture 80 (1): 69–75. doi:10.1007/s11240-004-8833-5.

Chen, C. C., & Chen, C. M. 1980. “Changes in Chromosome Number in Microspore Callus of Rice during Successive Subcultures.” *Canadian Journal of Genetics and Cytology* 22 (4). NRC Research Press: 607–14. doi:10.1139/g80-066.

Chen, C. C., Tsay, H. S., & Huang, C. R. 1991. “Factors Affecting Androgenesis in Rice (*Oryza Sativa* L.).” In *Rice*, edited by Y P S Bajaj, 193–215. Berlin, Heidelberg: Springer Berlin Heidelberg. doi:10.1007/978-3-642-83986-3_14.

Chen, Q F, C L Wang, Y M Lu, M Shen, R Afza, M V Duren, and H Brunner. 2002. “Anther Culture in Connection with Induced Mutations for Rice Improvement.” In *Mutations, In Vitro and Molecular Techniques for Environmentally Sustainable Crop Improvement*, 83–92. Springer.

Chu, C-C. 1975. “Establishment of an Efficient Medium for Anther Culture of Rice through Comparative Experiments on the Nitrogen Sources.” *Scientia Sinica* 18: 659–68.

Cousin, A.;, K.; Heel, W.A.; Cowling, and M.N.; Nelson. 2009. “An Efficient High-Throughput Flow Cytometric Method for Estimating DNA Ploidy Level in Plants.” *Cytometry* 75: 1015–19. doi:10.1002/cyto.a.20816.

Ferrie, A M R, K I Irmen, A D Beattie, and B G Rossnagel. 2014. “Isolated Microspore Culture of Oat (*Avena Sativa* L.) for the Production of Doubled Haploids: Effect of Pre-Culture and Post-Culture Conditions.” *Plant Cell, Tissue and Organ Culture (PCTOC)* 116 (1). Springer: 89–96.

Fltzgerald, T. J. 1976. “Molecular Features of Colchicine Associated with Antimitotic Activity and Inhibition of Tubulin Polymerization.” *Biochemical Pharmacology* 25 (12): 1383–87. doi:10.1016/0006-2952(76)90108-8.

Forster, B. P., Heberle-Bors, E., Kasha, K. J., and Touraev, A. 2007. “The Resurgence of Haploids in

Higher Plants." *Trends in Plant Science* 12 (8): 368–75. doi:10.1016/j.tplants.2007.06.007.

Gallone, A., Hunter A., and Douglas, G. C. 2014. "Polyploid Induction in Vitro Using Colchicine and Oryzalin on Hebe '*Oratia Beauty*': Production and Characterization of the Vegetative Traits." *Scientia Horticulturae* 179: 59–66. doi:http://dx.doi.org/10.1016/j.scienta.2014.09.014.

Germanà, M. A. 2011. "Anther Culture for Haploid and Doubled Haploid Production." *Plant Cell, Tissue and Organ Culture (PCTOC)* 104 (3): 283–300. doi:10.1007/s11240-010-9852-z.

Hansen, N J P, and S Bode Andersen. 1998. "In Vitro Chromosome Doubling with Colchicine during Microspore Culture in Wheat (*Triticum Aestivum* L.)." *Euphytica* 102 (1). Springer: 101–8.

He, T, Y Yang, S B Tu, M Q Yu, and X F Li. 2006. "Selection of Interspecific Hybrids for Anther Culture of Indica Rice." *Plant Cell, Tissue and Organ Culture* 86 (2). Springer: 271–77.

Herath, H M I, D C Bandara, and P K Samarajeewa. 2010. "Effect of Culture Media for Anther Culture of Indica Rice Varieties and Hybrids of Indica and Japonica." *Tropical Agricultural Research and Extension* 10. Faculty of Agriculture, University of Ruhuna.

Herath, H M I, D C Bandara, P K Samarajeewa, and D S A Wijesundara. 2008. "The Effect of Plant Growth Regulators on Anther Culture Response and Plant Regeneration in Selected Sri Lankan Indica Rice Varieties, Japonica Varieties and Their Inter-Sub Specific Hybrids." Postgraduate Institute of Agriculture, University of Peradeniya: Peradeniya.

Jensen, C J. 1974. "Chromosome Doubling Techniques in Haploids." The University of Guelph.

Kasha, Ken J. 2005. "Chromosome Doubling and Recovery of Doubled Haploid Plants." *Haploids in Crop Improvement II*. Springer, 123–52.

Kaushal, L., Balachandran, S. M., Ulaganathan, K., & Shenoy, V. 2014. "Effect of Culture Media on Improving Anther Culture Response of Rice (*Oryza Sativa* L.)." *International Journal of Agriculture Innovations and Research* 3 (1). International Journal of Agriculture Innovations and Research: 218–24.

Khanna, H K, and S K Raina. 1998. "Genotype X Culture Media Interaction Effects on Regeneration Response of Three Indica Rice Cultivars." *Plant Cell, Tissue and Organ Culture* 52 (3): 145–53. doi:10.1023/A:1006032303195.

Kumari, M., Clarke, H. J., Small, I., & Siddique, K. H. 2009. "Albinism in Plants: A Major Bottleneck in Wide Hybridization, Androgenesis and Doubled Haploid Culture." *Critical Reviews in Plant Science* 28 (6). Taylor & Francis: 393–409.

Lentini, Z., Roca, W. M., and Martinez, C. P. 1997. *Cultivo de Anteras de Arroz En El Desarrollo de Germoplasma*. Vol. 293. CIAT.

Maluszynska, J. 2003. "Cytogenetic Tests for Ploidy Level Analyses—chromosome Counting." In *Doubled Haploid Production in Crop Plants*, 391–95. Springer.

Mishra, R., & Rao, G. J. N. 2016. "In-Vitro Androgenesis in Rice: Advantages, Constraints and Future Prospects." *Rice Science* 23 (2). Elsevier: 57–68.

Möllers, C, M C M Iqbal, and G Röbbelen. 1994. "Efficient Production of Doubled Haploid *Brassica Napus* Plants by Colchicine Treatment of Microspores." *Euphytica* 75 (1): 95–104. doi:10.1007/BF00024536.

Moloney, M. M., Walker, J. M., & Sharma, K. K. 1989. "High Efficiency Transformation of *Brassica Napus* Using Agrobacterium Vectors." *Plant Cell Reports* 8 (4). Springer: 238–42.

Murashige, T., and Skoog F.. 1962. "A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures." *Physiologia Plantarum* 15 (3). Wiley Online Library: 473–97.

Naik, N., Rout, P., Umakanta, N., Verma, R. L., Katara, J. L., Sahoo, K. K., ... & Samantaray, S. 2017.

"Development of Doubled Haploids from an Elite Indica Rice Hybrid (BS6444G) Using Anther Culture." *Plant Cell, Tissue and Organ Culture (PCTOC)* 128 (3). Springer: 679–89.

Navarro-Alvarez, W, P S Baenziger, K M Eskridge, M Hugo, and V D Gustafson. 1994. "Addition of Colchicine to Wheat Anther Culture Media to Increase Doubled Haploid Plant Production." *Plant Breeding* 112 (3). Wiley Online Library: 192–98.

Niizeki, H., and Oono, K. 1968. "Induction of Haploid Rice Plant from Anther Culture." *Proceedings of the Japan Academy* 44 (6). The Japan Academy: 554–57.

Obert, B., & Barnabás, B. 2004. "Colchicine Induced Embryogenesis in Maize." *Plant Cell, Tissue and Organ Culture* 77 (3): 283–85. doi:10.1023/B:TICU.0000018399.60106.33.

Omidbaigi, R, M Mirzaee, M E Hassani, and M Sedghi Moghadam. 2012. "Induction and Identification of Polyploidy in Basil (*Ocimum Basilicum* L.) Medicinal Plant by Colchicine Treatment." *International Journal of Plant Production* 4 (2): 87–98.

Pauk, J, M Jancsó, and I Simon-Kiss. 2009. "Rice Doubled Haploids and Breeding." In *Advances in Haploid Production in Higher Plants*, 189–97. Springer.

Pickett-Heaps, J D. 1967. "The Effects of Colchicine on the Ultrastructure of Dividing Plant Cells, Xylem Wall Differentiation and Distribution of Cytoplasmic Microtubules." *Developmental Biology* 15 (3). Elsevier: 206–36.

Raina, S. K., and F. J. Zapata. 1997. "Enhanced Anther Culture Efficiency of Indica Rice (*Oryza Sativa* L.) through Modification of the Culture Media." *Plant Breeding* 116 (4). Blackwell Publishing Ltd: 305–15. doi:10.1111/j.1439-0523.1997.tb01004.x.

Reiffers, I., and Adelson B F.. 1990. "Production of Doubled Haploid Rice Plants (*Oryza Sativa* L.) by Anther Culture." *Plant Cell, Tissue and Organ Culture* 21 (2): 165–70. doi:10.1007/BF00033437.

Rukmini, M, G J N Rao, and R N Rao. 2013. "Effect of Cold Pretreatment and Phytohormones on Anther Culture Efficiency of Two Indica Rice (*Oryza Sativa* L.) Hybrids-Ajay and Rajalaxmi." *J. Exp. Biol. Agr. Sci* 2: 69–76.

Saisingtong, S, J E Schmid, P Stamp, and B Büter. 1996. "Colchicine-Mediated Chromosome Doubling during Anther Culture of Maize (*Zea Mays* L.)." *Theoretical and Applied Genetics* 92 (8). Springer: 1017–23.

Sarathum, S, M Hegele, S Tantiviwat, and M Nanakorn. 2010. "Effect of Concentration and Duration of Colchicine Treatment on Polyploidy Induction in *Dendrobium Scabrilingue* L." *European Journal of Horticultural Science* 75 (3). Verlag Eugen Ulmer KG: 123–27. http://www.jstor.org.sire.ub.edu/stable/24126421.

Serrat, X, M Cardona, J Gil, A M Brito, L Moysset, S Nogués, and E Lalanne. 2014. "A Mediterranean Japonica Rice (*Oryza Sativa*) Cultivar Improvement through Anther Culture." *Euphytica* 195 (1): 31–44. doi:10.1007/s10681-013-0955-6.

Soriano, M., Cistué L., Vallés M. P., and Castillo A. M. 2007. "Effects of Colchicine on Anther and Microspore Culture of Bread Wheat (*Triticum Aestivum* L.)." *Plant Cell, Tissue and Organ Culture* 91 (3). Springer: 225–34.

Thompson, D.M., K. Chalmers, R. Waugh, B.P. Forster, W.T.B. Thomas, P.D.S. Caligari, and W. Powell. 1991. "The Inheritance of Genetic Markers in Microspore-Derived Plants of Barley *Hordeum Vulgare* L." *Theoretical and Applied Genetics* 81 (4): 487–92. doi:10.1007/BF00219438.

Tian, Q Q, C M Lu, X Li, and X W Fang. 2015. "Low Temperature Treatments of Rice (*Oryza Sativa* L.) Anthers Changes Polysaccharide and Protein Composition of the Anther Walls and Increases

Pollen Fertility and Callus Induction." *Plant Cell, Tissue and Organ Culture (PCTOC)* 120 (1). Springer: 89–98.

Touraev, A., Forster, B. P., & Jain, S. M. 2009. *Advances in Haploid Production in Higher Plants*. Springer.

Trejo-Tapia, G., Amaya, U. M., Morales, G. S., Sánchez, A. D. J., Bonfil, B. M., Rodríguez-Monroy, M., & Jiménez-Aparicio, A. 2002. "The Effects of Cold-Pretreatment, Auxins and Carbon Source on Anther Culture of Rice." *Plant Cell, Tissue and Organ Culture* 71 (1). Springer: 41–46.

Weber, S, F Ünker, and W Friedt. 2005. "Improved Doubled Haploid Production Protocol for *Brassica Napus* Using Microspore Colchicine Treatment in Vitro and Ploidy Determination by Flow Cytometry." *Plant Breeding* 124 (5). Wiley Online Library: 511–13.

Xie, J., Gao, M., Cai, Q., Cheng, X., Shen, Y., & Liang, Z. 1995. "Improved Isolated Microspore Culture Efficiency in Medium with Maltose and Optimized Growth Regulator Combination in Japonica Rice (*Oryza Sativa*)." *Plant Cell, Tissue and Organ Culture* 42 (3): 245–50. doi:10.1007/BF00029994.

Yi, G., Lee, H. S., & Kim, K. M. 2015. "Improved Marker-Assisted Selection Efficiency of Multi-Resistance in Doubled Haploid Rice Plants." *Euphytica* 203 (2): 421–28. doi:10.1007/s10681-014-1303-1.

Zapata-Arias, F. J. 2003. "Laboratory Protocol for Anther Culture Technique in Rice." In *Doubled Haploid Production in Crop Plants*, 109–16. Springer.

4

GENERAL DISCUSSION

Rice (*Oryza sativa* L.) is without doubt one of the major crops worldwide, as its consumption is continuously increasing, especially in low- and lower-middle-income countries where it is the most important staple (McLean et al. 2013; FAO 2018b). This cereal has been domesticated for a long time, fruit of which several species and varieties are now available. Moreover, it was key during the green revolution where its production more than doubled and has a deep cultural background in all the regions it is grown. Hence, it is of uttermost importance for researchers and breeders to broaden and expand the knowledge we have on this cereal on all the study and research areas, especially now that we are living in the 21st century, which is marked by climate change. This phenomenon is one of the most menacing as it will reduce the quantity and quality of arable land due to salinization of soil as well as water scarcity which is the single most important factor that determines global crop yields (Dunwell 2010; Mickelbart et al. 2015; Shrivastava and Kumar 2015). In this sense, this thesis addressed three important topics on rice:

(i) Analysis of salinity tolerant rice plants subjected to high salt concentration through a combined approach of shotgun proteomics and physiological characterization for the identification of new key proteins involved in the tolerance to this stress.

(ii) Phytohormones analysis in dwarf varieties through a broad phytohormone profiling method developed during this thesis for the characterization of phytohormone levels during the development of plants with contrasting heights that will allow have more information for developing new dwarf mutant varieties.

(iii) Improvement and enhancement of anther culture protocols in rice for obtaining higher rates of stabilized green double haploid plants using different cold-pretreatments, hormones and antimitotics in the growing media for their subsequent commercialization.

As each chapter has an in-depth discussion of each topic discussed, this general discussion will have a different approach that will consist on critically examining the results obtained in each chapter in relation to our aim, which was to study different techniques for evaluating and producing new improved rice varieties consulting the corresponding literature. Finally, an integration of the three chapters that aims to complement all the achievements and give future perspectives on rice in the forthcoming years will be carried out.

6.1 CHAPTER 1 - Proteome profiling in shoots and roots of the FL478 genotype of rice (*Oryza sativa* L. ssp. *indica*) by shotgun proteomics during early salinity stress

As discussed in the introductions section, salinity will be the main abiotic factor affecting rice production as it is the most salt-sensitive cereal worldwide and will therefore lower yields in all rice producing areas (Sahi et al. 2006; Genua-Olmedo et al. 2016; Reddy et al. 2017; Lakra et al. 2018). In fact, a detailed study analyzed different scenarios of soil salinization in regard to rice production and rice profit in the Ebro Delta (one of Spain main rice producing deltas), and even in the less severe rice production will be reduced from 6512 to profit is still affected (Figure 4.1). Even though several rice strategies for salinity stress tolerance have

been studied and put into evidence which can be grouped in three main mechanisms [Figure 1.12: tissue tolerance, osmotic tolerance and ion exclusion; Roy et al. (2014) and Reddy et al. (2017)], there is still a lot that we do not know. In fact, the *Saltol* region itself is a clear example of this as rice salinity tolerance has been studied during several years only at the beginning of the 2000 this major QTL was discovered [Figure 1.13, Bonilla et al. (2002) and Lin et al. (2004)]. In this context, our study in FL478, a highly salinity tolerant rice variety is of crucial interest for identifying novel candidate protein biomarkers for this stress.

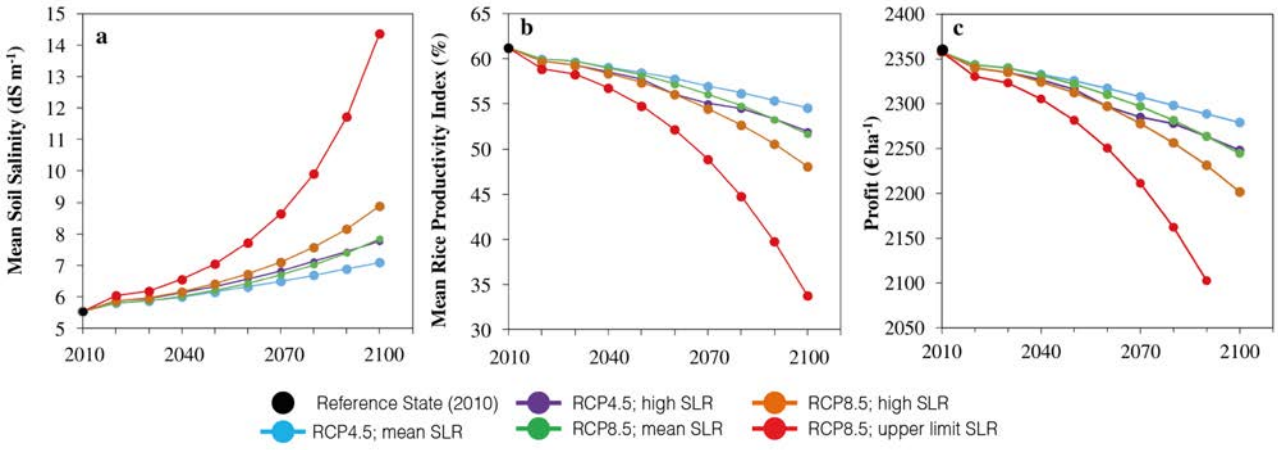


Figure 4.1. Estimated mean value of soil salinity (a); mean RPI (Rice Production Index) (b); and mean profit (c) along the 21st century under the simulated SLR (Sea Level Rise). RCP: Representative Concentration Pathways; 4.5: mitigation scenario; 8.5: "business as usual" scenario. Adapted from Genua-Olmedo et al. (2016).

Before analyzing the proteome and physiological characterization of this rice variety, it is crucial to highlight that this study stands out among other studies that analyzed the rice proteome under salinity as a shotgun proteomics approach was used. This technique, in addition to allowing a large-scale and high-throughput identification, it overcomes the several limitations of the widely used 2D-PAGE such as: low representations of proteins with high molecular weights, high isoelectric points (pIs), hydrophobic domains and those of low

abundance (Mirzaei et al. 2012; Gupta et al. 2015). Analyzing roots, in addition to shoots, is of crucial importance as it is the first tissue directly exposed to the salinity stress (Yan et al. 2005), in both a hydroponic culture and rice paddy fields (although shoot could be also affected due to the water column in which rice is commonly grown). This being said, when the literature was consulted prior to performing this study, the low availability of proteome studies in roots was evident. Therefore, the results obtained from the first chapter are of great importance to researchers and breeders analyzing salinity tolerance in rice as both shoots and roots were analyzed through a shotgun proteomics approach, in which more than 2000 proteins combined were identified to *Oryza sativa* L. spp *indica*. Only 35.7% of all these proteins were matched with annotated proteins suggesting that the rest are unidentified proteins, which could be key in understanding the salinity tolerance of FL478. In fact, Figure 3.1.3 and Figure 3.1.4 for shoot and root respectively show 149 proteins that are significantly different [$p < 0.05$ and a low false discovery rate ($q < 0.15$)] between the mock treatment (0 mM NaCl) and the salinity treatment (100 mM NaCl). Within this subset, nearly half of the proteins are unknown (i.e. not annotated proteins) confirming that there is still much research to do in order to properly identify them.

A schematic summary of some key mechanisms for shoots and roots is presented in Figure 4.2. As a general overview, root changes as observed in the figure are less evident as this tissue is more prepared for facing salinity stress, and therefore its cell machinery is not randomly disrupted and even more keeps its normal functioning, especially for amino acid biosynthesis. More in detail, when analyzing shoots, our results suggest that this tissue is less adapted for tolerating high salinity levels as their response compared to roots is highly unorganized and slower, as evidenced in the volcano plots in Figure 3.1.2. In addition,

physiological characterization agrees with this statement as during salinity stress in root the length is increased, there are no changes in water content and the Na^+/K^+ ratio is increased. Regarding shoot proteins, those related to photosynthesis were down-regulated during the salinity treatment with a concomitant reduction in the relative chlorophyll values (SPAD) suggesting a halt in the photosynthetic processes. These results are in agreement with the study by Moradi et al. (2007) in which in rice plants subjected to salinity the CO_2 fixation, stomatal conductance (gs) and transpiration decreased substantially.

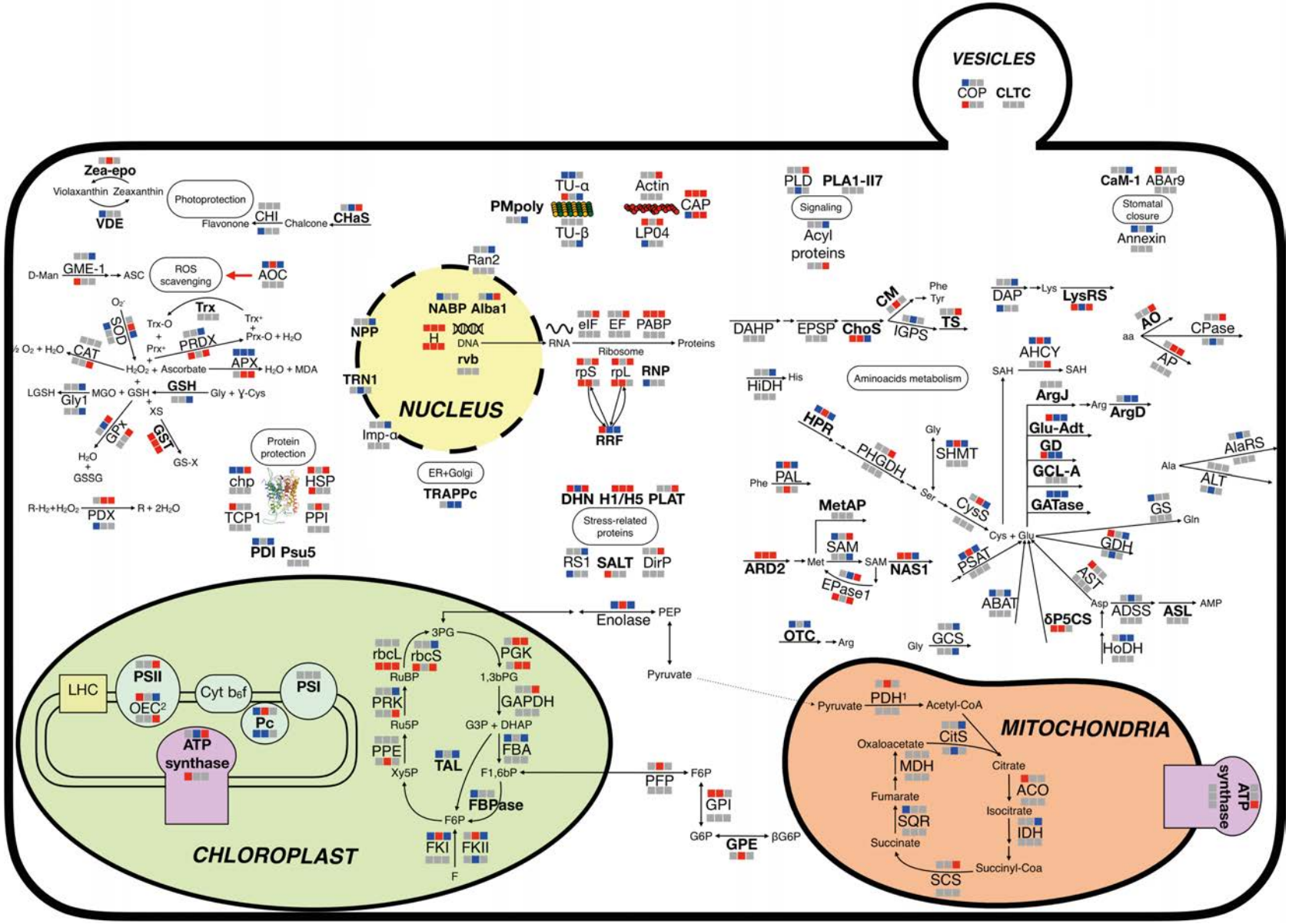
A salinity stress imposes both an osmotic and an ionic stress to the plants, therefore all plants have developed the ability to sense both components of the stress (Moradi and Ismail 2007; Deinlein et al. 2014; Maathuis 2014), nevertheless not all the molecules and enzymes have been fully identified (Deinlein et al. 2014; Roy et al. 2014; Reddy et al. 2017). In this regard, we identified enzymes related to Ca^{2+} sensing [e.g. calmodulin and annexin; Kim et al. (2009a), Jami et al. (2012) and Qiao et al. (2015)]. In fact, a recently published articles performed a genome-wide association study (GWAS) on 235 panel and showed that there was a high number of associations linked to candidate genes involved in calcium-mediated ion homeostasis highlights pathways, which could be explored to understand the salinity tolerance of the accessions (Frouin et al. 2018).

As in several other stresses, salinity also imposes and oxidative stress that should be scavenged by the antioxidant machinery of the cell (Reddy et al. 2017), hence it was evidenced under salinity conditions that FL478 that enzymes involved in this task [APX, CAT, PDX, SOD and GME-1 (L-ascorbic acid biosynthesis)] were up-regulated although with different patterns which is indicative of differential responses involvement. In this line of protection, enzymes involved in protein protection were also up-regulated during salinity such as HSPs, peptidylprolyl isomerase and chaperonins; although this response was more

marked in shoots. Both responses are common to salinity stress and have been evidenced in several works (Dooki et al. 2006; Li et al. 2011; Abdollah Hosseini et al. 2015; Lakra et al. 2018).

Unexpectedly, the *OsHKT1;5* protein was not detected even when using a highly efficient and precise method such as shotgun proteomics, and therefore its presence, at the protein level, still remains in doubt (Singh and Jwa 2013; Abdollah Hosseini et al. 2015; Lakra et al. 2018). Nevertheless, the proteins SalT and PDX, identified within the *Saltol* region were identified, which confirms the presence of this major QTL proteins, which are present in the *Saltol* QTL region. Furthermore, as said before this study provides a huge protein database, in both shoot and root, for future studies of the salinity-stress response in rice varieties harboring the *Saltol* region. Moreover, the protein-protein interaction networks (Figures Figure 3.1.3 and Figure 3.1.4) allow identification of key novel candidates that should have a major role in the tolerance to salinity stress and further detail about these will broaden our knowledge of this stress in rice. Finally, the information presented here expands our knowledge on adaptive processes under high salinity in rice plants and notably, in rice roots.

Figure 4.2 (page 243). Cell diagram of the proteins involved in salinity tolerance in shoots and roots of the salt-tolerant rice variety FL478 (abbreviations are according to Table SM.1.3 and Table SM.1.4 in pages 296 and 323, respectively). Bold abbreviations correspond to proteins exclusively in shoots or roots. The three squares above each protein correspond to the abundance at each time (6, 24 and 48 h), calculated as the fold change in the salinity/mock (100/0 mM NaCl) treatments. Colors in the square correspond to the fold-change: blue denotes down-regulated proteins (<0.9), grey denotes no change (0.9-.1.1) and red denotes up-regulated proteins (<1.1). *abundance corresponds to the average of proteins with the same abbreviation; [1]corresponds to the average of proteins comprising the pyruvate dehydrogenase complex; [2]corresponds to proteins comprising the oxygen evolving complex; [3]corresponds to a mitochondrial protein; [4]corresponds to xenobiotic substrates; [5]corresponds to the peroxidase reaction with its optimal substrate (H_2O_2).


VESICLES
COP CLTC
Zea-epo
Violaxanthin Zeaxanthin
VDE
Photoprotection
Flavonone CHI Chalcone CHaS
PMpoly
TU-α
TU-β
Actin
CAP
LP04
PLD PLA1-II7
Signaling
Acyl proteins
CaM-1 ABAr9
Stomatal closure
Annexin
D-Man GME-1 ASC
ROS scavenging
AOC
Ran2
NABP Alba1
NPP
H
DNA
rvb
TRN1
NUCLEUS
Imp-α
ER+Golgi
TRAPPc
RNA eIF EF PABP Proteins
Ribosome
rpS rpL RNP
RRF
Trx
SOD
PRDX
CAT
APX
GSH
GPx
GST
PDX
Protein protection
chp
HSP
TCP1
PPI
PDI Psu5
DHN H1/H5 PLAT
Stress-related proteins
RS1 SALT DirP
DAHP EPSP ChoS IGPS TS
CM
Phe Tyr
DAP Lys LysRS
AO CPase AP
HiDH His
Aminoacids metabolism
AHCY SAH
ArgJ Arg ArgD
Glu-Adt
GD
GCL-A
GATase
HPR PHGDH Ser CysS
Gly SHMT
Phe PAL
AlaRS Ala ALT
GS Gln
GDH
MetAP
SAM
ARD2 Met SAM NAS1
EPase1
PSAT
AST
ABAT
δP5CS
Asp ADSS ASL AMP
HoDH
OTC Arg
Gly GCS
Cys + Glu
LHC
PSII
OEC2
Cyt b6f
PSI
Pc
ATP synthase
CHLOROPLAST
Enolase PEP
Pyruvate
3PG
rbcL rbcS
RuBP
PGK
1,3bPG
PRK
Ru5P
GAPDH
G3P + DHAP
PPE
Xy5P
TAL
FBA
F1,6bP
FBPase
F6P
FKI FKII
F
PFP
F6P
GPI
G6P
GPE
βG6P
MITOCHONDRIA
Pyruvate PDH1 Acetyl-CoA
Oxaloacetate
CitS
Citrate
MDH
ACO
Fumarate
SQR
Isocitrate
IDH
Succinate
SCS
Succinyl-Coa
ATP synthase

6.2 CHAPTER 2 - Phytohormone profiling method for rice: effects of GA20ox mutation on the gibberellin content of Mediterranean *japonica* rice varieties

In this chapter, three Mediterrean *japonica* rice varieties were analyzed for their gibberellin content and a GA20ox mutation, it is important to re-emphasize that the dwarf-varieties discovered during the green evolution were and still are of great interest as they are far more productive than their wild-type counterpart, hence the name of High Yielding Varieties (HYVs) that was coined to them (Zeng et al. 2014). These HYVs allowed to obtain more than 200% of production with only a 20% crop area increase (Figure 1.4 and Figure 1.5), however, they are associated with reduced germination rates due to prolonged dormancy (Lo et al. 2008). Regarding the varieties used in chapter 2, the *Generalitat de Catalunya* (Spain) classifies Bomba as having medium germination but NRVC980385 as a high germinating variety (Generalitat de Catalunya 2012). Regarding *dwarf*-Bomba, as it is a *GA20ox*-mutated variety its germination is medium likewise for Gleva (i.e. a widely cultivated variety in the region; Català et al. (2007). This being said, *dwarf*-Bomba, Gleva and NRVC980385 have similar heights and productions but the fact that NRVC980385 germinates very well has made it one of the most cultivated crops since 2001, whereas Gleva was only introduced in 2005 (Català et al. 2007). Therefore, studying the levels of the GAs in their biosynthesis pathways is of crucial importance to understand these agricultural differences. In regard to this, it is crucial to highlight that to our knowledge our work is pioneer as it is the first time that such a high number of gibberellins (13 in total) are analyzed in one single run, therefore we have established a new protocol for analyzing rice plants that can give insights for rice variety improvement (Figure 4.3). In addition to the 13 GAs our method allows to detect ABA, JA and IAA, important phytohormones for plant development and stress tolerance (Davies 2010). In

addition, it was also important to select the most adequate rice development system for a proper selection of rice stages to carry this study, and after a broad literature revision the method proposed by Counce et al. (2000) was chosen.

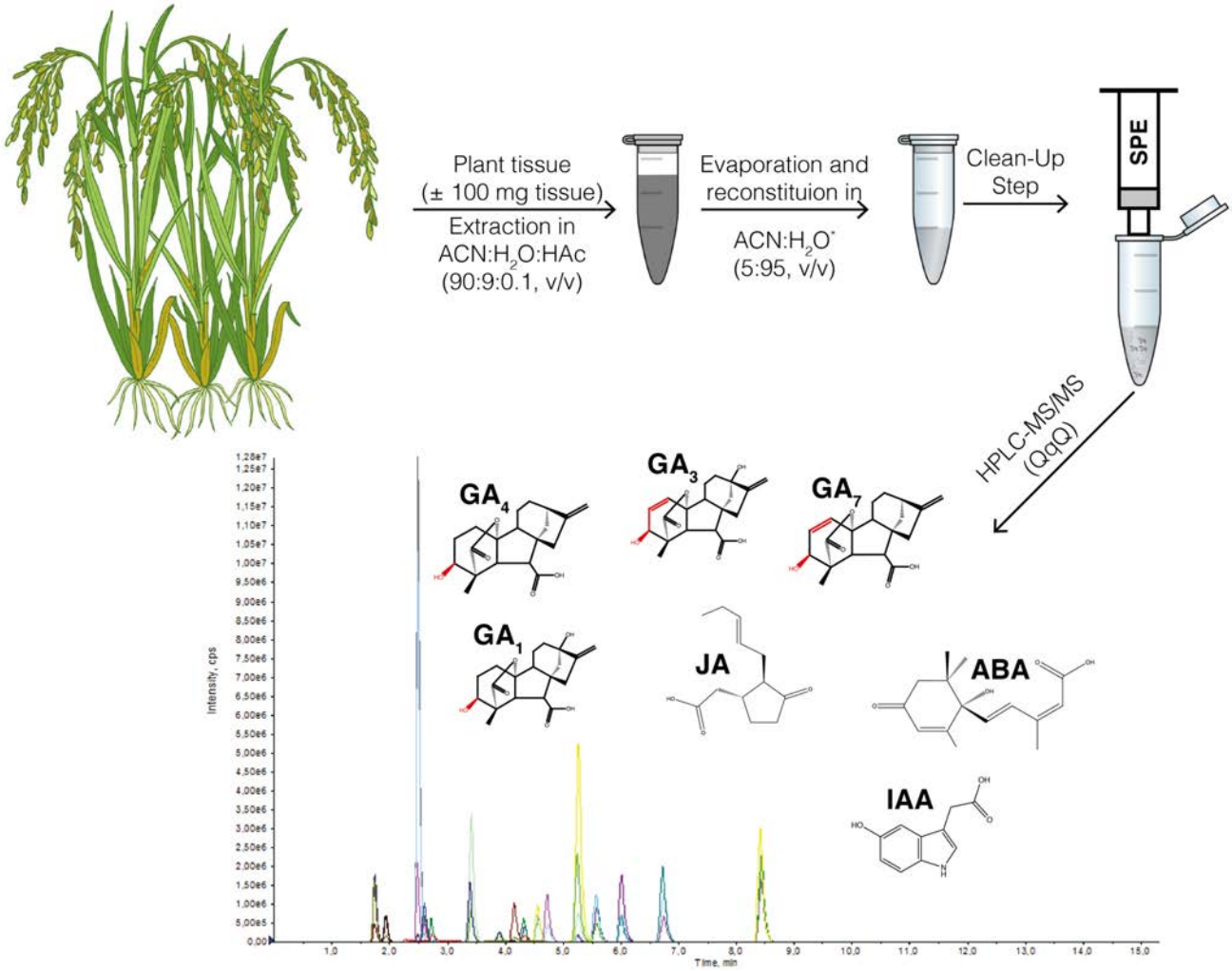


Figure 4.3. Rapid and broad method for the extraction, detection and quantification of 13 gibberellins (bioactive GAs are shown) and ABA, JA and IAA.

Interestingly, we observed that the levels of the four bioactive GAs was very contrasting between them but similar in the three assayed varieties (even the variety that had the Calrose mutation, *dwarf*-Bomba). GA_1 in 7-day-old coleoptiles was completely absent independent of the variety analyzed, suggesting that this GA is only needed for seed germination and not

coleoptile elongation. Contrarily, the other bioactive GAs (GA_3, GA_4 and GA_7) were detected in all tissues with only few exceptions. Notoriously, GA_4 stand among this three as the one displaying the higher concentrations throughout the rice plant development suggesting that in rice this GA has a predominant role. Despite this, GA_{19} which is a precursor of GA_3 and GA_7 was highly increased; hence these two GAs could be involved in specific responses to stress as their constitutive presence is low. In fact, some GAs have also been shown to be involved in tolerance to abiotic stress such as salinity which can severely affect yield (Iqbal et al. 2012; Sahoo et al. 2014; Reddy et al. 2017). Sahoo et al. (2014) and Tuteja et al (2013) demonstrated that *OsSUV3* over-expressing transgenic rice has higher endogenous levels of GA_3 and higher yield under salinity than wild-type plants, suggesting a role of gibberellins in salinity tolerance.

In all varieties, higher GA_4 levels were observed in the nodes compared to the internodes, which is in accordance with Kojima et al. (2009). Moreover, Kaneko et al. (2003) showed that nodes of elongating stems had higher activity of *OsGA3ox2* and *OsGA20ox2*. The exception was *dwarf*-Bomba that displayed high levels of all the 4 bioactive GAs at the flag leaf node (pNF) but also at the internode between flag leaf and previous leaf (pNF, Figure 3.2.5). This high content of bioactive GAs could explain its increased growth between week 11^{th} and 13^{th} of its development (Figure 3.2.3).

6.3 CHAPTER 3 - An improved anther culture procedure for obtaining new commercial Mediterranean temperate *japonica* rice (*Oryza sativa*) genotypes & Antimitotic and hormone effects on green double haploid plant production

Lastly, both subchapters (3.1 and 3.2) lead to two publications that revolve around anther culture in rice, technique that allows to rapidly obtain through an *in-vitro* procedure double haploid plants which are pure and stable (i.e. homozygous lines) in only one year, contrarily to more than 6 years that the pedigree process takes (Miah et al. 1985; Agache et al. 1989; López-Cristoffanini et al. 2018). Both publications are important as their main achievement is the improvement of anther culture protocols in temperate *japonica* rice varieties, which are scarce in the literature as the majority reported work well on tropical *japonica* and indica rice varieties (Miah et al. 1985; Yan et al. 1996; Herath et al. 2007; Mishra and Rao 2016). Moreover, we carried the anther-derived rice lines to the fields for line selection according to several agricultural and production traits such as resistance to biotic stresses and yield (kg/ha).

As it has been stated in a highly cited review by Germanà (2011) and one focused on *in-vitro* androgenesis in rice by Mishra and Rao (2016), genotype is one of the major factors reported so far that greatly affects the efficiency of anther culture protocols. Hence, researchers have been improving all the stages of this *in-vitro* procedure regularly in order to attain the higher rate of green double haploid plants, which is the last goal of this procedure if it is applied with the aim of producing new commercial varieties Mishra and Rao (2016), Table 1.2]. In regard to this, we first reported that a cold-pretreatment during 10 days at 5ºC produced the higher rates of *callus* induction (CI) for NRVC980385 as it was proposed by several other authors (Chen et al. 1986; Trejo-Tapia et al. 2002a, b). Nevertheless, in our second study we reported

that a cold-pretreatment during 9 days at 10ºC yields more than 12-fold the *callus* induction (Figure 4.4). The fact that a cold-pretreatment at 10ºC in contrast to 5ºC, as the duration is the same, is probably due as this is temperate *japonica* variety and the literature that suggested 5ºC corresponds to tropical *japonica* varieties. Moreover, it seems that the cold-pretreatment also induces higher frequencies of green double haploid plantlets (Figure 4.4). *Callus* induction is a crucial factor as a higher rate will allow for a higher regeneration of green double haploid plants which ultimately ensures more plantlets with differential genotypes, as plantlets regenerated from the same *callus* are more likely to be clones (Hooghvorst et al. 2018). This is of high importance, as from a breeder's point of view this means that more potentially commercial lines there are to assay and select.

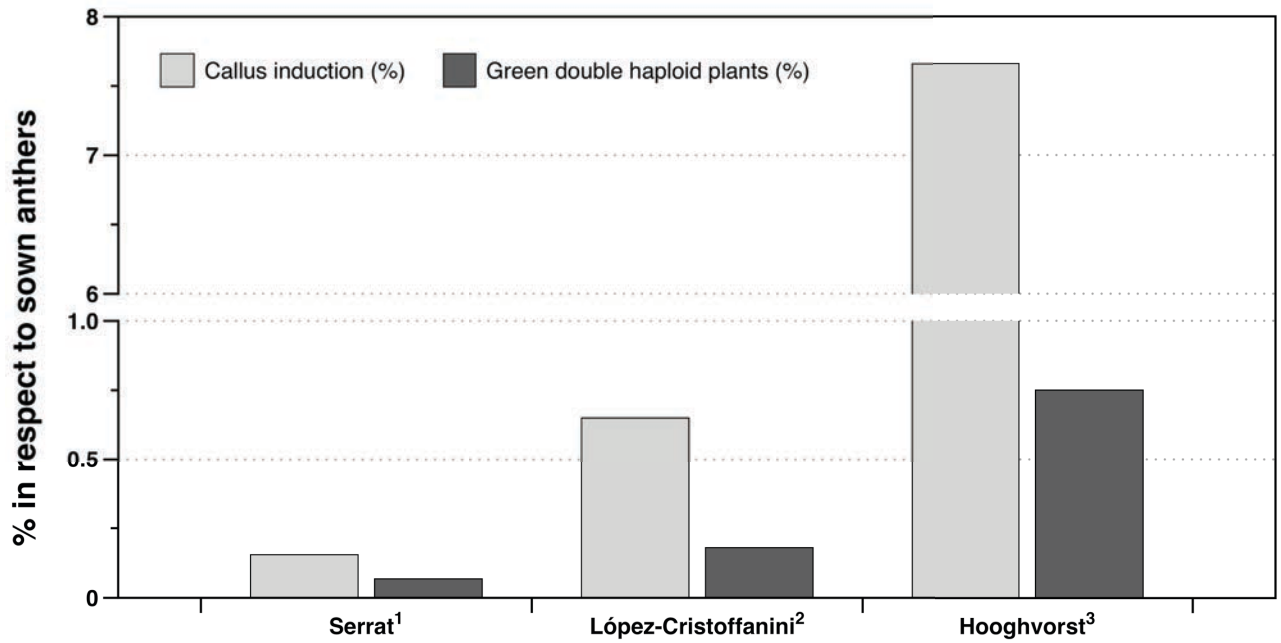


Figure 4.4. *Callus* induction and green double haploid percentage in respect to sown anther (percentage). [1]Values for NRVC980385 with a cold-pretreatment of 7-12 days at 7ºC, adapted from [1]Serrat et al. (2014); [2]Values for two crosses with NRVC980385 with a cold-pretreatment of 9 days at 5ºC, adapted from López-Cristoffanini et al. (2018); and [3]values for NRVC980385 with a cold-pretreatment of 9 days at 10ºC and a 500 $mg \cdot L^{-1}$ colchicine treatment during 48 hours, adapted from Hooghvorst et al. (2018).

As for the use of antimitotics in the anther culture, it was put in evidence that depending on the genotype, they can be beneficial for increasing the *callus* induction rates since H28 had a higher CI in all the colchicine-supplemented media, which is in accordance with Alemanno and Guiderdoni (1994). Moreover, for this same rice line and in agreement with the reported authors, the addition of colchicine in the media also increased the number of green plantlets but not double haploid plantlets as reported by other authors (Barnabás et al. 1991).

As in an anther culture procedure, a high number of regenerated plants can be obtained (which in the case of Chapter 3.2 was more than 250), using the predominant method for ploidy analysis which is razor-chopping could prove to be the limiting factor (Cousin et al. 2009). Therefore, to our knowledge, we are the first to have ever introduce a medium throughput method for ploidy analysis using a bead-beating procedure modified from the one published by Cousin et al. (2009), which allowed to reduce in a third the time needed for the analysis as 24 samples can be homogenized simultaneously (Figure 4.5). Finally, as several of the green regenerated plantlets did not spontaneously doubled their ploidy, a post anther diplodization protocol could be applied to increase the anther culture efficiency in terms of green double haploid plantlet obtaining. In this regard, we have, although with poor rates, demonstrated that a treatment during only 5 hours in 250 $mg \cdot L^{-1}$ colchicine or 1.25 $mg \cdot L^{-1}$ oryzalin can force haploid plants to double their chromosomes and develop into double haploid plants, which can be helpful for projects involved in new varieties development.

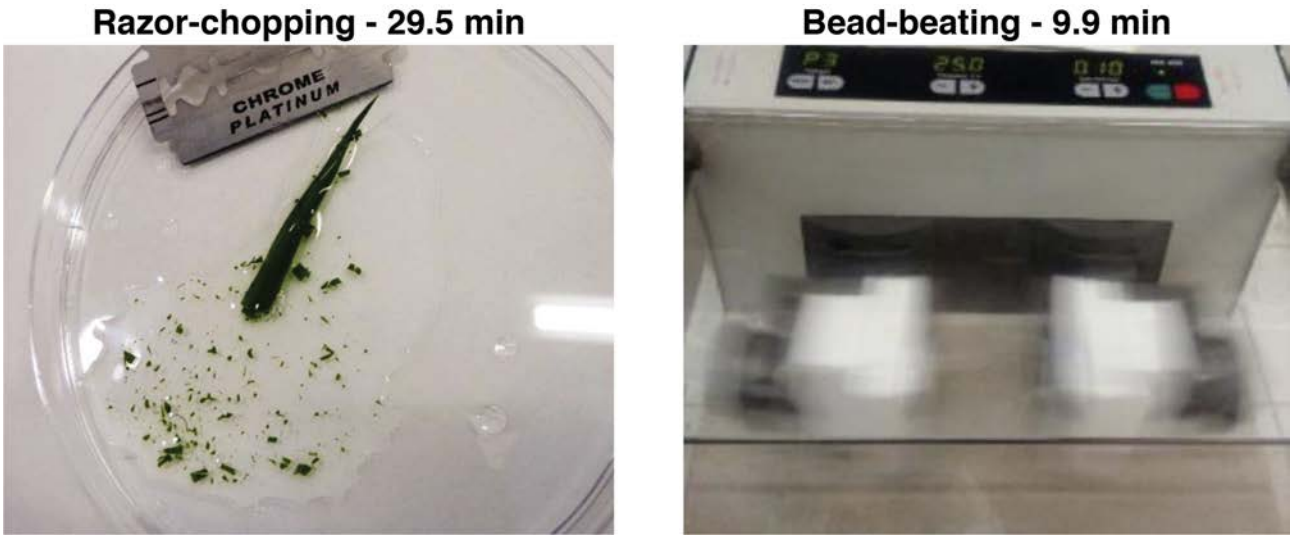


Figure 4.5. Ploidy determination protocols with their duration in minutes.

Finally, all the results and discussion presented in this thesis contribute greatly to the information and knowledge we have on rice, this cereal that is proving to be key for the increasing population. Moreover, due to climate change and shortage of proper arable lands humanity will have to improve rice varieties in order to attain higher yields with lower resources, especially low water availability. Nowadays, breeder's program are not focused on improving one or two traits but multiple traits at the same time, as is the case of the Green Super Rice (GSR), an idea already proposed ten years ago (Zhang 2007; Wing et al. 2018). The key attributes of this conceptual variety include improvements at all levels such as: reduced requirements for fertilizers, pesticides and water; increased yield; more palatable and nutritious grains; the ability to grow on marginal lands; and reduced greenhouse gas emissions. In addition, as mentioned in the General Introduction, we need to combine and dispose of all the omics approaches, information and biotechnological tools available for developing this SGR. All the methods/protocols and results/findings exposed in this thesis contribute greatly to this end of being able to have all the information for producing greatly enhanced and improved rice varieties for their cultivation continuity. In conclusion, we have

given insightful information on tolerance to salinity which is a threat that is largely affecting crops worldwide and that will not go away in the near future. Moreover, we have developed a protocol for analyzing gibberellin content for deeply characterizing dwarf varieties and enhanced anther culture protocols which could be used to produce a (slightly) super Mediterranean rice variety.

CONCLUSIONS

- Shoots of FL478, the salt tolerant variety harbouring the *Saltol* region, display lower Na+/K+ ratio than roots suggesting a protecting role of the root. In concordance with these results, roots of FL478 also display higher activation of antioxidant-related protein than shoots.
- FL478 roots, but not shoots, continue to grow when subjected to 100 mM NaCl as roots are exploring their growing environment for less salinized areas.
- FL478 proteome under salinity stress includes SalT (roots) and PDX (shoots and roots) but not OSHKT1;5, which are the genes that have been characterized in the *Saltol* region. Moreover, under salinity stress, the proteins related to cellular functions of FL478 roots are similar to the control, situation not observed for shoots.

- A fast and broad phytohormonal extraction and detection protocol was developed, for the first time, that allows identifying 13 gibberellins and ABA, JA and IAA in several tissues at different phenological stages of rice.
- The gibberellin GA1 is not a crucial gibberellin for rice coleoptiles elongation neither in more advanced phenological stages, as its levels are low in all the three assayed

varieties. Contrarily, GA19 seems to have a key role in bioactive gibberellins availability in rice as its levels were much higher than all the other gibberellins in all tissues.

- The GA20ox-2 mutation is not the only factor affecting height in rice, as a mutated variety had an increased growth during flag leaf collar formation stage (R2).
- It was observed for bioactive gibberellins that GA3 and GA7 (and GA1) are present at low levels in the majority of rice tissues, and contrarily, GA4 is present at high concentrations in all of the tissues, suggesting that it is a key gibberellin for rice development.

- We have shown that our improved anther culture protocols can be successfully applied in different F2 rice genotypes between temperate *japonica* rice genotypes to obtain green double haploid plants.
- A cold-pretreatment of 9 days at 10ºC has shown to be the most successful condition for the anther-derived *callus* induction in the temperate *japonica* Mediterranean rice lines NRVC980385.
- Colchicine on the induction medium increases the green double haploid plantlet production in all treatments of concentration and time assayed. If no colchicine is added to the media, the best efficiency is obtained using 2,4-D at 2 $mg \cdot L^{-1}$ and kinetin at 1 $mg \cdot L^{-1}$.
- Overall, our results highlight the importance of the genotype and media interactions effects on the anther culture efficiency in rice, and therefore more studies are needed in order to understand the main mechanism of interaction between genotype and induction media.

- In addition to this, we have introduced a fast method for ploidy-determination in rice as well as a post anther culture diploidization protocol, for the first time, that although needs to be optimized has good prospects for increasing overall anther culture efficiency.
- In our first rice anther study, 7 of the 9 lines evaluated in the field showed good qualities at the agricultural and production level. Therefore, these varieties are suited to be submitted to direct seeded medium scale assays before registry for their subsequent commercialization.
- Finally, our rice anther culture protocol is therefore highly applicable to rice genotypes at the F2 generation for producing new lines that could be registered and commercialized as new varieties.

6

REFERENCES

(General Introduction and General Discussion)

3000 rice genomes project T (2014) The 3,000 rice genomes project. Gigascience 3:7 . doi: 10.1186/2047-217X-3-7

Abbasi FM, Komatsu S (2004) A proteomic approach to analyze salt-responsive proteins in rice leaf sheath. Proteomics 4:2072–2081 . doi: 10.1002/pmic.200300741

Abdollah Hosseini S, Gharechahi J, Heidari M, et al (2015) Comparative proteomic and physiological characterisation of two closely related rice genotypes with contrasting responses to salt stress. Funct Plant Biol 42:527–542 . doi: 10.1071/FP14274

Agache S, Bachelier B, Buyser J, et al (1989) Genetic analysis of anther culture response in wheat using aneuploid, chromosome substitution and translocation lines. Theor Appl Genet 77:7–11 . doi: 10.1007/bf00292308

Agrawal GK, Jwa NS, Rakwal R (2009) Rice proteomics: Ending phase I and the beginning of phase II. Proteomics 9:935–963 . doi: 10.1002/pmic.200800594

Agrawal GK, Rakwal R (2011) Rice proteomics: A move toward expanded proteome coverage to comparative and functional proteomics uncovers the mysteries of rice and plant biology. Proteomics 11:1630–1649 . doi: 10.1002/pmic.201000696

Ahloowalia BS, Maluszynski M (2001) Induced mutations in plant breeding. Euphytica 118:8 . doi: 10.1007/978-3-319-22521-0_11

Ahloowalia BS, Maluszynski M, Nichterlein K (2004) Global impact of mutation-derived varieties. Euphytica 135:187–204 . doi: 10.1023/B:EUPH.0000014914.85465.4f

Alemanno L, Guiderdoni E (1994) Increased doucled haploid plant regeneration from rice (*Oryza sativa* L.) anthers cultured on colchicine-supplemented media. Plant Cell Rep 13:432–436

Alonso-Ramirez A, Rodriguez D, Reyes D, et al (2009) Evidence for a role of gibberellins in salicylic acid-modulated early plant responses to abiotic stress in arabidopsis seeds. Plant Physiol 150:1335–1344 . doi: 10.1104/pp.109.139352

Anderson JP (2004) Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in arabidopsis. Plant Cell Online 16:3460–3479 . doi: 10.1105/tpc.104.025833

Araki M, Ishii T (2015) Towards social acceptance of plant breeding by genome editing. Trends Plant Sci 20:145–149 . doi: 10.1016/j.tplants.2015.01.010

Ashikari M, Sasaki A, Ueguchi-Tanaka M, et al (2002) Loss-of-function of a rice gibberellin biosynthetic gene, GA20 oxidase (GA20ox-2), led to the rice 'Green Revolution'. Breed Sci 52:143–150 . doi: 10.1270/jsbbs.52.143

Babu NN, Krishnan SG, Vinod KK, et al (2017) Marker aided incorporation of *Saltol*, a major QTL

asociated with seedling stage salt tolerance, into *Oryza sativa* 'Pusa Basmati 1121'. Front Plant Sci 8:1–14 . doi: 10.3389/fpls.2017.00041

Barnabás B, Pfahler PL, Kovacs G (1991) Direct effect of colchicine on the microspore embryogenesis to produce dihaploid plants in wheat (*Triticum aestivum* L.). TAG Theor Appl Genet 81:675–678

Belhaj K, Chaparro-Garcia A, Kamoun S, et al (2015) Editing plant genomes with CRISPR/Cas9. Curr Opin Biotechnol 32:76–84 . doi: 10.1016/j.copbio.2014.11.007

Bielach A, Hrtyan M, Tognetti VB (2017) Plants under stress: Involvement of auxin and cytokinin. Int J Mol Sci 18: . doi: 10.3390/ijms18071427

Binenbaum J, Weinstain R, Shani E (2018) Gibberellin localization and transport in plants. Trends Plant Sci 23:410–421 . doi: 10.1016/j.tplants.2018.02.005

Bonilla P, Dvorak J, Mackill D, et al (2002) RFLP and SSLP mapping of salinity tolerance genes in chromosome 1 of rice (*Oryza sativa* L.) using recombinant inbred lines. Philipp Agric Sci 85:68–76

Bortesi L, Fischer R (2015) The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnol Adv 33:41–52 . doi: http://dx.doi.org/10.1016/j.biotechadv.2014.12.006

Britt AB, May GD (2003) Re-engineering plant gene targeting. Trends Plant Sci 8:90–95 . doi: 10.1016/S1360-1385(03)00002-5

Cai HW, Morishima H (2002) QTL clusters reflect character associations in wild and cultivated rice. Theor Appl Genet 104:1217–1228 . doi: 10.1007/s00122-001-0819-7

Capilla-Perez L, Solier V, Portemer V, et al (2018) The HEM Lines: A new library of homozygous *Arabidopsis thaliana* EMS mutants and its potential to detect meiotic phenotypes. Front Plant Sci 9:1–9 . doi: 10.3389/fpls.2018.01339

Català MM, Jardí M, Pla E (2007) Evolución de las variedades de arroz cultivadas en el Delta del Ebro durante los últimos 20 años. Agric Vergel 4

Català MM, Tomàs N, Martínez M, Pla E (2009) Valoración agronómica de nuevas variedades de arroz. Ensayos en el Delta del Ebro: 2004-2008. Agrícola vergel 327:161–166

Chait BT (2006) Mass Spectrometry: Bottom-Up or Top-Down? Science (80-) 314:2 . doi: 10.1126/science.1133987

Chandramouli K, Qian P-Y (2009) Proteomics: challenges, techniques and possibilities to overcome biological sample complexity. Hum Genomics Proteomics 2009:1–22 . doi: 10.4061/2009/239204

Chang T-T, Bardenas EA, del Rosario AC (1965) The morphology and varital characteristics of the rice plant. Los Baños, Laguna, The Philippines

Chen C-C, Tsay H-S, Huang C-R (1986) Rice (*Oryza sativa* L.): factors affecting androgenesis. In: Biotechnology in Agriculture Forestry, 1st edn. Springer Berlin Heidelberg, Heidelberg, Alemania, p 16

Chen ML, Fu XM, Liu JQ, et al (2012) Highly sensitive and quantitative profiling of acidic phytohormones using derivatization approach coupled with nano-LC-ESI-Q-TOF-MS analysis. J Chromatogr B Anal Technol Biomed Life Sci 905:67–74 . doi: 10.1016/j.jchromb.2012.08.005

Chen QF, Wang CL, Lu YM, et al (2002) Anther culture in connection with induced mutations for rice improvement. In: Mutations, In Vitro and Molecular Techniques for Environmentally Sustainable Crop Improvement. Springer, pp 83–92

Contreras-Porcia L, López-Cristoffanini C (2012) Proteomics in seaweeds: ecological interpretations. In: Magdeldin S (ed) Gel Electrophoresis - Advanced Techniques, 1st edn. InTech, Rijeka, Croatia, p 31

Counce PA, Keisling TC, Mitchell AJ (2000) A uniform, objective, and adaptive system for expressing rice development. Crop Sci 40:436–443 . doi: 10.2135/cropsci2000.402436x

Courtois B (1993) Comparison of single seed descent and anther culture-derived lines of 3 single crosses of rice. Theor Appl Genet 85:625–631

Cousin A, Heel K, Cowling WA, Nelson MN (2009) An efficient high-throughput flow cytometric method for estimating DNA ploidy level in plants. Cytom Part A 75A:1015–1019 . doi: 10.1002/cyto.a.20816

Das P, Nutan KK, Singla-Pareek SL, Pareek A (2015) Understanding salinity responses and adopting 'omics-based' approaches to generate salinity tolerant cultivars of rice. Front Plant Sci 6:712 . doi: 10.3389/fpls.2015.00712
Davies PJ (2010) Plant hormones: biosynthesis, signal transduction, action!, 3rd edn. Springer, London, New York
Deinlein U, Stephan AB, Horie T, et al (2014) Plant salt-tolerance mechanisms. Trends Plant Sci 19:371–379 . doi: 10.1016/j.tplants.2014.02.001
Dooki AD, Mayer-Posner FJ, Askari H, et al (2006) Proteomic responses of rice young panicles to salinity. Proteomics 6:6498–6507 . doi: 10.1002/pmic.200600367
Dunwell J (2010) Crop biotechnology : prospects and opportunities. J Agric Sci 11 . doi: 10.1017/S0021859610000833
Eriksson D (2018) The evolving EU regulatory framework for precision breeding. Theor Appl Genet. doi: 10.1007/s00122-018-3200-9
European Commission A and RD (2018) Balance sheets for cereals, oilseeds, proteins and rice
Eyidogan F, Oz M, Yucel M, Oktem H (2012) Signal transduction of phytohormones under abiotic stresses. In: Khan NA, Nazar R, Iqbal N, Anjum NA (eds) Phytohormones and Abiotic Stress Tolerance in Plants, 1sr edn. Springer, p 48
Fahad S, Hussain S, Matloob A, et al (2015) Phytohormones and plant responses to salinity stress: a review. Plant Growth Regul 75:391–404 . doi: 10.1007/s10725-014-0013-y
FAO (2018a) FAOSTAT. In: FAO. http://www.fao.org/faostat/en/. Accessed 23 Nov 2018
FAO (2018b) Rice Market Monitor - April 2018. XXI:38
Feist P, Hummon AB (2015) Proteomic challenges: Sample preparation techniques for Microgram-Quantity protein analysis from biological samples. Int J Mol Sci 16:3537–3563 . doi: 10.3390/ijms16023537
Ferrie AMR, Irmen KI, Beattie AD, Rossnagel BG (2014) Isolated microspore culture of oat (Avena sativa L.) for the production of doubled haploids: effect of pre-culture and post-culture conditions. Plant Cell, Tissue Organ Cult 116:89–96
Fountas S, Carli G, Sørensen CG, et al (2015) Farm management information systems: current situation and future perspectives. Comput Electron Agric 115:40–50 . doi: 10.1016/j.compag.2015.05.011
Franquet Bernis JM, Borràs Pàmies C (2004) Variedades y mejora del arroz (*Oryza sativa* L.), 1st editio. Tortosa, Spain
Frouin J, Languillaume A, Mas J, et al (2018) Tolerance to mild salinity stress in japonica rice: a genome-wide association mapping study highlights calcium signaling and metabolism genes. PLoS One 13:1–27 . doi: 10.1371/journal.pone.0190964
Gapper NE, Giovannoni JJ, Watkins CB (2014) Understanding development and ripening of fruit crops in an 'omics' era. Hortic Res 1:1–10 . doi: 10.1038/hortres.2014.34
García-Gonzáles R, Quiroz K, Carrasco B, Caligari P (2010) Plant tissue culture: current status, opportunities and challenges Rolando. Cienc e Investig Agrar 37:26 . doi: 10.1007/s00013-013-0564-6
Garris AJ, Tai TH, Coburn J, et al (2005) Genetic structure and diversity in *Oryza sativa* L. Genetics 169:1631–1638 . doi: 10.1534/genetics.104.035642
Generalitat de Catalunya (2012) Pliego de condiciones de la denominación de origen protegida 'arroz del Delta del Ebro' o 'arròs del Delta de l'Ebre'
Genua-Olmedo A, Alcaraz C, Caiola N, Ibáñez C (2016) Sea level rise impacts on rice production: The Ebro Delta as an example. Sci Total Environ 571:1200–1210 . doi: 10.1016/j.scitotenv.2016.07.136
Gepts P (2002) A comparison between crop domestication, classical plant breeding and genetic engineering. Crop Sci 42:11 . doi: 10.2135/cropsci2002.1780
Germanà MA (2011) Anther culture for haploid and doubled haploid production. Plant Cell Tissue Organ Cult 104:18 . doi: 10.1007/s11240-010-9852-z
Gupta B, Huang B (2014) Mechanism of salinity tolerance in plants: Physiological, biochemical, and

molecular characterization. Int J Genomics 2014: . doi: 10.1155/2014/701596
Gupta R, Wang Y, Agrawal GK, et al (2015) Time to dig deep into the plant proteome: a hunt for low-abundance proteins. Front Plant Sci 6:1–3 . doi: 10.3389/fpls.2015.00022
Gururani MA, Mohanta TK, Bae H (2015) Current understanding of the interplay between phytohormones and photosynthesis under environmental stress. Int J Mol Sci 16:19055–19085 . doi: 10.1080/09515070.2017.1300135
Ha S, Vankova R, Yamaguchi-Shinozaki K, et al (2012) Cytokinins: Metabolism and function in plant adaptation to environmental stresses. Trends Plant Sci 17:172–179 . doi: 10.1016/j.tplants.2011.12.005
Hansen NJP, Andersen SB (1998) In vitro chromosome doubling with colchicine during microspore culture in wheat (*Triticum aestivum* L.). Euphytica 102:101–108
Hasan MM, Rafii MY, Ismail MR, et al (2015a) Marker-assisted backcrossing: A useful method for rice improvement. Biotechnol Biotechnol Equip 29:237–254 . doi: 10.1080/13102818.2014.995920
Hasan MM, Rafii MY, Ismail MR, et al (2015b) Marker-assisted backcrossing: a useful method for rice improvement. Biotechnol Biotechnol Equip 29:237–254 . doi: 10.1080/13102818.2014.995920
Haun J (1973) Visual Quantification of Wheat Development. Agron J 65:4
Haynes PA, Roberts TH (2007) Subcellular shotgun proteomics in plants: looking beyond the usual suspects. Proteomics 7:2963–2975 . doi: 10.1002/pmic.200700216
Hedden P (2003) The genes of the Green Revolution. Trends Genet 19:5–9
Hedden P, Phillips AL (2000) Gibberellin metabolism: New insights revealed by the genes. Trends Plant Sci 5:523–530 . doi: 10.1016/S1360-1385(00)01790-8
Hedden P, Thomas SG (2012) Gibberellin biosynthesis and its regulation. Biochem J 444:11–25 . doi: 10.1042/BJ20120245
Heenan D, Lewin L, McCaffery D (1988) Salinity tolerance in rice varieties at different growth stages. Aust J Exp Agric 28:343–349 . doi: 10.1071/EA9880343
Herath HMI, Bandara DC, Samarajeewa PK (2007) Effect of culture media for anther culture of indica rice varieties and hybrids of indica and japonica. Trop Agric Res Ext 10:17–22 . doi: 10.4038/tare.v10i0.1866
Herath HMI, Bandara DC, Samarajeewa PK, Wijesundara DSA (2009) The effect of plant growth regulators on anther culture response and plant regeneration in selected Sri Lankan Indica rice varieties, Japonica varieties and their inter-sub specific hybrids. Trop Agric Res 20:243–250
Hirano H, Islam N, Kawasaki H (2004) Technical aspects of functional proteomics in plants. Phytochemistry 65:1487–1498 . doi: 10.1016/j.phytochem.2004.05.019
Hooghvorst I, Ramos-Fuentes E, López-Cristofannini C, et al (2018) Antimitotic and hormone effect on green double haploid plant production through anther culture of Mediterranean japonica rice. Plant Cell Tissue Organ Cult
Horváth E, Gabriella S, Janda T (2007) Induction of abiotic stress tolerance by salicylic acid signaling. J Plant Growth Regul 26:290–300 . doi: 10.1007/s00344-007-9017-4
Hospital F, Charcosset A (1997) Marker-assisted introgression of quantitative trait loci. Genetics 147:1469–1485
Hu X, Wang W (2016) Proteomics driven research of abiotic stress responses in crop plants. In: Rehman Hakeem K, Tombuloğlu H, Tombuloğlu G (eds) Plant Omics: Trends and Applications, 1st edn. Springer, Selangor, pp 351–362
Hussain A, Ahmed I, Nazir H, Ullah I (2012) Plant tissue culture: current status and opportunities. In: Leva A, Rinald LMR (eds) Recent Advances in Plant in vitro Culture, 1st edn. InTech Open, p 28
Ikeda A, Ueguchi-Tanaka M, Sonoda Y, et al (2001) slender rice, a constitutive gibberellin response mutant, is caused by a null mutation of the *SLR1* gene, an ortholog of the height-regulating gene *GAI/RGA/RHT/D8*. Plant Cell 13:999–1010 . doi: 10.1105/tpc.13.5.999
Ikeuchi M, Ogawa Y, Iwase A, Sugimoto K (2016) Plant regeneration: cellular origins and molecular mechanisms. Development 143:1442–1451 . doi: 10.1242/dev.134668

Iqbal N, Masood A, Khan NA (2012) Phytohormones in sailinity tolerance: ethylene and gibberellins cross talk. In: Khan NA, Nazar R, Iqbal N, Anjum NA (eds) Phytohormones and abiotic stress tolerance in plants, 1st editio. Springer, London New York, pp 77–98
IRGSP (2005) The map-based sequence of the rice genome. Nature 436:793–800 . doi: 10.1038/nature03895
Itoh T, Kawahara Y, Tanaka T (2018) Databases for rice omics studies. In: Sasaki T, Ashikari M (eds) Rice Genomics, Genetics and Breeding, 1st edn. Singapore, p 14
Jain M, Khurana JP (2009) Transcript profiling reveals diverse roles of auxin-responsive genes during reproductive development and abiotic stress in rice. FEBS J 276:3148–3162 . doi: 10.1111/j.1742-4658.2009.07033.x
Jami SK, Clark GB, Ayele BT, et al (2012) Identification and characterization of annexin gene family in rice. Plant Cell Rep 31:813–825 . doi: 10.1007/s00299-011-1201-0
Javid MG, Sorooshzadeh A, Moradi F, et al (2011) The role of phytohormones in alleviating salt stress in crop plants. Aust J Crop Sci 5:726–734
Juan Antonio Vizcaíno, Harald Barsnes, Henning Hermjakob (2015) Proteomics data visualisation. Proteomics 15:1339–1340
Kaneko M, Itoh H, Inukai Y, et al (2003) Where do gibberellin biosynthesis and gibberellin signaling occur in rice plants? Plant J 35:104–115 . doi: 10.1046/j.1365-313X.2003.01780.x
Khan MH, Panda SK (2008) Alterations in root lipid peroxidation and antioxidative responses in two rice cultivars under NaCl-salinity stress. Acta Physiol Plant 30:81–89 . doi: 10.1007/s11738-007-0093-7
Khan MIR, Syeed S, Nazar R, Anjum NA (2012) An insight into the role of salicylic acid and jasmonic acid in salt stress tolerance. In: Khan NA, Nazar R, Iqbal N, Anjum NA (eds) Phytohormones and Abiotic Stress Tolerance in Plants, 1st edn. Springer, London New York, p 24
Kim DW, Rakwal R, Agrawal GK, et al (2005) A hydroponic rice seedling culture model system for investigating proteome of salt stress in rice leaf. Electrophoresis 26:4521–4539 . doi: 10.1002/elps.200500334
Kim MC, Chung WS, Yun D-J, Cho MJ (2009a) Calcium and calmodulin-mediated regulation of gene expression in plants. Mol Plant 2:13–21 . doi: 10.1093/mp/ssn091
Kim S-H, Bhat P, Cui X, et al (2009b) Detection and validation of single feature polymorphisms using RNA expression data from a rice genome array. BMC Plant Biol 9:1–10 . doi: 10.1186/1471-2229-9-65
Kim ST, Kim SG, Agrawal GK, et al (2014) Rice proteomics: a model system for crop improvement and food security. Proteomics 14:593–610 . doi: 10.1002/pmic.201300388
Kojima M, Kamada-Nobusada T, Komatsu H, et al (2009) Highly sensitive and high-throughput analysis of plant hormones using ms-probe modification and liquid chromatographytandem mass spectrometry: An application for hormone profiling in *Oryza sativa*. Plant Cell Physiol 50:1201–1214 . doi: 10.1093/pcp/pcp057
Komatsu S, Tanaka N (2005) Rice proteome analysis: a step toward functional analysis of the rice genome. Proteomics 5:938–949 . doi: 10.1002/pmic.200401040
Kong D, Peng B, Peng Y, et al (2018) Effect of sowing date on grain endosperm chalkiness of different rice varieties. 6:41–51 . doi: 10.11648/j.jps.20180602.12
Korver RA, Koevoets IT, Testerink C (2018) Out of shape during stress: a key role for auxin. Trends Plant Sci 23:783–793 . doi: 10.1016/j.tplants.2018.05.011
Kovach MJ, Sweeney MT, McCouch SR (2007) New insights into the history of rice domestication. Trends Genet 23:578–587 . doi: 10.1016/j.tig.2007.08.012
Krishnamurthy SL, Sharma SK, Kumar V, et al (2016) Analysis of genomic region spanning Saltol using SSR markers in rice genotypes showing differential seedlings stage salt tolerance. J Plant Biochem Biotechnol 25:331–336 . doi: 10.1007/s13562-015-0335-5
Kudo T, Akiyama K, Kojima M, et al (2013) UniVIO: A multiple omics database with hormonome and transcriptome data from rice. Plant Cell Physiol 54:1–12 . doi: 10.1093/pcp/pct003
Kumar Dubey S, Pandey A, Singh Sangwan R (2017) Current developments in biotechnology and

bioengineering: crop modification, nutrition, and food production, 1st editio. Elsevier, Amsterdam, Netherlands

Kumar K, Kumar M, Kim S-R, et al (2013) Insights into genomics of salt stress response in rice. Rice 6:27 . doi: 10.1186/1939-8433-6-27

Kumar V, Singh A, Mithra SVA, et al (2015) Genome-wide association mapping of salinity tolerance in rice (*Oryza sativa*). DNA Res 22:133–145 . doi: 10.1093/dnares/dsu046

Kumari M, Clarke HJ, Small I, Siddique KHM (2009) Albinism in plants: a major bottleneck in wide hybridization, androgenesis and doubled haploid culture. CRC Crit Rev Plant Sci 28:393–409 . doi: 10.1080/07352680903133252

Lakra N, Kaur C, Anwar K, et al (2017) Proteomics of contrasting rice genotypes: identification of potential targets for raising crops for saline environment. Plant Cell Environ 1: . doi: 10.1111/pce.12946

Lakra N, Kaur C, Anwar K, et al (2018) Proteomics of contrasting rice genotypes: Identification of potential targets for raising crops for saline environment. Plant Cell Environ 41:947–969 . doi: 10.1111/pce.12946

Lancashire PD, Bleiholder H, Vandenboom T, et al (1991) A uniform decimal code for growth stages of crops and weeds. Ann Appl Biol 119:561–601 . doi: 10.1111/j.1744-7348.1991.tb04895.x

Langridge P, Fleury D (2011) Making the most of 'omics' for crop breeding. Trends Biotechnol 29:33–40 . doi: 10.1016/j.tibtech.2010.09.006

Lateef DD (2015) DNA marker technologies in plants and applications for crop improvements. J Biosci Med 03:7–18 . doi: 10.4236/jbm.2015.35002

Li C, Zhou A, Sang T (2006) Rice domestication by reducing shattering. Science (80-) 311:1936–1939

Li J, Assmann SM (2000) Mass spectrometry. An essential tool in proteome analysis. Plant Physiol 123:807–9 . doi: 10.1104/PP.123.3.807

Li XJ, Yang MF, Zhu Y, et al (2011) Proteomic Analysis of Salt Stress Responses in Rice Shoot. J Plant Biol 54:384–395 . doi: 10.1007/s12374-011-9173-8

Lin HX, Zhu MZ, Yano M, et al (2004) QTLs for Na^+ and K^+ uptake of the shoots and roots controlling rice salt tolerance. Theor Appl Genet 108:253–260 . doi: 10.1007/s00122-003-1421-y

Liu F, Wang P, Zhang X, et al (2018) The genetic and molecular basis of crop height based on a rice model. Planta 247:1–26 . doi: 10.1007/s00425-017-2798-1

Lo S-F, Yang S-Y, Chen K-T, et al (2008) A novel class of gibberellin 2-oxidases control semidwarfism, tillering, and root development in rice. Plant Cell 20:17 . doi: 10.1105/tpc.108.060913

López-Cristoffanini C, Bundó M, Serrat X, et al (2019) Proteome profiling in shoots and roots of the FL478 genotype of rice (Oryza sativa L. ssp. *indica*) by shotgun proteomics during early salinity stress. PROTEOMICS (submitted)

López-Cristoffanini C, Ramos E, Serrat X, et al (2016) Técnicas biotecnológicas aplicadas a la mejora del arroz en Latinoamérica. In: Cárdenas Neira C, López-Cristofannini C, Cares Mardones C, et al. (eds) Socializar conocimientos Nº3. América latina en diálogo: Oportunidades para hoy y mañana, 1st edn. RedINCHE, Barcelona

López-Cristoffanini C, Serrat X, Ramos-Fuentes E, et al (2018) An improved anther culture procedure for obtaining new commercial mediterranean temperate *japonica* rice (*Oryza sativa*) genotypes. Plant Biotechnol 35:161–166 . doi: 10.5511/plantbiotechnology.18.0409a

Loyola-Vargas VM, Ochoa-Alejo N (2012) An introduction to plant cell culture: the future ahead. In: Loyola-Vargas VM, Ochoa-Alejo N (eds) Plant Cell Cu, 3rd edn. Humana Press, p 8

Lutts S, Kinet JM, Bouharmont J (1995) Changes in plant response to NaCl during development of rice (*Oryza sativa* L.) varieties differing in salinity resistance. J Exp Bot 46:1843–1852

Maathuis FJM (2014) Sodium in plants: perception, signalling, and regulation of sodium fluxes. J Exp Bot 65:849–858 . doi: 10.1093/jxb/ert326

Macías JM, Pournavab RF, Reyes-Valdés MH, Benavides-Mendoza A (2014) Development of a rapid and efficient liquid chromatography method for determination of gibberellin A4 in plant tissue,

with solid phase extraction for purification and quantification. Am J Plant Sci 2014:573–583
McLean J, Hardy B, Hettel G (2013) Rice Almanac, 4th edition, 4th editio. Los Baños, Laguna, The Philippines
Mehraj SS, Kamili AN, Nazir R, et al (2018) Comparative evaluation of extraction methods for total proteins from *Crocus sativus* L. (Saffron). Saudi J Biol Sci 25:1603–1608 . doi: 10.1016/j.sjbs.2016.04.011
Miah MAA, Earle ED, Khush GS (1985) Inheritance of callus formation ability in anther cultures of rice, *Oryza sativa* L. Theor Appl Genet 70:113–116 . doi: 10.1007/BF00275308
Mickelbart M V., Hasegawa PM, Bailey-Serres J (2015) Genetic mechanisms of abiotic stress tolerance that translate to crop yield stability. Nat Rev Genet 16:237–251 . doi: 10.1038/nrg3901
Mirzaei M, Soltani N, Sarhadi E, et al (2012) Shotgun proteomic analysis of long-distance drought signaling in rice roots. J Proteome Res 11:348–358 . doi: 10.1021/pr2008779
Mishra R, Rao GJN (2016) In-vitro androgenesis in rice: advantages, constraints and future prospects. Rice Sci 23:12 . doi: 10.1016/j.rsci.2016.02.001
Mohanty S (2013) Trends in global rice consumption. Rice Today 44–45
Monna L, Kitazawa N, Yoshino R, et al (2002) Positional cloning of rice semidwarfing gene, sd-1: rice 'Green Revolution Gene' encodes a mutant enzyme involved in gibberellin synthesis. DNA Res 9:7 . doi: 10.1093/dnares/9.1.11
Moradi F, Ismail AM (2007) Responses of photosynthesis, chlorophyll fluorescence and ROS-scavenging systems to salt stress during seedling and reproductive stages in rice. Ann Bot 99:1161–1173 . doi: 10.1093/aob/mcm052
Munemasa S, Hauser F, Park J, et al (2015) Mechanisms of abscisic acid-mediated control of stomatal aperture. Curr Opin Plant Biol 28:154–162 . doi: 10.1016/j.pbi.2015.10.010
Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annu Rev Plant Biol 59:651–681 . doi: 10.1146/annurev.arplant.59.032607.092911
Negrão S, Almadanim MC, Pires IS, et al (2013) New allelic variants found in key rice salt-tolerance genes: an association study. Plant Biotechnol J 11:87–100 . doi: 10.1111/pbi.12010
Neilson KA, George IS, Emery SJ, et al (2007) Analysis of rice proteins using SDS-PAGE shotgun proteomics. In: Plant Proteomics. pp 1–253
Nesvizhskii AI, Vitek O, Arbersold R (2007) Analysis and validation of proteomic data generated by tandem mass spectrometry. Nat Methods 4:11
Niizeki H, Oono K (1968) Induction of haploid rice plant from anther culture. In: Proceedings of the Japan Academy. pp 554–557
Oikawa A, Matsuda F, Kusano M, et al (2008) Rice metabolomics. Rice 1:63–71 . doi: 10.1007/s12284-008-9009-4
Okuno A, Hirano K, Asano K, et al (2014) New approach to increasing rice lodging resistance and biomass yield through the use of high gibberellin producing varieties. PLoS One 9: . doi: 10.1371/journal.pone.0086870
Olszewski N, Sun T-P, Gubler F (2002) Gibberellin signaling: biosynthesis, catabolism, and response pathways. Plant Cell 14 Suppl:S61–S80 . doi: 10.1105/tpc.010476.GAs
Ookawa T, Inoue K, Matsuoka M, et al (2014) Increased lodging resistance in long-culm, low-lignin *gh2* rice for improved feed and bioenergy production. Sci Rep 4:1–9 . doi: 10.1038/srep06567
Oveland E, Muth T, Rapp E, et al (2015) Viewing the proteome: how to visualize proteomics data? Proteomics 15:1341–1355 . doi: 10.1002/pmic.201400412
Parihar P, Singh S, Singh R, et al (2015) Effect of salinity stress on plants and its tolerance strategies: a review. Environ Sci Pollut Res 22:4056–4075 . doi: 10.1007/s11356-014-3739-1
Peleg Z, Blumwald E (2011) Hormone balance and abiotic stress tolerance in crop plants. Curr Opin Plant Biol 14:290–295 . doi: 10.1016/j.pbi.2011.02.001
Pla E, Catalá MM, Tomás N (2017) Variedades de arroz registradas en España entre los años 2013 y 2016. Vida Rural 1:4
Platten JD, Cotsaftis O, Berthomieu P, et al (2006) Nomenclature for *HKT* transporters, key determinants of plant salinity tolerance. Trends Plant Sci 11:372–374 . doi:

10.1016/j.tplants.2006.06.001
Platten JD, Egdane J, Ismail A (2013) Salinity tolerance, Na^+ exclusion and allele mining of HKT1;5 in *Oryza sativa* and *O. glaberrima*: many sources, many genes, one mechanism? BMC Plant Biol 13:32
Plaza-Wüthrich S, Blösch R, Rindisbacher A, et al (2016) Gibberellin deficiency confers both lodging and drought tolerance in small cereals. Front Plant Sci 7:1–14 . doi: 10.3389/fpls.2016.00643
Qiao B, Zhang Q, Liu D, et al (2015) A calcium-binding protein, rice annexin *OsANN1*, enhances heat stress tolerance by modulating the production of H2O2. J Exp Bot 66:5853–5866 . doi: 10.1093/jxb/erv294
Rakwal R, Agrawal GK (2003) Rice proteomics: Current status and future perspectives. Electrophoresis 24:3378–3389 . doi: 10.1002/elps.200305586
Ray A, Langer M (2002) Homologous recombination: ends as the means. Trends Plant Sci 7:435–440 . doi: 10.1016/S1360-1385(02)02327-0
Reddy INBL, Kim BK, Yoon IS, et al (2017) Salt tolerance in rice: focus on mechanisms and approaches. Rice Sci 24:123–144 . doi: 10.1016/j.rsci.2016.09.004
Ren ZH, Gao JP, Li LG, et al (2005) A rice quantitative trait locus for salt tolerance encodes a sodium transporter. Nat Genet 37:1141–1146 . doi: 10.1038/ng1643
Roy SJ, Negrão S, Tester M (2014) Salt resistant crop plants. Curr Opin Biotechnol 26:115–124 . doi: 10.1016/j.copbio.2013.12.004
Sahi C, Singh A, Kumar K, et al (2006) Salt stress response in rice: genetics, molecular biology, and comparative genomics. Plant Mol Biol 6:263–284 . doi: 10.1007/s10142-006-0032-5
Sahoo RK, Ansari MW, Tuteja R, Tuteja N (2014) *OsSUV3* transgenic rice maintains higher endogenous levels of plant hormones that mitigates adverse effects of salinity and sustains crop productivity. Rice 7:1–3 . doi: 10.1186/s12284-014-0017-2
Salekdeh GH, Siopongco J, Wade LJ, et al (2002) A proteomic approach to analyzing drought- and salt-responsiveness in rice. F Crop Res 76:199–219 . doi: 10.1016/S0378-4290(02)00040-0
Sang T, Ge S (2007) The puzzle of rice domestication. J Integr Plant Biol 49:760–768 . doi: 10.1111/j.1744-7909.2007.00510.x
Sangha JS, Yolanda HC, Kaur J, et al (2013) Proteome analysis of rice (*Oryza sativa* L.) mutants reveals differentially induced proteins during brown planthopper (*Nilaparvata lugens*) infestation. Int J Mol Sci 14:3921–3945 . doi: 10.3390/ijms14023921
Santner A, Estelle M (2009) Recent advances and emerging trends in plant hormone signalling. Nature 459:1071–1078 . doi: 10.1038/nature08122
Sasaki A, Ashikari M, Ueguchi-Tanaka M, et al (2002) Green revolution: a mutant gibberellin-synthesis gene in rice. Nature 416:701–702 . doi: 10.1038/416701a
Serrat X, Cardona M, Gil J, et al (2014) A Mediterranean japonica rice (*Oryza sativa*) cultivar improvement through anther culture. Euphytica 195:31–44 . doi: 10.1007/s10681-013-0955-6
Shakirova FM, Sakhabutdinova AR, Bezrukova M V, et al (2003) Changes in the hormonal status of wheat seedlings induced by salicylic acid and salinity. Plant Sci 164:6 . doi: https://doi.org/10.1016/S0168-9452(02)00415-6
Shaw MM, Riederer BM (2003) Sample preparation for two-dimensional gel electrophoresis. Proteomics 3:1408–1417 . doi: 10.1002/pmic.200300471
Shrivastava P, Kumar R (2015) Soil salinity: a serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. Saudi J Biol Sci 22:123–131 . doi: 10.1016/j.sjbs.2014.12.001
Singh R, Jwa NS (2013) Understanding the responses of rice to environmental stress using proteomics. J Proteome Res 12:4652–4669 . doi: 10.1021/pr400689j
Singh RK, Gregorio GB, Jain RK (2007) QTL Mapping for salinity tolerance in rice. Physiol Mol Biol Plants 13:87–99
Skirycz A, Hannah MA (2012) The use of 'Omics' approaches in *Arabidopsis* for the improvement of abiotic stress tolerance. In: Improving Crop Resistance to Abiotic Stress, 1st edn. Wiley Online Library, p 20

Smith SM, Maughan PJ (2014) SNP genotyping using KASPar assays. Plant Genotyping, Methods Mol Biol 1245: . doi: https://doi.org/10.1007/978-1-4939-1966-6_18
Spielmeyer W, Ellis MH, Chandler PM (2002) Semidwarf (*sd-1*), 'green revolution' rice, contains a defective gibberellin 20-oxidase gene. Proc Natl Acad Sci U S A 99:9043–9048 . doi: 10.1073/pnas.132266399
Sposito G (2008) The chemistry of soils, 2nd editio. Oxford University Press, New York
Steele KA, Quinton-Tulloch MJ, Amgai RB, et al (2018) Accelerating public sector rice breeding with high-density KASP markers derived from whole genome sequencing of *indica* rice. Mol Breed 38: . doi: 10.1007/s11032-018-0777-2
Sumner LW, Mendes P, Dixon RA (2003) Plant metabolomics: large-scale phytochemistry in the functional genomics era. Phytochemistry 62:817–836 . doi: 10.1016/S0031-9422(02)00708-2
Sweeney M, McCouch S (2007) The complex history of the domestication of rice. Ann Bot 100:951–957 . doi: 10.1093/aob/mcm128
Szklarczyk D, Morris JH, Cook H, et al (2017) The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. Nucleic Acids Res 45:D362–D368 . doi: 10.1093/nar/gkw937
Thelen JJ, Peck SC (2007) Quantitative proteomics in plants: choices in abundance. Plant Cell Online 19:3339–3346 . doi: 10.1105/tpc.107.053991
Thomson M, de Ocampo M, Egdane J, et al (2010) Characterizing the *Saltol* quantitative trait locus for salinity tolerance in rice. Rice 3:148–160 . doi: 10.1007/s12284-010-9053-8
Thorpe T (2012) History of plant tissue culture. In: Loyola-Vargas VM, Ochoa-Alejo N (eds) Plant Cell Culture Protocols, 3rd edn. Humana Press, p 19
Thrall PH, Bever JD, Burdon JJ (2010) Evolutionary change in agriculture: the past, present and future. Evol Appl 3:405–8 . doi: 10.1111/j.1752-4571.2010.00155.x
Tian QQ, Lu CM, Li X, Fang XW (2015) Low temperature treatments of rice (Oryza sativa L.) anthers changes polysaccharide and protein composition of the anther walls and increases pollen fertility and callus induction. Plant Cell, Tissue Organ Cult 120:89–98 . doi: 10.1007/s11240-014-0582-5
Touraev A, Forster BP, Jain SM (2009) Advances in haploid production in higher plants. Springer
Trade and Markets Division F (2018) Food Outlook - Biannual report on global food markets / July 2018. Rome
Trejo-Tapia G, Amaya UM, Morales GS, et al (2002a) The effects of cold-pretreatment, auxins and carbon source on anther culture of rice. Plant Cell Tissue Organ Cult 71:41–46 . doi: 10.1023/a:1016558025840
Trejo-Tapia G, Maldonado-Amaya U, Jiménez-Aparicio A, et al (2002b) Effect of time at low temperature treatment and growth regulators on regeneration of plants from anthers of rice Oryza sativa L. (Japonica H2005 cultivar). Agrociencia 36:441–449
Trewavas A (2009) What is plant behaviour? Plant, Cell Environ 32:606–616 . doi: 10.1111/j.1365-3040.2009.01929.x
Tuteja N, Sahoo RK, Garg B, Tuteja R (2013) *OsSUV3* dual helicase functions in salinity stress tolerance by maintaining photosynthesis and antioxidant machinery in rice (Oryza sativa L. cv. IR64). Plant J 76:115–127 . doi: 10.1111/tpj.12277
United Nations (2017) World population prospects. New York
Urbanová T, Tarkowská D, Novák O, et al (2013) Analysis of gibberellins as free acids by ultra performance liquid chromatography-tandem mass spectrometry. Talanta 112:85–94 . doi: 10.1016/j.talanta.2013.03.068
Van Emon JM (2016) The Omics revolution in agricultural research. J Agric Food Chem 64:36–44 . doi: 10.1021/acs.jafc.5b04515
Visscher PM, Haley CS, Thompson R (1996) Marker-assisted introgression in backcross breeding programs. Genetics 144:1923–1932
Wang R, Perez-Riverol Y, Hermjakob H, Vizcaíno JA (2015) Open source libraries and frameworks for biological data visualisation: A guide for developers. Proteomics 15:1356–1374 . doi:

10.1002/pmic.201400377
Wang W-Q, Jensen ON, Møller IM, et al (2018) Evaluation of sample preparation methods for mass spectrometry-based proteomic analysis of barley leaves. Plant Methods 14:72 . doi: 10.1186/s13007-018-0341-4
Wang Y, Zhao J, Lu W, Deng D (2017) Gibberellin in plant height control: old player, new story. Plant Cell Rep 36:391–398 . doi: 10.1007/s00299-017-2104-5
Wani SH, Kumar V, Shriram V, Sah SK (2016) Phytohormones and their metabolic engineering for abiotic stress tolerance in crop plants. Crop J 4:162–176 . doi: 10.1016/j.cj.2016.01.010
Wen F ping, Zhang Z hui, Bai T, et al (2010) Proteomics reveals the effects of gibberellic acid (GA_3) on salt-stressed rice (Oryza sativa L.) shoots. Plant Sci 178:170–175 . doi: 10.1016/j.plantsci.2009.11.006
Wing RA, Purugganan MD, Zhang Q (2018) The rice genome revolution: from an ancient grain to Green Super Rice. Nat Rev Genet 19:505–517 . doi: 10.1038/s41576-018-0024-z
Wolt JD, Wang K, Yang B (2016) The regulatory status of genome-edited crops. Plant Biotechnol J 14:510–518 . doi: 10.1111/pbi.12444
Xiong LZ, Liu KD, Dai XK, et al (1999) Identification of genetic factors controlling domestication-related traits of rice using an F2 population of a cross between *Oryza sativa* and *O. rufipogon*. Theor Appl Genet 98:243–251 . doi: 10.1007/s001220051064
Xue W, Xing Y, Weng X, et al (2008) Natural variation in *Ghd7* is an important regulator of heading date and yield potential in rice. Nat Genet 40:761–767
Yadav SP (2007) The wholeness in suffix *-omics*, *-omes*, and the word *om*. J Biomol Tech 18:277 . doi: 18/5/277 [pii]
Yamaguchi S (2008) Gibberellin metabolism and its regulation. Annu Rev Plant Biol 59:225–251 . doi: 10.1007/s003440010039
Yan J, Xue Q, Zhu J (1996) Genetic studies of anther culture ability in rice (*Oryza sativa*). Plant Cell Tissue Organ Cult 45:253–258
Yan S, Tang Z, Su W, Sun W (2005) Proteomic analysis of salt stress-responsive proteins in rice root. Proteomics 5:235–244 . doi: 10.1002/pmic.200400853
Zadoks J, Chang T, Konzak C (1974) A decimal code for growth stages of cereals. Weede Res 14:7
Zapata-Arias FJ (2003) Laboratory protocol for anther culture technique in rice. In: Doubled Haploid Production in Crop Plants. Springer, pp 109–116
Zeng N, Zhao F, Collatz GJ, et al (2014) Agricultural Green Revolution as a driver of increasing atmospheric CO_2 seasonal amplitude. Nature 515:394–397 . doi: 10.1038/nature13893
Zhang Q (2007) Strategies for developing Green Super Rice. Proc Natl Acad Sci 104:8 . doi: 10.1073/pnas.95.4.1663
Zhang SP, Song WY, Chen LL, et al (1998) Transgenic elite *Indica* rice varieties, resistant to *Xanthomonas oryzae* pv. *oryzae*. Mol Breed 4:551–558 . doi: 10.1023/a:1009601520901
Zhang Y, Fonslow BR, Shan B, et al (2015) Protein analysis by shotgun/bottom-up proteomics. Chem Rev 113:213–223 . doi: 10.1007/978-1-62703-673-3

ANNEX – Published articles

CHAPTER 3.1 - An improved anther culture procedure for obtaining new commercial Mediterranean temperate *japonica* rice (*Oryza sativa*) genotype

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Short Communication

An improved anther culture procedure for obtaining new commercial Mediterranean temperate *japonica* rice (*Oryza sativa*) genotypes

Camilo López-Cristoffanini[1,*], Xavier Serrat[1], Eduardo Ramos-Fuentes[1,2], Isidre Hooghvorst[1,3], Roser Llaó[2], Marta López-Carbonell[1], Salvador Nogués[1]

[1]Departament de Biologia Evolutiva, Ecologia i Ciencies Ambientals, Secció de Fisiologia Vegetal, Universitat de Barcelona, 08028 Barcelona, España; [2]Càmara Arrossera del Montsià i Secció de Crèdit SCCL, 43870 Amposta, Tarragona, España; [3]ROCALBA S.A., c/Barcelona, 15 PO BOX 156, 17001 Girona, España
*E-mail: camilo.lopez.cr.puc@gmail.com Tel: +34-934-033-714 Fax: +XX-XX-XX-XXX

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Abstract Rice is one of the greatest calorie supply for the world population, especially since its production is almost entirely destined to direct human consumption and its demand will increase along with the world population. There are efforts worldwide to increase rice yields by obtaining new improved and stabilized rice lines. The rice anther culture, a fast and cheap technique, allows to obtain double haploid lines in less than one year. We report its application with an improved protocol in four Mediterranean *japonica* rice genotypes at F_2 generation. We performed a screening test for cold-pretreatment at 5.0±0.1°C and concluded that the optimum duration was 9 days as it produced the higher rate of anther-derived *callus* induction. This revised protocol was successfully applied to the four genotypes, obtaining good results in all the procedure's steps. At the end, more than 100 of double haploid green plants were generated. Moreover, 9 lines obtained from the anther culture procedure showed good qualities for the Spanish market at the growing, farming and grain production level during the field assays. Therefore, we report an improved anther culture procedure for obtaining double haploid lines from temperate *japonica* rice genotypes showing high commercialization expectance.

Key words: anther culture, cold-pretreament, field assays, Mediterranean rice, *Oryza sativa*.

Rice is a highly important cereal cultivar in the world, with a total of 490.9 million tonnes (milled equivalent) produced in 2015/16 of which more than 80% is destined to direct human consumption (FAO Trade and Market Division 2017). In addition, it has been proposed that rice will be one of the main calorie supplies in the forthcoming years (FAO Rice Market Monitor 2016). Thus, there are efforts worldwide to accelerate the development of new rice varieties either to attain higher yielding rates and/or to obtain higher quality grains (Guimaraes 2009; Khush 2005; Moon et al. 2003; Peng et al. 2008; Zeng et al. 2017). Despite the efforts made, rice breeders' seed suffer recurrent deteriorations due to successive annual cultivation (IRRI 1988; Serrat et al. 2014). Programs for ensuring rice breeders' seeds stability are laborious and time-consuming (Briggs and Knowles 1967; Jennings et al. 1979; Serrat et al. 2014). In addition, selecting and stabilizing new rice lines from an F_1 cross is a long process that usually takes about 8 years minimum (Martínez et al. 1996; Serrat et al. 2014).

The anther culture technique, first developed in rice by Niizeki and Oono (1968), allows to obtain stabilized double haploid (DH) plants bypassing the inbreeding process. Moreover, it is the fastest method to obtain DH rice plants as can be performed in less than one year (Agache et al. 1989; Miah et al. 1985). Roughly, this technique is a two-step process from the initial development of *calli* to the subsequent regeneration of green plants from embryogenic *calli* (Mishra and Rao 2016). This technique has been used to obtain pure parental lines and to speed up descendant's selection after an artificial cross (Courtois 1993; Mishra and Rao 2016). Over the years, it has been shown that it is much easier to apply this technique on tropical *japonica* varieties, since they are more responsive at the *callus* formation and plant regeneration stages than Mediterranean *japonica* or *indica* varieties (Chen et al. 1986; Herath et al. 2007; Hu 1985; Miah et al. 1985; Mishra and Rao 2016; Serrat et al. 2014; Yan et al. 1996). Despite of that, we have previously reported an anther culture technique adaptation for a Mediterranean temperate *japonica* rice (*Oryza sativa*) cultivar (NRVC980385) to produce a new commercial cultivar (NRVC20110077; Serrat el al. (2014)), which however showed a very poor anther-derived *callus*

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induction.

Therefore, the main aim of this study is to test for the first time and improved anther culture procedure on F_2 rice genotypes coming from self-pollination of four crosses between different temperate Mediterranean *japonica* rice varieties. In addition, a secondary aim was to test the effect of a colder cold-pretreatment performed at different days of exposure for increasing the anther induction rate. This will allow to establish a standard and fast technique for obtaining commercial DH plants, with a high anther induction efficiency, from any temperate Mediterranean *japonica* rice line in development.

For testing this improved protocol, four different F_2 rice genotypes that resulted from self-pollination of an F_1 generation generated by crosses between Mediterranean temperate *japonica* rice cultivars were used (Table 1; germplasm rice genotypes are coded according to La Càmara cooperative seed producer simplified coding system). We employed the F_2 generation as characters segregation is maximum and plants will therefore provide high variability when obtaining the double haploid green plants (Guimaraes 2009). Plants were grown in greenhouse conditions at the Experimental Fields Service at the University of Barcelona (Barcelona, Spain) on four litre plastic containers filled with rice substrate as described in Serrat et al. (2014).

The anther culture procedure was performed similar to Serrat et al. (2014). The cold-pretreament was modified in order to enhance the anther-derived *callus* induction stage according to (Chen et al. 1986; Trejo-Tapia et al. 2002a, 2002b). We performed a screening test at 5.0±0.1°C during 8 to 12 days to select the best cold-pretreatment duration for using it for the anther protocol. Haploid *calli* spontaneously double their ploidy during the plantlet regeneration step, and thus develop into double haploid (DH) plants but could also develop into haploid, triploid or polyploid plants (Alemanno and Guiderdoni 1994). Further, the ploidy level before was analysed with the aim of reducing greenhouse space and costs since haploid plants are sterile. The ploidy determination was performed by flow cytometry following the protocol described in Serrat et al. (2014). Dihaploid plants were cultured in greenhouse until seed-set, and seeds were harvested for the subsequent field assays.

For comparing the suitability of the improved anther protocol, several parameters were analysed in the four F_2 rice genotypes tested and NRCV980385 cultivar used in Serrat et al. (2014). These were: *callus* induction percentage (CI%)=number of anthers producing *calli*/number of plated anthers×100; *callus* production ratio (CP_{ratio})=number of produced *calli*/number of anthers producing *calli*; green plant percentage (GR%)=number of green plant regenerated/number of transferred *calli*×100; green double haploid plant percentage (GRDH%)=number of green DH plants regenerated/number of transferred *calli*×100. For comparing data among rice genotypes, we used two approximations: (i) visually, we calculated the confidence intervals (CI) using the following formula

$$CI = \% \pm 1.96 \times \sqrt[2]{\frac{\% \times (1 - \%)}{\text{number of observations}}}$$

and used them as a mean of standard error; and (ii) statistically, we performed a Chi-squared test with Yates correction (Zar 2010). No visual nor statistical approximations were used for CP_{ratio}, since due to its nature neither CI nor Chi-squared test with Yates correction were possible to calculate. Please note that due to the experimental procedure of the anther culture, we did not use replicates, thus total values of several parameters for each genotype assayed were used instead.

Finally, for testing rice genotypes with commercial interest, we performed a general field assay on 70 double haploid in order to screen overall diseases resistance and production estimates. Selected genotypes were assayed in small scale field assays in La Càmara experimental fields (Amposta, Tarragona, Spain). For this, two designs were used: (i) plant agronomical trait evaluation: 25 plants per genotype assayed were planted in row as to have 20 cm between each plant and 50 cm between rows; (ii) plant production evaluation: 80 plants per genotype assayed were planted in row as to have 20 cm between each plant and 25 cm between rows. Plant agronomical traits such as plant height (i.e., from the base of the plant to the top

Table 1. Parental cultivar (P1 and P2) and F_2 rice genotypes produced by P1×P2 cross are listed using the simplified code system according to La Càmara seed producer. Anther culture in vitro results are show: number of plated anthers, number of anthers producing *calli*, number of *calli* generated, *callus* induction percentage (CI,%), *callus* production ratio (CP_{ratio}) and number of green double haploid plants regenerated in the four rice genotypes assayed. The error for CI(%) corresponds to the confidence interval.

Parental cultivar 1 (P1)[a]	Parental cultivar 2 (P2)[a]	F_2 rice genotype (P1×P2→F_1→F_2)	No of plated anthers	No of anther producing *calli*	No of produced *calli*	*Callus* induction (CI, %)	CP_{ratio}	No of green double haploid plants regenerated
rG3	NRCV980385 (rG0)	F_2-30	20185	27	160	0.133±0.050	5.93	30
rG4	NRCV980385 (rG0)	F_2-40	21301	99	547	0.465±0.091	5.53	70
rG4	rG2	F_2-42	18880	37	152	0.196±0.063	4.11	7
rG4	rG5	F_2-45	17456	72	360	0.412±0.095	5	17
—	—	NRVC980385[b]	42660	4	66	0.009±0.009	16.5	29

[a]rG: Rice genotype. [b]Data for NRCV980385 was obtained from supplementary material in Serrat et al. (2014).

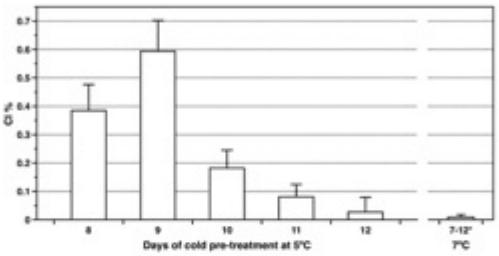

Figure 1. Effect of the cold pre-treatment on the callus induction percentage (CI,%) in the four rice lines assayed. The star (*) data on the right side of the graph corresponds to NRCV980385 retrieved from supplementary material in Serrat et al. (2014), in which the cold pre-treatment was performed at 7°C during 7–12 days. Error bars correspond to the confidence intervals.

of the panicle), susceptibility to rice stem borers and resistance to blast and brown spot (Mew and Gonzales 2002), number of spikes per plant and inter-homogeneity (homogeneity between plants of the same genotype) were recorded 120 days after sowing. For plant production traits, the parameters evaluated were humidity (%) at the time of data recollection, 1,000-grains weight, percentage of whole grains (unshattered milled grains/total milled grains×100) and yield (kg of grains per hectare, kg/ha). NRVC980385 was used as a control to monitor field behaviour as it is a parental cultivar for F_2-30 and F_2-40, and is also common variety cultivated in the region (Català et al. 2007; Serrat et al. 2014).

Results of the cold-pretreatment duration test at 5.0±0.1°C as well as that performed at 7°C by Serrat et al. 2014 is shown in Figure 1. It was observed that for the *callus* induction (CI, %), there were significant differences between the duration in days of the cold-pretreament ($\chi^2_{Yates}(4)=94.0699$, $p<0.0001$; Figure 1), being 9 days the optimum for anther-derived *callus* induction. Moreover, it was also observed that a cold-pretreament at 5°C during 9 days instead of 7°C during 7–12 days had a higher CI% in all the days tested, being 0.254±0.072 in average and 0.009±0.009, respectively. Our results differ to those of Kaushal et al. (2014b) where the optimum is for 5 days at 12°C. These contrasting results can be explained by the fact that Kaushal et al. (2014b) used *indica* varieties, whereas the ones used in our experiment are Mediterranean temperate *japonica* genotypes. Trejo-Tapia et al. (2002a, 2002b) also studied the effect of cold-pretreament in anther-derived *callus* induction, but at 4°C. In their first study, 14 days was the best for the majority of the cultivars (tropical *japonica* sub-species) (Trejo-Tapia et al. 2002a), whereas in their second study 7 days was the best for H2500 cultivar (tropical *japonica* sub-species) (Trejo-Tapia et al. 2002b). Our cold-pretreament duration is situated between both works, suggesting that 9 days is an ideal time for this stage of the anther culture procedure to enhance the *callus* production in Mediterranean temperate rice *japonica* varieties. Moreover, the cold-pretreament at 5°C during 9 days radically increases the CI%, as the lowest CI% value reported in this study is almost 15 times higher than the one reported with NRCV980385 (Serrat et al. 2014).

Regarding the anther culture procedure for obtaining new rice line, an average of approximately 19,500±1,600 anthers was plated for each of the four F_2 rice genotypes used in this experiment (details for each genotype in Table 1). *Calli* were produced from all of the four assessed genotypes differing in the CI%, but in average was higher in average than the one reported by Serrat et al. (2014). More in detail, it was observed that the genotype F_2-30, the one with the lowest CI%, had an anther-derived *callus* induction 12 times greater than NRCV980385 (Table 1). On the other hand, genotypes F_2-40 and F_2-45 displayed a CI% 51.7 and 45.8 times higher, respectively, when compared to NRCV980385. It is worth noting that the three of the F_2 genotypes in which the cultivar 4 was one of the used parental, yielded the highest CI%, being the higher the genotype F_2-40, cross between cultivar 4 and NRCV980385, with 0.465%. Statistical analysis showed significant differences between all the five rice genotypes (four F_2 rice genotypes and NRCV980385 cultivar) ($\chi^2_{Yates}(4)=193.9229$, $p<0,0001$). In the literature, CI% vary between as low as 0.2% to up to 77.9%, being the genotype the most important factor that determines these percentages (Bishnoi et al. 2000; Herath et al. 2007; Kaushal et al. 2014a, 2014b; Shahnewaz et al. 2004). Our CI% is situated in the lower ones, and it is probably due to the genotype of our F_2 rice genotypes, which do not favour *callus* formation as also observed in the study performed by Serrat et al. 2014. Several studies support this affirmation, since most of the differences can be explained by the genotype factor (Herath et al. 2007; Kaushal et al. 2014a; Khanna and Raina 1998; Shahnewaz et al. 2004; Yan et al. 1996). Despite the low CI% reported in this study when compared to the majority of the literature, the number of *callus* obtained was higher than that reported by other authors (Shahnewaz et al. 2003, 2004).

The higher anther-derived *callus* induction translated in a higher number of *calli*, 1,219 in total for the four F_2 genotypes assayed (Table 1). Moreover, the number of *calli* produced was much higher in this study compared to NRCV980385. The *callus* production ratio (CP_{ratio}) was similar in the four rice genotypes, being F_2-30 and F_2-40 the ones that showed higher values of 5.9 and 5.5, respectively. In average, the CP_{ratio} only corresponded to 31.2% of the displayed by NRCV980385 (Table 1) though we expected, as seen for CI%, that this parameter would be higher. This can be explained by considering the following: (i) average of anthers producing *callus* for our experiment was 59±17 whereas for Serrat et al. (2014)

only four anthers were used for *callus* production; (ii) average of *callus* produced were 305±94 and 66 in our experiment and in Serrat et al. (2014), respectively. Thus, although having in average a low CP_{ratio} in our study, we expect to have a higher chromosomal variability of the rice genotypes since an elevated number of *calli* coming from a larger number of anthers was obtained. It is worth nothing that not much data is available in the literature regarding CP_{ratio} which in turn does not allow for much comparison. Nevertheless, it is of high importance since it gives information if regenerated plants come from several or few *calli*, thus we propose that this ratio should be regularly given.

The green plant percentage (GR,%) was in average 89.6±5.9% among the four rice genotypes tested as seen in Figure 2, which is similar to our prior results using NRCV980385. But, it is noteworthy that the GR% was considerably higher than in other articles (ranging from 2 to even 16 times more) (Herath et al. 2007; Kaushal et al. 2014a, 2014b; Shahnewaz et al. 2003, 2004; Trejo-Tapia et al. 2002a, 2002b). Statistical analysis also showed that there are significant differences between the 5 rice genotypes ($\chi^2_{Yates}(4)=6.3447$, $p=0.1478$; Figure 2). The green double haploid plant percentage (GRDH,%) reported in this study ranges from 11.5 to 47.1% of that reported with NRVC980385, being the genotypes F_2-30 and F_2-40 the ones displaying the higher GRDH% among our four F_2 rice genotypes (20.7 and 15.5%, respectively). In this study, more than 75 plants for each genotype were analysed, whereas in Serrat et al. (2014) only 43 in total were analysed. The GRDH% was significantly different among the 5 varieties ($\chi^2_{Yates}(4)=67.6942$, $p<0.0001$; Figure 2). This parameter is of the uttermost importance since those plants are viable and suited for field assay evaluations, which is the final purpose of this procedure. No data for this is available in the literature thus we cannot further compare. The last stage of the process was to acclimatize in vitro plants to greenhouse conditions in rice substrate. We transplanted a total of 547 green double haploid plants produced from the 4 F_2 genotypes, of which in average 89±4% were successfully grown to maturity (data not shown), which is greater than the 67±8% in average that is reported by Herath et al. (2007). Similarly, only 12±8% of the total of the transplanted plants showed more than 5% of sterility (data not shown), which is a better success rate than 24±31%, value observed in the work by Herath et al. (2007).

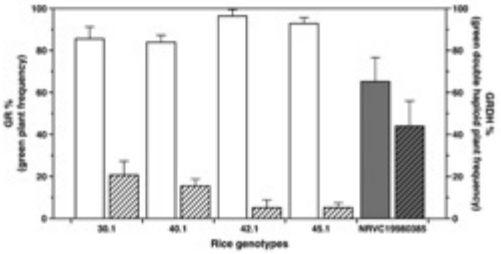

Figure 2. Total percentage of green regenerated plants (GR%) and total percentage of green double haploid regenerated plants (GRDH%) in the four rice lines assayed (white bars). Data for NRCV980385 was retrieved from supplementary material in Serrat et al. (2014) (grey bars). Solid bars correspond to GR% and hatched bars to GRDH%. Error bars correspond to the confidence intervals.

During the general field assay of 70 double haploid (DH) lines, it was observed that all DH plants coming from F_2-42 and F_2-45 along with some DH plants of F_2-30 and F_2-40 lacked agronomic and commercial interest (data not shown). Therefore, a total of 9 lines were selected for agronomical and production traits, which showed high inter-homogeneity and a high tillering activity (more than 40 tillers per plant; data not shown). The average height of the lines was 70.7±2.2 cm, which is in the range of those cultivated in the Ebro Delta. Moreover, 7 of them were shorter than Gleva, the shortest cultivated variety in the region (Pla

Table 2. Agronomical and production traits evaluation for the lines assayed in the field assays.

Anther-derived rice line	Height (cm)[a]	Plant agronomical traits evaluation			Plant production traits evaluation[d]			
		Fungal disease resistance[b]		Rice stem borers resistance[b,c]	Humidity (%)	1,000-grain weight (g)	Whole grains (%)	Yield (kg/ha)
		Blast[c]	Brown spot[c]					
F_2-30.C1	68.5±1.9	+++	+++	++	13.8	27.5	67.3	5740
F_2-30.C2	70.5±0.6	+++	+++	++	14	28.8	66.7	5828
F_2-40.C15	66.3±4.1	+++	+++	++	13.9	32.5	67.8	11393
F_2-40.D37	68.5±1.3	+++	+++	+	13.8	35	63.3	11598
F_2-40.D39	62.8±2.1	+++	+++	+++	13.6	35	61.4	10338
F_2-40.D118	65.3±0.5	+++	+++	+	13.8	35	66.9	11328
F_2-40.D173	59.0±2.7	+++	+++	++	13.9	30	63	11670
F_2-40.D174	61.5±1.3	+++	+++	++	13.9	32.5	65.9	11760
F_2-40.D266	90.3±4.4	+++	+	−	14.1	35	63.9	13553
NRVC980385	94.0±2.7	+++	+++	+	14.2	27.5	65.3	11325

[a] The value shown correspond to the mean of 25 plants and the SD. [b] Resistance scale is the following: −: sensible; +: low resistance; ++: medium resistance; +++: high resistance. [c] Blast and brown spot diseases are caused by *Magnaporthe oryzae* and *Helminthosporium* sp. respectively, and rice stem borers *Chilo suppresalis*. [d] Production traits evaluation data for NRCV980385 was recorded at the same time of the F_2 rice lines assayed.

et al. 2017). Overall, all evaluated lines showed a high resistance to fungal diseases and medium resistance to rice stem borers (Table 2), except for F_2-40.D266 plants which was promising in terms of production during the general field assay. Regarding blast resistance, it was in general higher in comparison to the varieties regularly cultivated in the region of the Ebro Delta (Català et al. 2009; Pla et al. 2017). No literature was available for comparing tolerance to brown spot disease and the rice stem borers in local field conditions. Despite this, resistance to brown spot was similar to that of the control variety (NRVC980385), and rice stem borers resistance was higher than NRVC980835 for several lines. The 1,000-grain weight (determined by grain length, width and thickness) of the lines was in average 31.6 g, comparable among them since the humidity range was 13.6–14.2%. Of the assayed lines, all of them with the exception of F_2-30.C1, as seen in Table 2, showed a 1,000-grain weight higher than NRVC980385 and other *indica* and *japonica* varieties (Fan et al. 2006; Koutroubas and Ntanos 2003). The whole grains percentage was variable among the 9 lines assayed but ranged between 60–70%, similar to NRVC980385 and several other Spanish varieties values (Català et al. 2009), thus these lines are suitable for large scale production. In terms of yield, lines of the F_2-30 genotype were below those reported for Gleva (most cultivated variety in the region) and NRVC980835 cultivar (Pla et al. 2017), and half of the values reported for F_2-40 genotype lines (Table 2). On the other hand, lines of the F_2-40 genotype displayed higher yields than Gleva (Pla et al. 2017). Furthermore, the observation that F_2-40.D266 was promising in terms of production was certain as its yield was the highest among the lines tested and higher than the Spanish rice varieties including the NRVC980835 cultivar (Pla et al. 2017). Nevertheless, to fully characterize and evaluate the assayed lines, direct seeded field assays should be performed in a medium (and maybe even large scale) in order to better assess for pathogens resistance, plant height and yield.

In conclusion, we have shown that the improved anther culture protocol can be successfully applied in different F_2 rice genotypes between temperate *japonica* rice genotypes to obtain green double haploid plant. Moreover, we have observed that genotype is one of the main factors that affects the anther culture protocol success. Despite this, we have determined that the cold-pretreatment improvement, 9 days at 5.0±0.1°C, greatly increases the anther-derived *callus* induction in temperate *japonica* Mediterranean rice crossed genotypes at the F_2 generation, since the number of green double haploid plants obtained at the end of the anther culture procedure was high. Furthermore, 7 of the 9 lines evaluated in the field showed good qualities at the agricultural and production level. Therefore, these varieties are suited to be submitted to direct seeded medium scale assays before registry for their subsequent commercialization. Thus, in conclusion, our proposed method for Mediterranean *japonica* rice is highly applicable to rice genotypes at the F_2 generation of different *japonica* rice cultivars for producing new lines that could be registered and commercialized as new varieties.

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Conflicts of interest

The authors declare that they have no competing interests.

References

Agache S, Bachelier B, Buyser J, Henry Y, Snape J (1989) Genetic analysis of anther culture response in wheat using aneuploid, chromosome substitution and translocation lines. *Theor Appl Genet* 77: 7–11

Alemanno L, Guiderdoni E (1994) Increased doubled haploid plant-regeneration from rice (*Oryza sativa* L.) anthers cultured on colchicine-supplemented media. *Plant Cell Rep* 13: 432–436

Bishnoi U, Jain RK, Rohilla JS, Chowdhury VK, Gupta KR, Chowdhury JB (2000) Anther culture of recalcitrant *indica* × Basmati rice hybrids. *Euphytica* 114: 93–101

Briggs FN, Knowles PF (1967) Introduction to plant breeding. Introduction to plant breeding. Reinhold Publishing Corporation, New York

Català MM, Jardí M, Pla E (2007) Evolución de las variedades de arroz cultivadas en el Delta del Ebro durante los últimos 20 años. *Agric Vergel* 303: 132–135

Català MM, Tomàs N, Martinez M, Pla E (2009) Valoración agronómica de nuevas variedades de arroz. Ensayos en el Delta del Ebro: 2004–2008. *Agric Vergel* 327: 161–166

Chen CC, Tsay HS, Huang CR (1986) Rice (*Oryza sativa* L.): factors affecting androgenesis. In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry, vol 2, Springer Berlin Heidelberg, Heidelberg, Germany, pp 123–128

Courtois B (1993) Comparison of single seed descent and anther culture-derived lines of three single crosses of rice. *Theor Appl Genet* 85: 625–631

Fan C, Xing Y, Mao H, Lu T, Han B, Xu C, Li X, Zhang Q (2006) GS3, a major QTL for grain length and weight and minor QTL for grain width and thickness in rice, encodes a putative transmembrane protein. *Theor Appl Genet* 112: 1164–1171

FAO Rice Market Monitor (2016) Rice market monitor. Trade and markets division. Food and agriculture organization of the United Nations

FAO Trade and Markets Division (2016) Food Outlook: Biannual

report on global food markets, November 2017. Food outlook, food and agriculture organization of the United Nations

Guimaraes EP (2009) Rice breeding. In: Carena MJ (ed) Cereals, The banks and the Italian economy. Springer-Verlag New York, New York, pp 99–126

Herath HMI, Bandara DC, Samarajeewa PK (2007) Effect of culture media for anther culture of *indica* rice varieties and hybrids of *Indica* and *Japonica*. *Trop Agric Res Ext* 10: 17–22

Hu H (1985) Use of haploids for crop improvement in China. *Genetic Manipulation in Crops Newsletter* 1: 11–23

IRRI (1988) Rice Seed Health. Proceedings of the International Workshop on Rice Seed Health. International Rice Research Institute, Manila

Jennings PR, Coffman WR, Kauffman HE (1979) Rice improvement. International Rice Research Institute (IRRI), Los Baños, Laguna, Philippines

Kaushal L, Balachandram SM, Ulaganathan K, Shenoy V (2014a) Effect of culture media on improving anther culture response of rice (*Oryza sativa* L.). *International Journal of Agriculture Innovations and Research* 3: 218–224

Kaushal L, Sharma R, Balachandram SM, Ulaganathan K, Shenoy V (2014b) Effect of cold pretreatment on improving anther culture response of rice (*Oryza sativa* L.). *J Exp Biol Agric Sci* 2(2S): 233–242

Khanna HK, Raina SK (1998) Genotype x culture media interaction effects on regeneration response of three *indica* rice cultivars. *Plant Cell Tissue Organ Cult* 52: 145–153

Khush GS (2005) What it will take to feed 5.0 billion rice consumers in 2030. *Plant Mol Biol* 59: 1–6

Koutroubas SD, Ntanos DA (2003) Genotypic differences for grain yield and nitrogen utilization in *Indica* and *Japonica* rice under Mediterranean conditions. *Field Crops Res* 83: 251–260

Martínez CP, Correa Victoria F, Amézquita MC, Tulande E, Lema G, Zeigler RS (1996) Comparison of rice lines derived through anther culture and the pedigree method in relation to blast (*Pyricularia grisea* Sacc.) resistance. *Theor Appl Genet* 92: 583–590

Mew TW, Gonzales P (2002) A handbook of rice seedborne fungi. IRRI, Los Baños, Philippines

Miah MAA, Earle ED, Khush GS (1985) Inheritance of *callus* formation ability in anther cultures of rice, *Oryza sativa* L. *Theor Appl Genet* 70: 113–116

Mishra R, Rao GJN (2016) In-vitro androgenesis in rice: Advantages, constraints and future prospects. *Rice Sci* 23: 57–68

Moon HP, Kang KH, Choi IS, Jeong OY, Hong HC, Choi SH, Choi HC (2003) Comparing agronomic performance of breeding populations derived from anther culture and single-seed descent in rice. In: Advances in Rice Genetics. Los Baños, Laguna, Philippines, pp 3–5

Niizeki H, Oono K (1968) Induction of haploid rice plant from anther culture. *Proc Jpn Acad* 44: 554–557

Peng S, Khush GS, Virk P, Tang Q, Zou Y (2008) Progress in ideotype breeding to increase rice yield potential. *Field Crops Res* 108: 32–38

Pla E, Català MM, Tomàs N (2017) Variedades de arroz registradas en España entre los años 2013 y 2016. *Vida Rural* 424: 14–18

Serrat X, Cardona M, Gil J, Brito AM, Moysset L, Nogués S, Lalanne E (2014) A Mediterranean *japonica* rice (*Oryza sativa*) cultivar improvement through anther culture. *Euphytica* 195: 31–44

Shahnewaz S, Bari MA, Siddique NA, Khatun N, Rahman MH, Haque ME (2003) Induction of haploid rice plants through *in vitro* anther culture. *Pak J Biol Sci* 6: 1250–1252

Shahnewaz S, Bari MA, Siddique NA, Rahman MH (2004) Effects of genotype on induction of *callus* and plant regeneration potential *in vitro* anther culture of rice (*Oryza sativa* L.). *Pak J Biol Sci* 7: 235–237

Trejo-Tapia G, Maldonado-Amaya U, Jiménez-Aparicio A, Blanqueto-Illescas M, Salcedo-Morales G, Martínez-Bonfil BP, De Jesús-Sánchez A (2002b) Effect of time at low temperatura treatment and growth regulators on regeneration of plants from andthers of rice *Oryza sativa* L. (*japónica* H2005 cultivar). *Agrociencia* 36: 441–449

Trejo-Tapia G, Maldonado Amaya U, Salcedo Morales G, De Jesús Sánchez A, Martínez Bonfil B, Rodríguez-Monroy M, Antonio JA (2002a) The effects of cold-pretreatment, auxins and carbon source on anther culture of rice. *Plant Cell Tissue Organ Cult* 71: 41–46

Yan J, Xue Q, Zhu J (1996) Genetic studies of anther culture ability in rice (*Oryza sativa*). *Plant Cell Tissue Organ Cult* 45: 253–258

Zar JH (2010) Biostatistical Analysis. Statistics and Mathematics, 5th ed. Prentice Hall, Upper Saddle River, NJ, USA

Zeng D, Tian Z, Rao Y, Dong G, Yang Y, Huang L, Leng Y, Xu J, Sun C, Zhang G, et al. (2017) Rational design of high-yield and superior-quality rice. *Nat Plants* 3: 17031

CHAPTER 3.2 - Antimitotic and hormone effects on green double haploid plant production

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ORIGINAL ARTICLE

Antimitotic and hormone effects on green double haploid plant production through anther culture of Mediterranean japonica rice

Isidre Hooghvorst[1,2] · Eduardo Ramos-Fuentes[1,3] · Camilo López-Cristofannini[1] · Mirari Ortega[1] · Raimon Vidal[1] · Xavier Serrat[1] · Salvador Nogués[1]

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Abstract
Rice double haploid (DH) plants are produced mainly through anther culture. In order to improve the anther culture protocol, microspores of two japonica rice genotypes (NRVC980385 and H28) were subjected to three growth regulator combinations and four colchicine treatments on induction medium. In addition, a post anther culture procedure using colchicine or oryzalin was tested to induce double haploid plantlets from haploid plantlets. A cold pre-treatment of microspores for 9 days at 10 °C increased callus induction 50-fold in the NRCV980385 genotype. For both genotypes, 2 mg L^{-1} 2,4-*D* and 1 mg L^{-1} kinetin on colchicine-free induction medium gave the best culture responses. The culturability of both genotypes changed on colchicine-supplemented induction media. A high genotype dependency was recorded for callus induction, callus regenerating green plantlets and regeneration of green double haploid plantlets. Colchicine at 300 mg L^{-1} for 48 h enhanced callus induction 100-fold in H28. Colchicine-supplemented media clearly improved green double haploid plantlet regeneration. We showed that the post-anther culture treatment of haploid plantlets at 500 mg L^{-1} of colchicine permitted fertile double haploid plantlets to be generated. Finally, an enhanced medium-throughput flow cytometry protocol for rice was tested to analyse all the plantlets from anther and post anther culture.

Keywords Mediterranean japonica rice · Anther culture · Hormones · Colchicine · Antimitotics · Double haploid

Introduction

Doubled haploid lines (DHs) are produced when spontaneous or induced chromosome duplication of haploid cells occurs. DH plants are complete homozygous individuals that can be produced within a year through anther or microspore culture. Therefore, the production of homozygous lines from heterozygous parents is feasible and shortens the time required to obtain them (Germanà 2011). Nowadays, anther culture is being used to produce DH plants in more than 250 species, including major cereals such as rice, wheat, maize, barley and also economically important trees, fruit crops and medicinal plants (Maluszynska 2003).

Rice DH plant production is mainly obtained through anther culture. Niizeki and Oono (1968) were the first to produce haploid rice plantlets through anther culture. Rice anther culture is a two-step process with initial callus development and subsequent green plantlet regeneration from embryogenic callus (Mishra and Rao 2016). Since the first report of anther culture, much research has aimed at optimizing the media used at each step in the process to enhance callus induction and callus regeneration (Herath et al. 2010; Pauk et al. 2009). This work has focused on overcoming limiting factors that reduce the efficiency of green DH plantlet production such as high genotypic dependency, low frequency of callus induction and plantlet regeneration, the low percentage of doubled haploids

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✉ Isidre Hooghvorst
isidrevander@gmail.com

[1] Departament de Biologia Evolutiva, Ecologia i Ciencies Ambientals, Secció de Fisiologia Vegetal, Universitat de Barcelona, 08028 Barcelona, Spain

[2] Rocalba S.A., c/Barcelona 15, PO Box 156, 17001 Girona, Spain

[3] Càmara Arrossera del Montsià i Secció de Crèdit SCCL, 43870 Amposta, Tarragona, Spain

produced and the high ratio of albino plantlets (Lentini et al. 1997). The application of stress during the developmental period of pollen grains, osmotic stress applied to cells during culture, the composition of the culture media, and the addition of antimitotic agents, gelling agents or growth regulators amongst many exogenous factors may affect the success of anther culture in rice (Mishra and Rao 2016). Endogenous factors such as the rice variety and genotype also affect anther culture success. Indica rice varieties have a limited response to anther culture due to early necrosis, poor callus proliferation and a high regeneration of albino plantlets (Chen et al. 1991), unlike japonica varieties where green DH plant production is more efficient (He et al. 2006).

Despite the improvements and progress achieved in every step of the anther culture procedure, there is still a need to optimize conditions for higher rates of green DH plant production while reducing the amount of work in each step. Colchicine is an antimitotic compound widely used in microspore culture and has been shown to improve results in terms of green double haploid plant production (Forster et al. 2007) in maize (Obert and Barnabás 2004), barley (Thompson et al. 1991), wheat (Barnabás et al. 1991), rapeseed (Weber et al. 2005), and other species. However, few authors have reported the use of colchicine in rice anther culture. Alemanno and Guiderdoni (1994) were the first to study a routine in vitro colchicine treatment to increase DH plant production in rice. In addition, post anther culture procedures have rarely been used in green haploid plantlets regenerated from anther culture. Finally, such a procedure can be undertaken either in vivo by treating tillers with antimitotic compounds such as colchicine in order to increase the DH recovery from haploid plantlets (Jensen 1974; Zapata-Arias 2003; Chen et al. 2002) or in vitro as explained in this work.

Among the auxins, 2,4-dichlorophenoxyacetic acid (2,4-*D*) and naphthaleneacetic acid (NAA) are the most used hormones for rice callus induction from anthers (Trejo-Tapia et al. 2002).

The aim of this study is to improve the anther culture efficiency in two japonica rice genotypes by assessing some factors that could improve the numbers of green double haploid plants. Thus we tested (i) the effect of different growth regulators (2,4-*D*, NAA and Kinetin) in the anther culture induction medium, (ii) the effect of different colchicine doses in the anther culture induction medium and (iii) a post anther culture procedure to increase plant DH production from haploid plantlets through colchicine and oryzalin in vitro treatments. We showed that colchicine-supplemented media increase green plantlet double haploid production and 500 mg L^{-1} of colchicine in a post-anther culture procedure enabled recovery of green double haploid plantlets from haploid plantlets to be maximized.

Materials and methods

Plant material and growth conditions

The commercial temperate japonica rice variety NRVC980385 and a temperate japonica F2 hybrid called H28, provided by the *Càmara Arrossera del Montsià SCCL* cooperative, were used as plant material. Plants were grown in greenhouse conditions at the *Servei de Camps Experimentals* at the University of Barcelona (Barcelona, Spain) in 4 L plastic containers filled with rice substrate as previously described (Serrat et al. 2014).

Anther culture procedure

Tillers were selected at the booting stage, when the distance from the flag leaf to the auricle of the penultimate leaf was 5–12 cm. The time of collection was from 8:00 to 9:30 as recommended by Chen et al. (1991). Collected tillers were soaked in 70% ethanol for 1 min, rinsed twice with distilled water and were then cold pre-treated for 9 days at 10 °C in polystyrene bags, prior to being surface disinfected again as above. Tillers were dissected to obtain the panicles in a laminar flow cabinet. Panicles were soaked in 70% ethanol for 1 min, rinsed twice and soaked in 10% sodium hypochlorite solution with Tween 20 (30 drops L^{-1}) and 35% HCl (50 drops L^{-1}) for 3 min, and rinsed five times in sterile distilled water. Anthers were obtained from the panicles and plated into 90 mm petri dishes (Sterilin LTD, Cambridge). Basal induction medium consisted of Chu N6 modified as follows: N6 standard salts and vitamins fortified with a combination of growth regulators, 1 g L^{-1} casein enzymatic hydrolysate, 250 mg L^{-1} L-proline, 2 mg L^{-1} 500 mg L^{-1} 2-(*N*-morpholino) ethanesulfonic acid (MES), 30 g L^{-1} sucrose and 3 g L^{-1} Gelrite.

The anther culture procedure was carried out in parallel for all the combinations of different growth regulators and their concentrations, colchicine concentrations and colchicine exposure times (Table 1). Regarding growth regulators, three combinations were used: (i) treatment D1, 1 mg L^{-1} 2,4-*D* and 1 mg L^{-1} kinetin; (ii) treatment D2, 2 mg L^{-1} 2,4-*D* and 1 mg L^{-1} kinetin, as used by Serrat et al. (2014) and Chen et al. (2002); and (iii) treatment NA, 2 mg L^{-1} NAA and 0,5 mg L^{-1} kinetin, as used by Alemanno and Guiderdoni (1994). Colchicine was assayed at 0 (control), 150 and 300 mg L^{-1}, each for the two exposure times of 24 and 48 h. Thus, five colchicine treatments were tested and named as concentration/exposure time: 0/0 (control), 150/24, 150/48, 300/24 and 300/48. Six petri dishes were sown with approximately 100 anthers

Table 1 Conditions for induction media assayed for both genotypes

Growth regulator			Colchicine treatment		Conditions
2,4-*D* (mg L^{-1})	NAA (mg L^{-1})	Kinetin (mg L^{-1})	Concentration (mg L^{-1})	Exposure time (h)	Concentration/time-hormone
1	0	1	0	0	0/0-D1
			150	24	150/24-D1
			150	48	150/48-D1
			300	24	300/24-D1
			300	48	300/48-D1
2	0	1	0	0	0/0-D2
			150	24	150/24-D2
			150	48	150/48-D2
			300	24	300/24-D2
			300	48	300/48-D2
0	2	0.5	0	0	0/0-NA
			150	24	150/24-NA
			150	48	150/48-NA
			300	24	300/24-NA
			300	48	300/48-NA

Three growth regulator treatments were tested: *D1* 1 mg L^{-1} 2,4-*D* and 1 mg L^{-1} kinetin, *D2* 2 mg L^{-1} 2,4-*D* and 1 mg L^{-1} kinetin; and treatment NA, 2 mg L^{-1} NAA and 0,5 mg L^{-1} kinetin; and three colchicine treatments concentrations were applied, 0 (control condition), 150 and 300 mg L^{-1}

for each condition. After 24 or 48 h, anthers inoculated in colchicine-supplemented media were transferred to exactly the same medium but colchicine-free.

Anthers were kept in the dark at 24 °C and analysed weekly for 8 weeks. Microspore-derived calluses of 1–2 mm diameter that emerged from anthers were transferred to callus regeneration medium as described by Serrat et al. (2014). Anthers that induced callus were removed to ensure a count of one callus per anther, to avoid overestimation of callus induction and to match the number of calluses and induced calli for data analysis. Calluses were transferred to plantlet regeneration medium containing Chu N6 (Chu 1975) standard salts and vitamins fortified with 1 g L^{-1} casein hydrolysate, 250 mg L^{-1} L-proline, 1 mg L^{-1} naphthaleneacetic acid, 2 mg L^{-1} kinetin, 500 mg L^{-1} MES, 30 mg L^{-1} sucrose and 3 g L^{-1} Gelrite. IWAKI 94 mm petri dishes (Asahi Techno Glass Corporation, Amagasaki) were filled with 25 mL of the medium. Calluses were transferred after 28 days onto fresh regeneration medium. Cultures were kept at 25 °C and illuminated with 50–70 µmol m^{-2} s^{-1} fluorescent light under a 16/8 h day/night photoperiod until plantlet formation occurred.

The tiny but fully formed albino and green plantlets (0.5-3 cm length) were transferred into tubes with hormone-free MS (Murashige and Skoog 1962) medium as described by Serrat et al. (2014). Subsequently, clearly sprouting individual plantlets were propagated under conditions as described for regeneration above.

All components of the media were supplied by Duchefa Biochemie BV (The Netherlands). Media were prepared using distilled water and the pH was adjusted to 5.7 by adding 1M KOH (Sigma-Aldrich Co). All components including growth regulators were added before standard autoclave sterilization (121 °C for 20 min).

Ploidy-level determination

The ploidy of green and albino regenerated plantlets was determined by flow cytometry following the procedure of Cousin et al. (2009) with slight modifications. About 5 mg of young leaves were collected and put into ice-cold 2 mL microcentrifuge tubes each with a single steel bead (3 mm diameter). To each tube, 300 µL of cold lysis buffer (0.1 M citric acid and 0.5% Triton X-100 in distilled water) were added. Tubes were cooled at − 20 °C for 10 min. Samples were shaken at 25 Hz for a total of 48 s in a MM 400 tissue lyser (Retsch, Haan, Germany). The aliquot from each tube was filtered through a 22 µm nylon filter (Sefar Maissa, Blacktown, Australia), gently vacuumed and transferred to a flow cytometry sample tube (Beckman Coulter Inc., Pasadena, California, USA). Afterwards, 150 µL of propidium iodide (PI) stain solution [0.25 mM Na_2HPO_4, 10 mL 10× stock (100 mM sodium citrate, 250 mM sodium sulfate) and 9 M PI made up to 100 mL with Milli-Q water] was added to each tube. Tubes were then sealed and kept on ice in the dark for 1 h before flow cytometry (FCM) analysis. The stained nuclei samples were analysed using a Gallios™ Flow Cytometer (Beckman Coulter Inc., Pasadena, California, USA) with a 488-nm laser at the Cytometry Unit (Scientific and Technological Centres, University of Barcelona) and a 32-well carrousel. One diploid control (NRVC 980385) sample was included every seven measurements. Samples analysed with a clearly defined peak as the reference ploidy control were classified as DH, whereas those producing half the fluorescence were classified as haploids. Flow cytometry data was analysed using Summit Software v4.3 (Beckman Coulter Inc., Pasadena, California, USA).

Diploidization of haploid green plantlets

Green haploid plantlets regenerated from anther culture were subjected to a post anther culture in vitro treatment with colchicine at 1000, 500 and 250 mg L^{-1}, or oryzalin at 5, 2.5 and 1.25 mg L^{-1}; both in a solution containing 1% DMSO and Tween 20 (4 drops·L^{-1}) in sterilized distilled water. Prior to the antimitotic treatment, plantlet stems and roots were trimmed to 3 cm in length and were incubated in the antimitotic solution for 5 h on a shaker at 120 rpm at 25 °C and maintained under sterile conditions in a laminar

flow cabinet. Thereafter, the plantlets were transferred to hormone-free MS medium as described before and by 3–4 weeks of growth plantlets that had survived and reached 10–15 cm in size were collected to perform flow cytometry analysis as described before.

Statistics

All parameters were divided by the number of anthers sown for each treatment and multiplied by 100 in order to obtain percentages: induced calluses (IC), number of calluses regenerating green plantlets (CRGP), number of calluses regenerating albino plantlets (CRALP), number of calluses regenerating green and albino plantlets (CRGAP), number of green plantlets regenerated (GPR), number of double haploid plantlets regenerated (DHPR) and number of green double haploid plantlets regenerated (GDHPR). The three concentrations of growth regulators in the colchicine-free media were compared with each other, and each colchicine treatment was also compared individually with the corresponding control medium (0/0) according to the growth regulator hormone concentration (D1, D2 and NA). Growth regulators and colchicine treatments were analysed separately for both genotypes. To determine significant differences between the conditions assayed, a Chi-Square ($P < 0.05$) test for homogeneity was used.

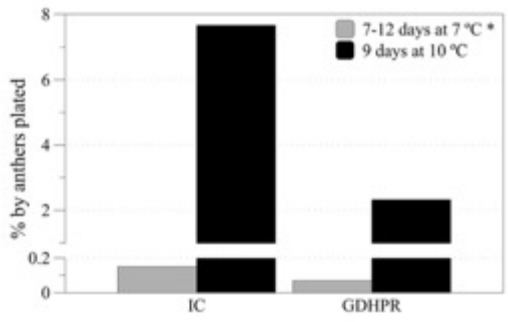

Fig. 1 Induced callus (IC) and green double haploid plantlets regenerated (GDHPR) for NRCV980385 in 0/0-D2 treatment with a cold pre-treatment of 7–12 days at 7 °C* and 9 days at 10 °C (this work). Reproduced with permission from Serrat et al. (2014)

Results

Effect of the cold pre-treatment on callus induction

The percentages of induced calluses (IC) and green double haploid plantlets regenerated (GDHPR) were compared according to the cold pre-treatment applied (Fig. 1) Cold pre-treatment was adjusted to 9 days at 10 °C for NRCV980385 (data not shown) as suggested by Serrat et al. (2014). Following cold pre-treatment for 9 days at 10 °C the IC and GDHPR percentages were 51 times and 33 times higher, respectively, than the 7–12 day pre-treatments at 7 °C.

Effect of different growth regulators on rice anther culture

The culturability of both genotypes with the different growth regulators in colchicine-free induction media is shown in Table 2. In D2 medium, the culturabilities of NRCV980385 and H28 were greater for all the parameters analysed.

For NRCV980385, there were no significant differences ($P > 0.05$) between the three growth regulator treatments (Table 2) for any of the anther culture parameters. Nevertheless, there was a tendency for D2 conditions (2 mg L^{-1} of 2,4-*D* and 1 mg L^{-1} kinetin) to yield higher values than the other treatments for callus induction (IC), callus that regenerates green plantlets (CRGP), regenerated green plantlets (RGP), regenerated double haploid plantlets (RDHP) and regenerated green double haploid plantlets (RGDHP). The values of the D1 and NA growth hormone regulator

Table 2 Culturability results for the temperate japonica rice variety, NRVC980385, and the temperate japonica F2 hybrid, H28, among three growth regulator treatments assayed without colchicine: *D1* 1 mg L^{-1} 2,4-*D* and 1 mg L^{-1} kinetin, *D2* 2 mg L^{-1} 2,4-*D* and 1 mg L^{-1} kinetin; and treatment NA, 2 mg L^{-1} NAA and 0,5 mg L^{-1} kinetin

Rice genotype	Hormone combination	Anther culture parameters				
		IC	CRGP	GRP	DHPR	GDHPR
NRCV 980385	D1	6.05	0.36	1.96	0.36	1.60
	D2	7.66	0.89	3.21	0.89	2.32
	NA	6.96	0.17	1.39	0.17	1.22
H28	D1	0.18*	0.00	0.00	0.00	0.00
	D2	4.18	0.70	1.57	0.70	0.87
	NA	5.10	0.17	0.51	0.17	0.34

Values followed by * show significant differences at the 5% level in a Chi square test for homogeneity when compared individually with the other two treatments. Induced callus (IC), number of calluses regenerating green plantlets (CRGP), number of green plantlets regenerated (GPR), number of double haploid plantlets regenerated (DHPR) and number of green double haploids plantlets regenerated (GDHPR)

treatments were similar, but the D2 treatment had slightly higher values for the majority of the parameters.

The percentage of induced calluses (IC) in H28 with the D1 treatment was significantly lower ($P < 0.05$) than the D2 and NA treatments. Therefore, in D1 treatment, parameters that are dependent on IC (CRGP, RGP, RGDHP and RGDHP) were zero, due to a low callus induction. No culture parameters between D2 and NA for H28 were statistically significantly ($P < 0.05$). Although D2 showed higher values than NA for callus that regenerates green plantlets (CRGP), regenerated green plantlets (RGP), regenerated double haploid plantlets (RDHP) and regenerated green double haploid plantlets (RGDHP), these differences were not significant ($P < 0.05$).

Effects of colchicine treatment on callus induction and plantlet regeneration

Callus induction was observed in almost all conditions assayed for both genotypes with the colchicine-supplemented treatments (Table 3). The only exceptions were the 150/24-NA and 300/24-NA media for NRCV980385, with both media used as 24 h colchicine treatments. On one hand, IC in NRCV980385 in the colchicine treatments was significantly lower ($P < 0.05$) than the control. On the other hand, IC for H28 seemed to increase with colchicine, showing significant differences ($P < 0.05$) in both colchicine treatments over 48 h. Moreover, the values were higher in comparison to their respective controls (0/0). Finally, several 24 h colchicine treatments in H28, such as 150/24, 300/24 for D1 and 150/24 for NA, had significantly higher percentages of induced calluses ($P < 0.05$) than their respective controls (0/0-D1 and 0/0-NA).

Regenerated plantlets were obtained from thirteen and fourteen out of fifteen different media for NRCV980385 and H28, respectively. The exceptions were the NA treatments supplemented with colchicine for 24 h (150/24-NA and 300/24-NA) in NRCV980385 and 0/0-D1 in H28. Plantlets regenerated from calluses were either albino or green, although some calluses were capable of regenerating both (Figs. 2, 3 for NRCV980385 and H28 respectively). There was a tendency for higher numbers of albino plantlets to be present when there was a high rate of plantlet regeneration. Albino plantlets regenerating calluses were the most

Table 3 Induced callus (IC) in all treatments assayed for the temperate japonica rice variety, NRVC980385, and the temperate japonica F2 hybrid, H28

	NRCV980385			H28		
	D1	D2	NA	D1	D2	NA
0/0	6.05	7.66	6.96	0.18	4.18	5.10
150/24	7.39	2.40*	0.00*	6.44*	2.73	8.67*
150/48	2.39*	3.51*	7.80	14.10*	17.73*	11.39*
300/24	5.23	3.44*	0.00*	10.55*	3.28	1.37*
300/48	5.81	5.12	8.56	18.75*	9.63*	12.54*

Values followed by * show significant differences at the 5% level in a Chi square test for homogeneity when compared with its hormone control colchicine-free medium (0/0)

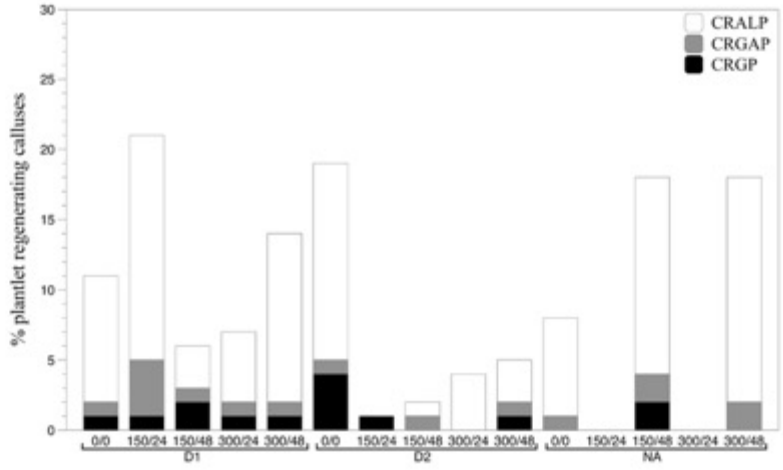

Fig. 2 Percentage of plantlets regenerating calluses for each induction media assayed in NRVC980385. *CRALP* callus regenerating albino plantlets, *CRGP* callus regenerating green plantlets, *CRGAP* callus regenerating green and albino plantlets

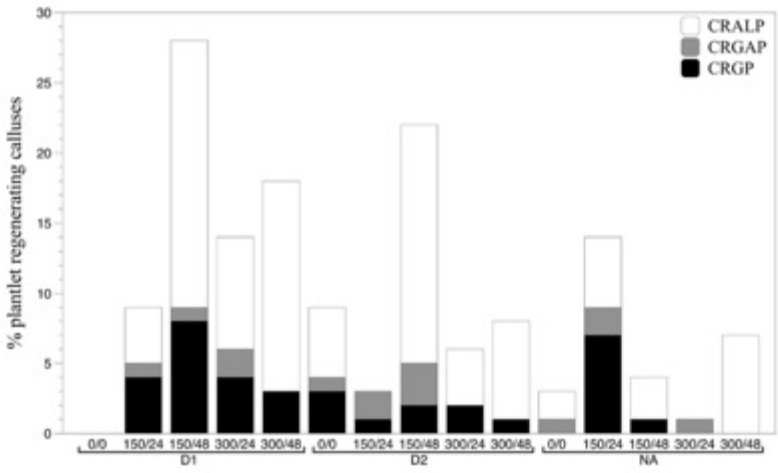

Fig. 3 Percentage of plantlets regenerating calluses for each induction media assayed in H28. *CRALP* callus regenerating albino plantlets, *CRGP* callus regenerating green plantlets, *CRGAP* callus regenerating green and albino plantlets

frequent, representing 77% of and 75% of NRCV980385 and H28 plantlets, respectively, when all media were grouped together.

NRCV980385 regenerated green plantlets in all D1 hormone media (1 mg L^{-1} of 2,4-*D* and 1 mg L^{-1} kinetin). In contrast, under D2 hormone conditions (2 mg L^{-1} of 2,4-*D* and 1 mg L^{-1} kinetin) the values for the total number of calluses regenerating plantlets were higher under colchicine-free conditions, with the number of green plantlets also being higher. Plantlets regenerated in the 150/24-D2 treatment were all green, unlike 300/24-D2 where all plantlets were albino. Finally, 0/0-NA and 300/48-NA conditions caused calluses to regenerate either albino plantlets or albino and green plantlets, but none of the calluses regenerated green plantlets alone.

The ability of H28 calluses to regenerate green plantlets was higher than NRCV980385 in almost all conditions (Fig. 3). Furthermore, colchicine treatments combined with D1 and NA hormone conditions displayed higher CRGP than their control treatments (0/0). Meanwhile, D2 hormone treatment without colchicine regenerated a higher number of green plantlets compared to colchicine treatments with this hormone, a tendency also observed for NRCV980385. Finally, 0/0-NA, 300/24-NA and 300/48-NA conditions had no calluses that regenerated green plantlets exclusively.

Effects of colchicine treatment on RDHP, G/A and RGDHP

The hormone factor has been grouped for both genotypes with the aim of analysing the colchicine effect on regenerated double haploid plantlets (RDHP), the green/albino plantlet ratio (G/A) and regenerated green double haploid plantlets (RGDHP) (Table 4). Values of RDHP and RGDHP for NRCV980385 were greater than for H28, but on average the G/A ratio was lower in NRCV980385 (Table 4). In addition, RDHP values for H28 in the four colchicine treatments at 48 h were higher than in the control, with both treatments showing significant differences.

Table 4 Regenerating double haploid plantlets per 100 anthers plated (RDHP), green/albino ratio (G/A) and regenerating green double haploid plantlets per 100 anthers plated (RGDHP) by genotypes according to colchicine treatment

Colchicine treatment	RDHP		G/A		RGDHP	
	NRCV 980385	H28	NRCV 980385	H28	NRCV 980385	H28
0/0	3.89	0.23	0.19	0.67	0.18	0.00
150/24	2.38	0.54	0.14	1.33	0.49*	0.22
150/48	1.77*	2.26*	0.28	0.55	0.22	0.29
300/24	1.80*	0.46	0.09	0.65	0.31	0.12
300/48	4.22	1.34*	0.12	0.20	0.75*	0.17

Three concentrations were assayed: 0 (control condition), 150 and 300 mg L^{-1}. For each colchicine concentration two exposure times, 24 and 48 h, were tested. In the end, 5 colchicine treatments were tested and named according to concentration/exposure time: 0/0 (control), 150/24, 150/48, 300/24 and 300/48. Hormone factors has been grouped. RDHP and RGDHP values followed by * are significantly different at the 5% level in a Chi square test for homogeneity in comparison with colchicine-free media (0/0)

On the most part, the G/A ratio for NRCV980385 and H28 was not significantly affected by the colchicine treatments. The one exception was H28 in the 150/24 colchicine treatment, which had a doubled G/A ratio in comparison to the colchicine-free control.

Calluses from all colchicine treatments regenerated green double haploid plantlets and the values were similar. The values for regenerated green double haploid plantlets (RGDHP) were always higher on colchicine treatments than on the colchicine-free treatment for both genotypes, with NRCV980385 having the highest values. For NRCV980385, the 150/24 and 300/48 colchicine treatments were significantly different ($P < 0.05$) from their 0/0 controls, with the number of regenerated green double haploid plantlets being 2.5 and 4 times higher respectively. For H28, the RGDHP values showed no significant differences when compared to its control due to the absence of regenerating green double haploid plantlets on 0/0. The best colchicine treatments for H28 were 150 mg L^{-1} at 24 and 48 h.

Haploid diploidization

Application of antimitotic agents to haploid plantlets obtained from the anther culture procedure hindered the rate of plantlet survival. After antimitotic treatment, most analysed plantlets were haploids (Table 5). Plantlets showing only double haploid ploidy were observed at the lower antimitotic concentrations (250 mg L^{-1} of colchicine and 1.25 mg L^{-1} of oryzalin). Within the plantlets that changed his chromosome content, mixiploids, including double haploid ploidy, were the majority. Moreover, when a higher antimitotic concentration was used the plantlet mortality rate increased, reaching 91.11% mortality with 1000 mg L^{-1} of colchicine. In the case of oryzalin, the mid-range concentration treatment (2.5 mg L^{-1}) showed the highest mortality, with a value of 34.78%. The percentage of plantlets that remained haploid after the treatment was higher when the antimitotic oryzalin was used (Table 5) at any concentration. The percentage of haploid plantlets and dead plantlets tended to increase with increases in the antimitotic concentrations.

Discussion

Anther culture is a powerful technique to produce rice DH plants. Nevertheless, the genotype effect is the major limiting factor, causing a differential response in callus induction as well as plantlet regeneration, ploidy and pigmentation previously reported by a number of authors (Mishra and Rao 2016; Herath et al. 2010; Khanna and Raina 1998; Raina and Zapata 1997; Moloney et al. 1989). Additionally, obtaining a high number of regenerating calluses is essential to increase the number of green double haploid plantlets displaying differential genotypes. Regenerated plantlets from the same callus or calluses from the same anther are more likely to be clones and therefore will have poor genetic variability. In addition, due to the fact that anther culture is a two-step process (i.e. initial development of calluses and subsequent regeneration of green plantlets from embryogenic calluses), researchers interested in obtaining new rice varieties from anther culture must avoid bottlenecks in the procedure. Low callus induction, low green plantlet regeneration and low double haploid regeneration can drastically limit the outcomes of anther culture. To minimize this, we proposed a workflow to study the response of the desired genotypes to anther culture over a six-month period using a range of induction media. Consequently, this study has focused on making preliminary assays to determine factors that could improve the yield of green double haploid plants. Additionally, the procedure has reported ways of reducing the amount of work to obtain DH plants in rice by: (i) reducing the time needed for the anther culture procedure, (ii) introducing a fast ploidy determination method, and (iii) a post anther culture diploidization protocol for rice.

Table 5 Post anther culture parameters of treated plantlets

Antimitotic	Concentration (mg L^{-1})	Post anther culture parameters					
		Total plants	% n	% 2n	% 2n mixploid	% mixploid	% dead
Control	0	20	100	0.00	0.00	0.00	0.00
Colchicine	250	47	38.30	2.13	29.79	2.13	27.66
	500	37	18.92	0.00	35.14	0.00	45.95
	1000	45	4.44	0.00	4.44	0.00	91.11
Oryzalin	1.25	31	77.42	6.45	3.23	0.00	12.90
	2.5	46	60.87	0.00	2.17	2.17	34.78
	5	47	70.21	0.00	6.38	0.00	23.40

Percentage n percentage of haploid plantlets with only a level of haploidy, *Percentage 2n* percentage of doubled haploid plantlets that with only a level of diploidy, *Percentage 2n mixiploid* percentage of double haploid plantlets with multiple ploidy levels, including 2n, *Percentage mixiploid* percentage of plantlets with multiple ploidy levels, excluding 2n

We included in our study the NRCV980385 genotype and the growth regulator 2,4-*D* in the induction medium at 2 mg L^{-1} in order to compare results with Serrat et al. (2014). In fact, the number of induced calluses was higher in our study than the previous one, which in turn resulted in a greater number of green double haploid plantlets. The cold pre-treatment was the main difference between the studies, which was changed from 7 to 10 °C and adjusted from a variable 7–12 days to a fixed 9 days. Another factor that could have affected the results is that in this experiment NRVC980385 genotype was a stabilized commercial genotype rather than a heterozygous seed batch as used previously in Serrat et al. (2014). Many authors have confirmed that cold pre-treatment has a stimulatory effect on androgenic response in several genotypes (Tian et al. 2015; Herath et al. 2010; Touraev et al. 2009). Moreover, a temperature of 10 °C is commonly used as a cold pre-treatment (Rukmini et al. 2013; Naik et al. 2017). Indeed, Naik et al. (2017) described that 7 days at 10 °C resulted in the best callus induction in a japonica cultivar. Therefore, the changes in cold pre-treatment enhanced the anther culture protocol with a higher rate of induced calluses.

Among the auxins, 2,4-dichlorophenoxyacetic acid (2,4-*D*) and naphthaleneacetic acid (NAA) are the most commonly used hormones for rice callus induction from anthers (Trejo-Tapia et al. 2002). The colchicine-free D2 treatment (2 mg L^{-1} of 2,4-*D* and 1 mg L^{-1} of kinetin), induced the best culture conditions overall for both the NRVC980385 and H28 genotypes. These results are in agreement with many authors who have determined that 2 mg L^{-1} of 2,4-*D* results in the best culturability results for many genotypes (Chen et al. 2002; Herath et al. 2008). The effect of 2,4-*D* may be the promotion of rapid cell proliferation and formation of non-embryogenic callus as described for spring wheat (Ball et al. 1993). In addition, this auxin at this concentration is widely used in rice anther culture, although regularly combined with other auxins or other cytokines to obtain the best results (Serrat et al. 2014; Afza et al. 2000; Kaushal et al. 2014; Chen et al. 2002). In contrast, the effect of NAA, which is also commonly used in rice anther culture, may be to induce direct androgenesis (Yi et al. 2015; Alemanno and Guiderdoni 1994; Reiffers and Freire 1990). Finally, many authors use combinations of 2,4-*D* and NAA, to obtain better results (He et al. 2006; Xie et al. 1995). Nevertheless, our results do not show clear patterns of rice anther culturability between the two growth regulators.

A differential anther culture response was observed when adding colchicine to the induction medium, which depended on the genotype cultured. NRCV980385 callus induction was negatively affected by colchicine. On the other hand, H28 induced more calluses on colchicine-supplemented induction media. In maize, an increase in embryo frequency has been reported in the presence of colchicine (Barnabás et al. 1999; Obert and Barnabás 2004). In wheat, an absence of effect of colchicine on microspore embryogenesis has been reported by Barnabás et al. (1991), and a reduction was reported by Navarro-Alvarez et al. (1994). In the present study, 75% of the colchicine-supplemented induction media assayed for H28 had significant callus induction enhancement. Moreover, H28 callus induction increased in both colchicine concentrations at 48 h for all of the growth regulators assayed. This effect is also in accordance with Alemanno and Guiderdoni (1994), who described a significant (50%) increase in rice anther callusing with 500 mg L^{-1} of colchicine at 24 and 48 h.

The ability to regenerate plantlets, and specifically green plantlets, was different between the genotypes and the media assayed. NRCV980385 in colchicine-supplemented induction media had no positive effects on green plantlet regeneration. Indeed, treatments with 2,4-*D* at 1 mg L^{-1} regenerated as efficiently with colchicine-supplementation as the colchicine-free control. The ability of H28 to regenerate plantlets from calluses was greater than NRCV980385. At 1 mg L^{-1} of 2,4-*D*, colchicine increased the percentage of regenerating calluses, which was defined as the ability to regenerate higher numbers of green plantlets. Alemanno and Guiderdoni (1994) doubled the number of green plantlet-regenerating calluses with 250 mg L^{-1} of colchicine for 24 h relative to the colchicine-free treatment using the Miara genotype. In contrast, colchicine in the regenerating medium at 30 mg L^{-1} increased 7 times the number of green plantlets in comparison to the control in the Zao jing 26 genotype (Chen et al. 2002). Like these earlier reports, the number of calluses that regenerate green plantlets in H28 genotype, was increased. It is clear from the current work and previous reports that there is a strong effect of genotype on the outcome of anther culture.

In our study, the incidence of albinism was high for both genotypes. Albinism in plants is characterized by a lack of chlorophyll pigments and/or incomplete differentiation of chloroplast membranes in normally green tissues. Many studies have suggested that the use of colchicine in anther culture reduces the albinism ratio (Kumari et al. 2009; Barnabás et al. 1991; Ferrie et al. 2014). In our study, a reduction in albinism was only observed with 150 mg L^{-1} of colchicine during the 24 h treatment of the H28 genotype. This observation is in agreement with other authors who have noted no increases in the proportions of green plantlets after colchicine treatment at the callus stage (Hansen and Andersen 1998; Alemanno and Guiderdoni 1994). Furthermore, the number of double haploid regenerated plantlets also seems to be unaffected by colchicine treatments, irrespective of the different concentrations and exposure times tested. These results are in contrast with reports of an increase in regenerating double haploid plantlets when using colchicine (Barnabás et al. 1991). In our work, the

ploidy of green and albino plantlets was analysed to obtain the parameter of regenerated double haploid plantlets (RDHP), whereas other authors have usually only considered the regenerated green plantlets. Nevertheless, in our study, colchicine-supplemented media increased the proportion of regenerated green double haploid plantlets (RGDHP), which is in accordance with other studies (Alemanno and Guiderdoni 1994; Barnabás et al. 1991; Weber et al. 2005). Finally, all colchicine treatments yielded higher proportions of regenerated green double haploid plantlets for both genotypes in comparison to the colchicine-free induction media. In NRCV980385, the 150 mg L^{-1} colchicine treatment for 24 h and the 300 mg L^{-1} treatment for 48 h gave the best results ($P < 0.05$) compared to 0/0 control, and the numbers of RGDHPs were 2.5 and 4 times greater than the control, respectively. The H28 genotype was not able to regenerate green double haploid plantlets in colchicine-free induction media, and because of that a statistical test was not possible. This lack of green double haploid plantlets from colchicine-free media may be due to a low endoreduplication or low ability for endomitosis in H28, which entails a spontaneous duplication of chromosomes from the haploid (Chen and Chen 1980).

In both genotypes assayed, colchicine seemed to affect the endomitosis rate in the treated microspores. Endomitosis is described as nuclear chromosome doubling due to a failure of the spindle during metaphase (Kasha 2005). C-mitosis is a form of endomitosis caused by colchicine, which has the ability to abort mitosis and inhibit tubulin polymerization in animal and plant cells (Fltzgerald 1976; Pickett-Heaps 1967; Kasha 2005), and this explains the high yield of green double haploid plantlets in rice. Our observed 2.5-fold increase in the proportion of calluses regenerating diploid green plantlets is in accordance with the work of Alemanno and Guiderdoni (1994). Chen et al. (2002) also observed an increase in regenerated green double haploid plantlets when using regeneration media fortified with 75 mg L^{-1} colchicine, although higher concentrations caused harmful effects on calluses and regenerated plantlets. For this reason we assayed 300 and 150 mg L^{-1} concentrations to test lower concentrations than the 500 mg L^{-1} used by Alemanno and Guiderdoni (1994) and delimit the best colchicine concentration while avoiding toxicity. Finally, colchicine is widely used to increase the number of green double haploid plantlets in anther culture of other species and has had positive results in wheat (Hansen and Andersen 1998; Soriano et al. 2007), maize (Saisingtong et al. 1996), oats (Ferrie et al. 2014) and rapeseed (Mollers et al. 1994; Weber et al. 2005). Despite our results and those reported in the literature, we suggest that further studies should be performed in rice to investigate the ability of colchicine to increase the numbers of regenerated green double haploids above albino double haploids.

The main limitation of anther culture is the unknown interaction that occurs between media and genotypes. Our results describe completely different responses for callus induction, regenerating calluses, plant albinism and the ploidy of regenerated plantlets that are dependent on the growth regulator, their concentrations and the exposure time. Many authors have reported that the genotype affects the androgenic response (Lentini et al. 1997; He et al. 2006; Bagheri and Jelodar 2008) and that changes in medium composition can alter the response of different rice cultivars (Trejo-Tapia et al. 2002; Chen et al. 2002; Herath et al. 2008, 2010). However, manipulation of colchicine in induction media has not been reported previously for rice.

A complementary way to obtain DH plants is to perform a post anther procedure treatment of green haploid plantlets with antimitotics. It has been widely reported that antimitotic treatments of plantlets may change their ploidy (Chen et al. 2002; Ascough et al. 2008; Gallone et al. 2014; Sarathum et al. 2010; Omidbaigi et al. 2012; de Carvalho et al. 2005). In this study, the in vitro production of double haploid plantlets from already formed haploid plantlets was achieved. Most of the plantlets that survived were mixiploid with levels of diploidy, and it was from these latter plants that we were able to obtain double haploid seed from those tillers that were double haploids. Colchicine at 500 mg L^{-1} was the best in vitro treatment to double the ploidy (35.14% of plantlets treated). This concentration of colchicine was also used previously in an in vivo treatment of tillers with an effectiveness of 11.5% (Chen et al. 2002). Ascough et al. (2008) reported that when lower antimitotic concentrations were used the number of surviving plantlets was higher, but on the other hand the level of diploidization was also lower. Omidbaigi et al. 2012 reported that high concentrations of oryzalin did not have much effect on the survival ratio.

Anther culture in rice has been studied in many genotypes to achieve the best method of maximizing green double haploid plantlet formation through different stresses. Taking this earlier work into account, we selected a range of stresses to formulate protocols for two Mediterranean japonica rice genotypes that will form the basis of an anther culture procedure for a wide range of genotypes. The genotypes trialled each had specific responses to the experimental conditions. We have demonstrated that cold pre-treatment at 10 °C for 9 days increases callus induction. Without colchicine in the induction medium, we recommend 2,4-*D* at 2 mg L^{-1} and kinetin at 1 mg L^{-1} to obtain the highest values for callus induction and green double haploid plantlet regeneration for Mediterranean japonica rice varieties. Colchicine-supplemented induction media may increase the level of callus induction, depending on the genotype. Colchicine on the induction medium increases the green double haploid plantlet production in all treatments of concentration and time assayed. We have shown that post-anther culture colchicine

treatment at 500 mg L^{-1} increases the green double haploid production from green haploid plantlets. Our results highlight the importance of the genotype and media interactions effects on the anther culture efficiency in rice. This study stands out the necessity to continue studying the response of rice to anther culture to better understand the main mechanism of interaction between genotype and induction media.

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References

Afza R, Shen M, Zapata-Arias FJ, Xie J, Fundi HK, Lee KS, Kodym A (2000) Effect of spikelet position on rice anther culture efficiency. Plant Sci 153(2):155–159

Alemanno L, Guiderdoni E (1994) Increased doubled haploid plant regeneration from rice (*Oryza sativa* L.) anthers cultured on colchicine-supplemented media. Plant Cell Rep 13(8):432–436. https://doi.org/10.1007/BF00231961

Ascough GD, Van Staden J, Erwin JE (2008) Effectiveness of colchicine and oryzalin at inducing polyploidy in *Watsonia lepida* NE brown. HortScience 43(7):2248–2251

Bagheri N, Jelodar NB (2008) Combining ability and heritability of callus induction and green plant regeneration in rice anther culture. Biotechnology 7(2):287–292

Ball ST, Zhou H, Konzak CF (1993) Influence of 2,4-D, IAA, and duration of callus induction in anther cultures of spring wheat. Plant Sci 90(2):195–200

Barnabás B, Pfahler PL, Kovacs G (1991) Direct effect of colchicine on the microspore embryogenesis to produce dihaploid plants in wheat (*Triticum aestivum* L.). TAG Theoret Appl Genet 81(5):675–678

Barnabás B, Obert B, Kovács G (1999) Colchicine, an efficient genome-doubling agent for maize (*Zea mays* L.) microspores cultured in anthero. Plant Cell Rep 18(10):858–862

Chen CC, Chen CM (1980) Changes in chromosome number in microspore callus of rice during successive subcultures. Can J Genet Cytol 22(4):607–614. https://doi.org/10.1139/g80-066

Chen CC, Tsay HS, Huang CR (1991) Factors affecting androgenesis in rice (*Oryza sativa* L.). In: YP S Bajaj (ed) Rice. Springer, Berlin, pp 193–215

Chen QF, Wang CL, Lu YM, Shen M, Afza R, Duren MV, Brunner H (2002) Anther culture in connection with induced mutations for rice improvement. In: Mutations, in vitro and molecular techniques for environmentally sustainable crop improvement, pp 83–92. Springer, New York

Chu C-C (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci Sin 18:659–668

Cousin A, Heel K, Cowling WA, Nelson MN (2009) An efficient high-throughput flow cytometric method for estimating DNA ploidy level in plants. Cytometry 75:1015–1019. https://doi.org/10.1002/cyto.a.20816

de Carvalho JFRP., de Carvalho CRDP., Otoni WC (2005) In vitro induction of polyploidy in annatto (*Bixa orellana*). Plant Cell Tissue Organ Cult 80(1):69–75. https://doi.org/10.1007/s11240-004-8833-5

Ferrie AMR, Irmen KI, Beattie AD, Rossnagel BG (2014) Isolated microspore culture of oat (*Avena sativa* L.) for the production of doubled haploids: effect of pre-culture and post-culture conditions. Plant Cell Tissue Organ Cult 116(1):89–96

Fltzgerald TJ (1976) Molecular features of colchicine associated with antimitotic activity and inhibition of tubulin polymerization. Biochem Pharmacol 25(12):1383–1387. https://doi.org/10.1016/0006-2952(76)90108-8

Forster BP, Heberle-Bors E, Kasha KJ, Touraev A (2007) The resurgence of haploids in higher plants. Trends Plant Sci 12(8):368–375. https://doi.org/10.1016/j.tplants.2007.06.007

Gallone A, Hunter A, Douglas GC (2014) Polyploid induction in vitro using colchicine and oryzalin on hebe '*Oratia Beauty*': production and characterization of the vegetative traits. Sci Hortic 179:59–66. https://doi.org/10.1016/j.scienta.2014.09.014

Germanà MA (2011) Anther culture for haploid and doubled haploid production. Plant Cell Tissue Organ Cult 104(3):283–300. https://doi.org/10.1007/s11240-010-9852-z

Hansen NJP, Bode Andersen S (1998) In vitro chromosome doubling with colchicine during microspore culture in wheat (*Triticum aestivum* L.). Euphytica 102(1):101–108

He T, Yang Y, Tu SB, Yu MQ, Li XF (2006) Selection of interspecific hybrids for anther culture of indica rice. Plant Cell Tissue Organ Cult 86(2):271–277

Herath HMI, Bandara DC, Samarajeewa PK, Wijesundara DSA (2008) The effect of plant growth regulators on anther culture response and plant regeneration in selected Sri Lankan indica rice varieties, japonica varieties and their inter-sub specific hybrids. Postgraduate Institute of Agriculture, University of Peradeniya: Peradeniya

Herath HM, I DC, Bandara, Samarajeewa PK (2010) Effect of culture media for anther culture of indica rice varieties and hybrids of indica and japonica. Tropical agriculture research extension, vol 10. Faculty of Agriculture, University of Ruhuna, Matara

Jensen CJ (1974) Chromosome doubling techniques in haploids. The University of Guelph, Guelph

Kasha KJ (2005) Chromosome doubling and recovery of doubled haploid plants. In: Haploids in crop improvement II. Springer, New York, pp 123–152

Kaushal L, Balachandran SM, Ulaganathan K, Shenoy V (2014) Effect of culture media on improving anther culture response of rice (*Oryza sativa* L.). Int J Agric Innovations Res 3(1):218–224

Khanna HK, Raina SK (1998) Genotype × culture media interaction effects on regeneration response of three indica rice cultivars. Plant Cell Tissue Organ Cult 52(3):145–153. https://doi.org/10.1023/A:1006032303195

Kumari M, Clarke HJ, Small I, Siddique KH (2009) Albinism in plants: a major bottleneck in wide hybridization, androgenesis and doubled haploid culture. Crit Rev Plant Sci 28(6):393–409

Lentini Z, Roca WM, Martinez CP (1997) Cultivo de anteras de arroz en el desarrollo de germoplasma, vol 293. CIAT, Cali

Maluszynska J (2003) Cytogenetic tests for ploidy level analyses—chromosome counting. In: Doubled haploid production in crop plants, pp 391–395. Springer, New York

Mishra R, Rao GJN (2016) In-vitro androgenesis in rice: advantages, constraints and future prospects. Rice Sci 23(2):57–68

Möllers C, Iqbal MCM, Röbbelen G (1994) Efficient production of doubled haploid *Brassica Napus* plants by colchicine treatment

of microspores. Euphytica 75(1):95–104. https://doi.org/10.1007/BF00024536
Moloney MM, Walker JM, Sharma KK (1989) High efficiency transformation of *Brassica Napus* using agrobacterium vectors. Plant Cell Rep 8(4):238–242
Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15(3):473–497
Naik N, Rout P, Umakanta N, Verma RL, Katara JL, Sahoo KK, Samantaray S (2017) Development of doubled haploids from an elite indica rice hybrid (BS6444G) using anther culture. Plant Cell Tissue Organ Cult 128(3):679–689
Navarro-Alvarez W, Baenziger PS, Eskridge KM, Hugo M, Gustafson VD (1994) Addition of colchicine to wheat anther culture media to increase doubled haploid plant production. Plant Breed 112(3):192–198
Niizeki H, Oono K (1968) Induction of haploid rice plant from anther culture. Proc Jpn Acad 44(6):554–557
Obert B, Barnabás B (2004) Colchicine induced embryogenesis in maize. Plant Cell Tissue Organ Cult 77(3):283–285. https://doi.org/10.1023/B:TICU.0000018399.60106.33
Omidbaigi R, Mirzaee M, Hassani ME, Sedghi Moghadam M (2012) Induction and identification of polyploidy in Basil (*Ocimum Basilicum* L.) medicinal plant by colchicine treatment. Int J Plant Prod 4(2):87–98
Pauk J, Jancsó M, Simon-Kiss I (2009) Rice doubled haploids and breeding. In: Advances in haploid production in higher plants. Springer, New York, pp 189–197
Pickett-Heaps JD (1967) The effects of colchicine on the ultrastructure of dividing plant cells, xylem wall differentiation and distribution of cytoplasmic microtubules. Dev Biol 15(3):206–236
Raina SK, Zapata FJ (1997) Enhanced anther culture efficiency of indica rice (*Oryza Sativa* L.) through modification of the culture media. Plant Breed 116(4):305–315. https://doi.org/10.1111/j.1439-0523.1997.tb01004.x
Reiffers I, Adelson BF (1990) Production of doubled haploid rice plants (*Oryza Sativa* L.) by anther culture. Plant Cell Tissue Organ Cult 21(2):165–170. https://doi.org/10.1007/BF00033437
Rukmini M, Rao GJN, Rao RN (2013) Effect of cold pretreatment and phytohormones on anther culture efficiency of two indica rice (*Oryza Sativa* L.) Hybrids-Ajay and Rajalaxmi. J Exp Biol Agr Sci 2:69–76
Saisingtong S, Schmid JE, Stamp P, Büter B (1996) Colchicine-mediated chromosome doubling during anther culture of maize (*Zea Mays* L.). Theor Appl Genet 92(8):1017–1023
Sarathum S, Hegele M, Tantiviwat S, Nanakorn M (2010) Effect of concentration and duration of colchicine treatment on polyploidy induction in *Dendrobium Scabrilingue* L. Eur J Hortic Sci 75(3):123–127. http://www.jstor.org.sire.ub.edu/stable/24126421
Serrat X, Cardona M, Gil J, Brito AM, Moysset L, Nogués S, Lalanne E (2014) A Mediterranean japonica rice (*Oryza Sativa*) cultivar improvement through anther culture. Euphytica 195(1):31–44. https://doi.org/10.1007/s10681-013-0955-6
Soriano M, Cistué L, Vallés MP, Castillo AM (2007) Effects of colchicine on anther and microspore culture of bread wheat (*Triticum Aestivum* L.). Plant Cell Tissue Organ Cult 91(3):225–234
Thompson DM, Chalmers K, Waugh R, Forster BP, Thomas WTB, Caligari PDS, Powell W (1991) The inheritance of genetic markers in microspore-derived plants of barley *Hordeum Vulgare* L. Theor Appl Genet 81(4):487–492. https://doi.org/10.1007/BF00219438
Tian QQ, Lu CM, Li X, Fang XW (2015) Low temperature treatments of rice (*Oryza Sativa* L.) anthers changes polysaccharide and protein composition of the anther walls and increases pollen fertility and callus induction. Plant Cell Tissue Organ Cult 120(1):89–98
Touraev A, Forster BP, Jain SM (2009) Advances in haploid production in higher plants. Springer, New York
Trejo-Tapia G, Amaya UM, Morales GS, Sánchez ADJ, Bonfil BM, Rodríguez-Monroy M, Jiménez-Aparicio A (2002) The effects of cold-pretreatment, auxins and carbon source on anther culture of rice. Plant Cell Tissue Organ Cult 71(1):41–46
Weber S, Ünker F, Friedt W (2005) Improved doubled haploid production protocol for *Brassica napus* using microspore colchicine treatment in vitro and ploidy determination by flow cytometry. Plant Breed 124(5):511–513
Xie J, Gao M, Cai Q, Cheng X, Shen Y, Liang Z (1995) Improved isolated microspore culture efficiency in medium with maltose and optimized growth regulator combination in japonica rice (*Oryza sativa*). Plant Cell Tissue Organ Cult 42(3):245–250. https://doi.org/10.1007/BF00029994
Yi G, Lee HS, Kim KM (2015) Improved marker-assisted selection efficiency of multi-resistance in doubled haploid rice plants. Euphytica 203(2):421–428. https://doi.org/10.1007/s10681-014-1303-1
Zapata-Arias FJ (2003) Laboratory protocol for anther culture technique in rice. In: Doubled haploid production in crop plants. Springer, New York, pp 109–116

SUPPLEMENTARY MATERIAL

CHAPTER 1 - Proteome profiling in shoots and roots of the FL478 genotype of rice (*Oryza sativa* L. ssp. *indica*) by shotgun proteomics during early salinity stress

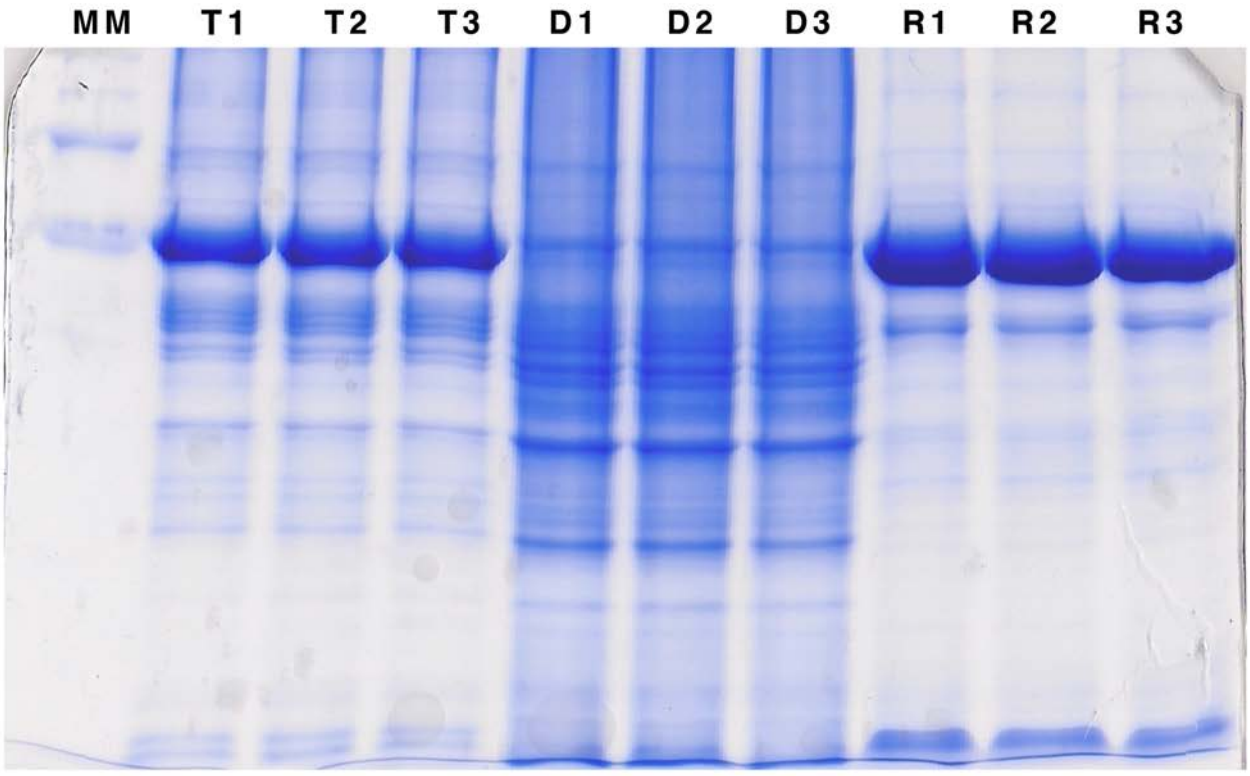
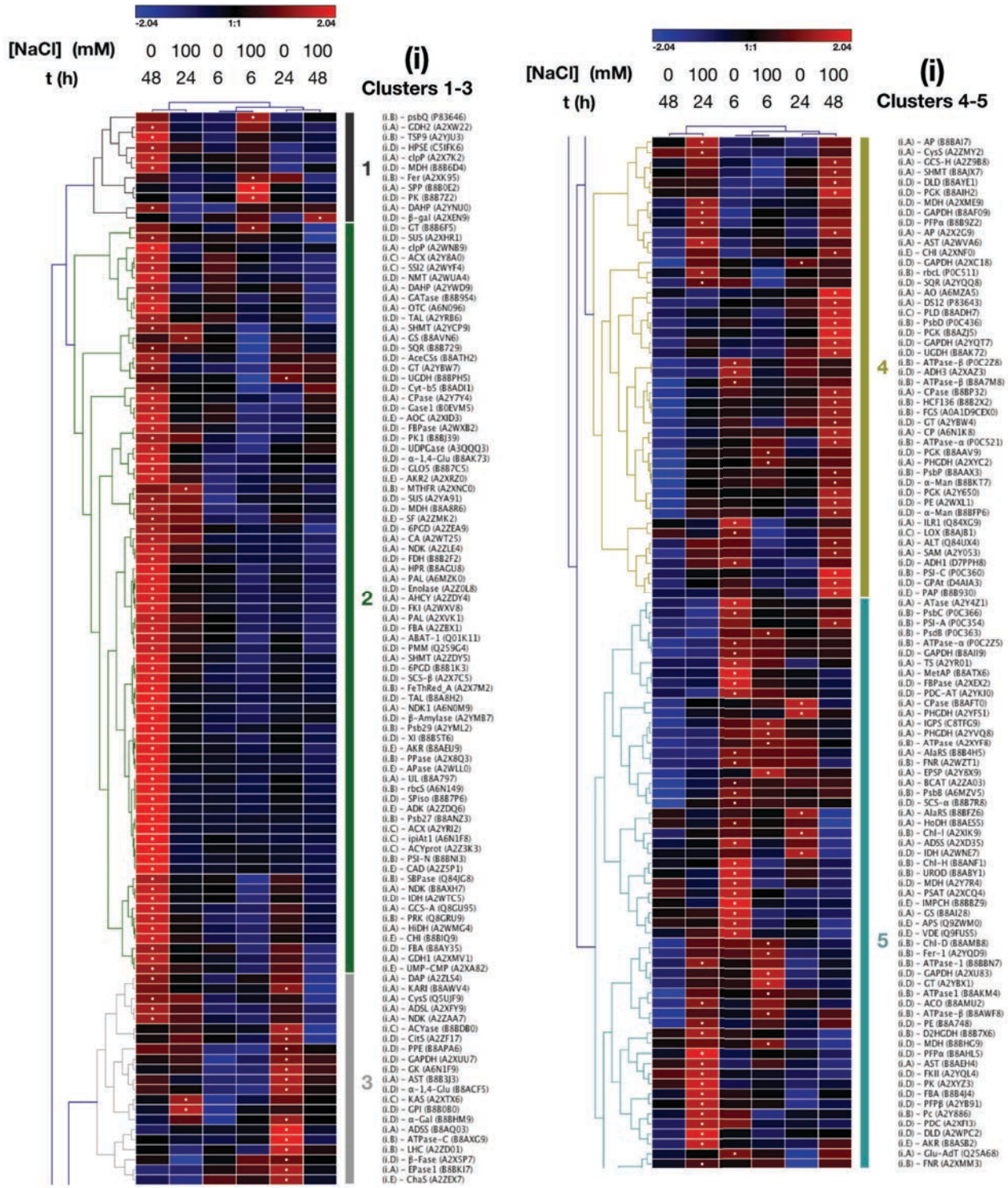
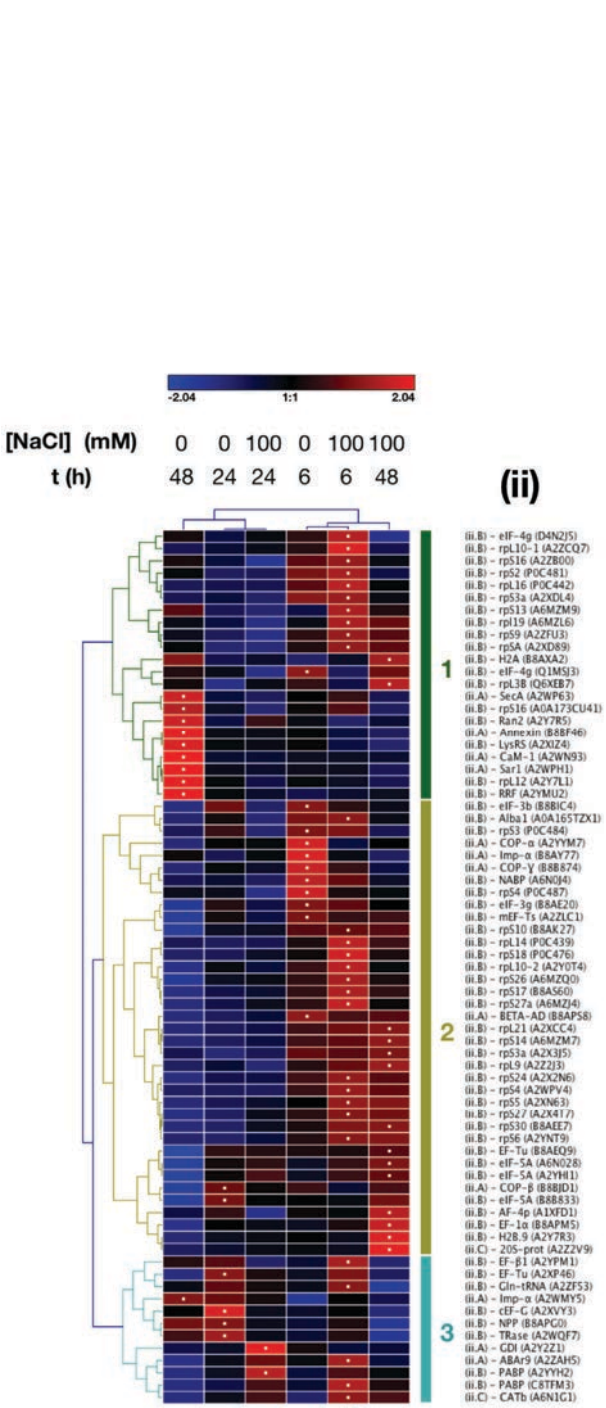


Figure SM.1.1. Representative SDS-PAGE of the RuBisCO depletion protocol performed in shoots of rice. MM: Molecular marker; T: total proteins; R: RuBisCO fraction; D: RuBisCO depleted fraction.

Figure SM.1.2 (pages 288 and 289). Heatmaps of the identified shoot proteins. The full list can be consulted in Table SM.1.3. Heatmaps are shown for each functional category: (i) energy and biomolecule metabolism (page 288); (ii) genetic and environmental information processing (page 289); (iii) antioxidant and defense function (page 289); (iv) cytoskeleton-related (page 289); (v) unassigned function (apparently not involved in tolerance to salinity stress, page 289). Identifiers for proteins in heatmaps are as follows: category.sub-category – protein abbreviation (UniProt accession). Both proteins and treatments were clustered using Pearson correlations, and clusters for proteins are displayed with colored bars and an assigned number. The color of each square corresponds to the relative expression in a log2 scale, which is shown in the colored box at the top of each heatmap. White circles within the squares denote the most distant expression for each protein between the six conditions.


-2.04
1:1
2.04
[NaCl] (mM) 0 100 0 100 0 100
t (h) 48 24 6 6 24 48
(i)
Clusters 1-3
1
2
3
(i.B) - psbQ (P83646)
(i.A) - GDH2 (A2XW22)
(i.B) - TSP9 (A2YJU3)
(i.D) - HPSE (C5IFK6)
(i.A) - clpP (A2X7K2)
(i.D) - MDH (B8B6D4)
(i.B) - Fer (A2XK95)
(i.A) - SPP (B8B0E2)
(i.D) - PK (B8B7Z2)
(i.A) - DAHP (A2YNU0)
(i.D) - β-gal (A2XEN9)
(i.D) - GT (B8B6F5)
(i.D) - SUS (A2XHR1)
(i.A) - clpP (A2WNB9)
(i.C) - ACX (A2Y8A0)
(i.C) - SSI2 (A2WYF4)
(i.D) - NMT (A2WUA4)
(i.A) - DAHP (A2YWD9)
(i.A) - GATase (B8B954)
(i.A) - OTC (A6N096)
(i.D) - TAL (A2YRB6)
(i.A) - SHMT (A2YCP9)
(i.A) - GS (B8AVN6)
(i.D) - SQR (B8B729)
(i.D) - AceCSs (B8ATH2)
(i.D) - GT (A2YBW7)
(i.D) - UGDH (B8BPH5)
(i.D) - Cyt-b5 (B8ADI1)
(i.A) - CPase (A2Y7Y4)
(i.D) - Gase1 (B0EVM5)
(i.E) - AOC (A2XID3)
(i.D) - FBPase (A2WXB2)
(i.D) - PK1 (B8BJ39)
(i.D) - UDPGase (A3QQQ3)
(i.D) - α-1,4-Glu (B8AK73)
(i.D) - GLO5 (B8B7C5)
(i.E) - AKR2 (A2XRZ0)
(i.B) - MTHFR (A2XNC0)
(i.D) - SUS (A2YA91)
(i.D) - MDH (B8A8R6)
(i.E) - SF (A2ZMK2)
(i.D) - 6PGD (A2ZEA9)
(i.A) - CA (A2WT25)
(i.A) - NDK (A2ZLE4)
(i.D) - FDH (B8B2F2)
(i.A) - HPR (B8ACU8)
(i.A) - PAL (A6MZK0)
(i.D) - Enolase (A2Z0L8)
(i.A) - AHCY (A2ZDY4)
(i.D) - FKI (A2WXV8)
(i.A) - PAL (A2XVK1)
(i.D) - FBA (A2ZBX1)
(i.A) - ABAT-1 (Q01K11)
(i.D) - PMM (Q259G4)
(i.A) - SHMT (A2ZDY5)
(i.D) - 6PGD (B8B1K3)
(i.D) - SCS-β (A2X7C5)
(i.B) - FeThRed_A (A2X7M2)
(i.D) - TAL (B8A8H2)
(i.A) - NDK1 (A6N0M9)
(i.D) - β-Amylase (A2YMB7)
(i.B) - Psb29 (A2YML2)
(i.D) - XI (B8B5T6)
(i.E) - AKR (B8AEU9)
(i.B) - PPase (A2X8Q3)
(i.E) - APase (A2WLL0)
(i.A) - UL (B8A797)
(i.B) - rbcS (A6N149)
(i.D) - SPiso (B8B7P6)
(i.E) - ADK (A2ZDQ6)
(i.B) - Psb27 (B8ANZ3)
(i.C) - ACX (A2YRI2)
(i.C) - ipiAt1 (A6N1F8)
(i.C) - ACYprot (A2Z3K3)
(i.B) - PSI-N (B8BNI3)
(i.E) - CAD (A2Z5P1)
(i.B) - SBPase (Q84JG8)
(i.A) - NDK (B8AXH7)
(i.D) - IDH (A2WTC5)
(i.A) - GCS-A (Q8GU95)
(i.B) - PRK (Q8GRU9)
(i.A) - HiDH (A2WMC4)
(i.E) - CHI (B8BIQ9)
(i.A) - FBA (B8AY35)
(i.A) - GDH1 (A2XMV1)
(i.E) - UMP-CMP (A2XA82)
(i.A) - DAP (A2ZLS4)
(i.A) - KARI (B8AWV4)
(i.A) - CysS (Q5UJF9)
(i.A) - ADSL (A2XFY9)
(i.A) - NDK (A2ZAA7)
(i.C) - ACYase (B8BDB0)
(i.D) - Cit5 (A2ZF17)
(i.D) - PPE (B8APA6)
(i.D) - GAPDH (A2XUU7)
(i.D) - GK (A6N1F9)
(i.A) - AST (B8B3J3)
(i.D) - α-1,4-Glu (B8ACF5)
(i.C) - KAS (A2XTX6)
(i.D) - GPI (B8B0B0)
(i.D) - α-Gal (B8BHM9)
(i.A) - ADSS (B8AQ03)
(i.B) - ATPase-C (B8AXG9)
(i.B) - LHC (A2ZD01)
(i.D) - β-Fase (A2X5P7)
(i.A) - EPase1 (B8BKI7)
(i.E) - ChaS (A2ZEX7)
-2.04
1:1
2.04
[NaCl] (mM) 0 100 0 100 0 100
t (h) 48 24 6 6 24 48
(i)
Clusters 4-5
4
5
(i.A) - AP (B8BAJ7)
(i.A) - CysS (A2ZMY2)
(i.A) - GCS-H (A2Z9B8)
(i.A) - SHMT (B8AJX7)
(i.D) - DLD (B8AYE1)
(i.D) - PGK (B8AIH2)
(i.D) - MDH (A2XME9)
(i.D) - GAPDH (B8AF09)
(i.D) - PFPα (B8B9Z2)
(i.A) - AP (A2X2C9)
(i.A) - AST (A2WVA6)
(i.E) - CHI (A2XNF0)
(i.D) - GAPDH (A2XC18)
(i.B) - rbcL (P0C511)
(i.D) - SQR (A2YQQ8)
(i.A) - AO (A6MZA5)
(i.A) - D512 (P83643)
(i.C) - PLD (B8ADH7)
(i.B) - PsbD (P0C436)
(i.D) - PGK (B8AZJ5)
(i.D) - GAPDH (A2YQT7)
(i.D) - UGDH (B8AK72)
(i.B) - ATPase-β (P0C2Z8)
(i.D) - ADH3 (A2XAZ3)
(i.B) - ATPase-β (B8A7M8)
(i.A) - CPase (B8BP32)
(i.B) - HCF136 (B8B2X2)
(i.B) - FCS (A0A1D9CEX0)
(i.D) - GT (A2YBW4)
(i.A) - CP (A6N1K8)
(i.B) - ATPase-α (P0C521)
(i.D) - PGK (B8AAV9)
(i.A) - PHGDH (A2XYC2)
(i.B) - PsbP (B8AAX3)
(i.D) - α-Man (B8BKT7)
(i.D) - PGK (A2Y650)
(i.D) - PE (A2WXL1)
(i.D) - α-Man (B8BFP6)
(i.A) - ILR1 (Q84XG9)
(i.C) - LOX (B8AJB1)
(i.A) - ALT (Q84UX4)
(i.A) - SAM (A2Y053)
(i.D) - ADH1 (D7PPH8)
(i.B) - PSI-C (P0C360)
(i.D) - GPAt (D4AIA3)
(i.E) - PAP (B8B930)
(i.A) - ATase (A2Y4Z1)
(i.B) - PsbC (P0C366)
(i.B) - PSI-A (P0C354)
(i.B) - PsbB (P0C363)
(i.B) - ATPase-α (P0C2Z5)
(i.D) - GAPDH (B8AII9)
(i.A) - TS (A2YR01)
(i.A) - MetAP (B8ATX6)
(i.D) - FBPase (A2XEX2)
(i.D) - PDC-AT (A2YKI0)
(i.A) - CPase (B8AFT0)
(i.A) - PHGDH (A2YFS1)
(i.A) - ICPS (C8TFG9)
(i.A) - PHGDH (A2YVQ8)
(i.B) - ATPase (A2XYF8)
(i.A) - AlaRS (B8B4H5)
(i.B) - FNR (A2WZT1)
(i.A) - EPSP (A2Y8X9)
(i.A) - BCAT (A2ZA03)
(i.B) - PsbB (A6MZV5)
(i.D) - SCS-α (B8B7R8)
(i.A) - AlaRS (B8BFZ6)
(i.A) - HoDH (B8AES5)
(i.B) - Chl-I (A2XIK9)
(i.A) - ADSS (A2XD35)
(i.D) - IDH (A2WNE7)
(i.B) - Chl-H (B8ANF1)
(i.B) - UROD (B8ABY1)
(i.D) - MDH (A2Y7R4)
(i.A) - PSAT (A2XCQ4)
(i.E) - IMPCH (B8BBZ9)
(i.A) - GS (B8AI28)
(i.E) - APS (Q9ZWM0)
(i.E) - VDE (Q9FUS5)
(i.B) - Chl-D (B8AMB8)
(i.B) - Fer-1 (A2YQD9)
(i.B) - ATPase-1 (B8BBN7)
(i.D) - GAPDH (A2XU83)
(i.D) - GT (A2YBX1)
(i.B) - ATPase1 (B8AKM4)
(i.D) - ACO (B8AMU2)
(i.B) - ATPase-β (B8AWF8)
(i.D) - PE (B8A748)
(i.B) - D2HGDH (B8B7X6)
(i.D) - MDH (B8BHC9)
(i.D) - PFPα (B8AHL5)
(i.A) - AST (B8AEH4)
(i.D) - FKII (A2YQL4)
(i.D) - PK (A2XYZ3)
(i.D) - FBA (B8B4J4)
(i.D) - PFPβ (A2YB91)
(i.B) - Pc (A2Y886)
(i.D) - PDC (A2XFI3)
(i.D) - DLD (A2WPC2)
(i.E) - AKR (B8ASB2)
(i.A) - Glu-AdT (Q25A68)
(i.B) - FNR (A2XMM3)


-2.04
1:1
2.04
[NaCl] (mM) 0 0 100 0 100 100
t (h) 48 24 24 6 6 48
(ii)
1
2
3

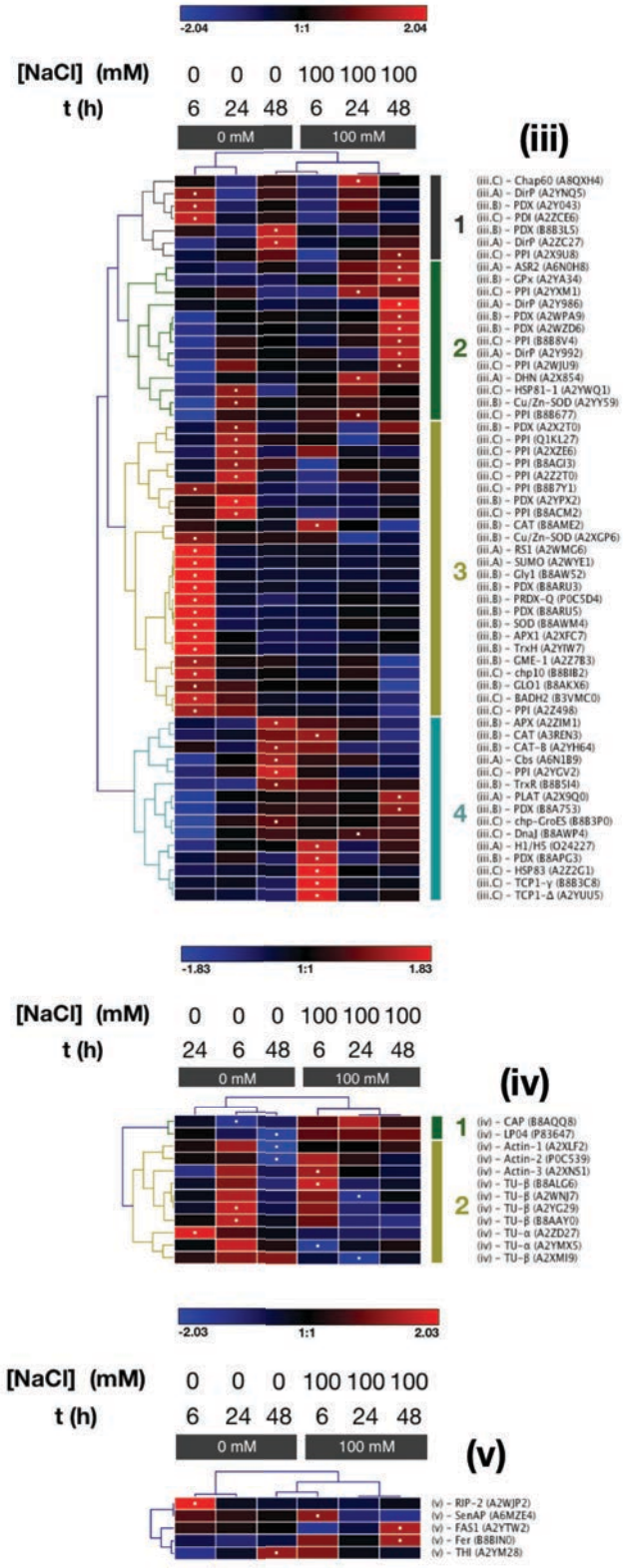
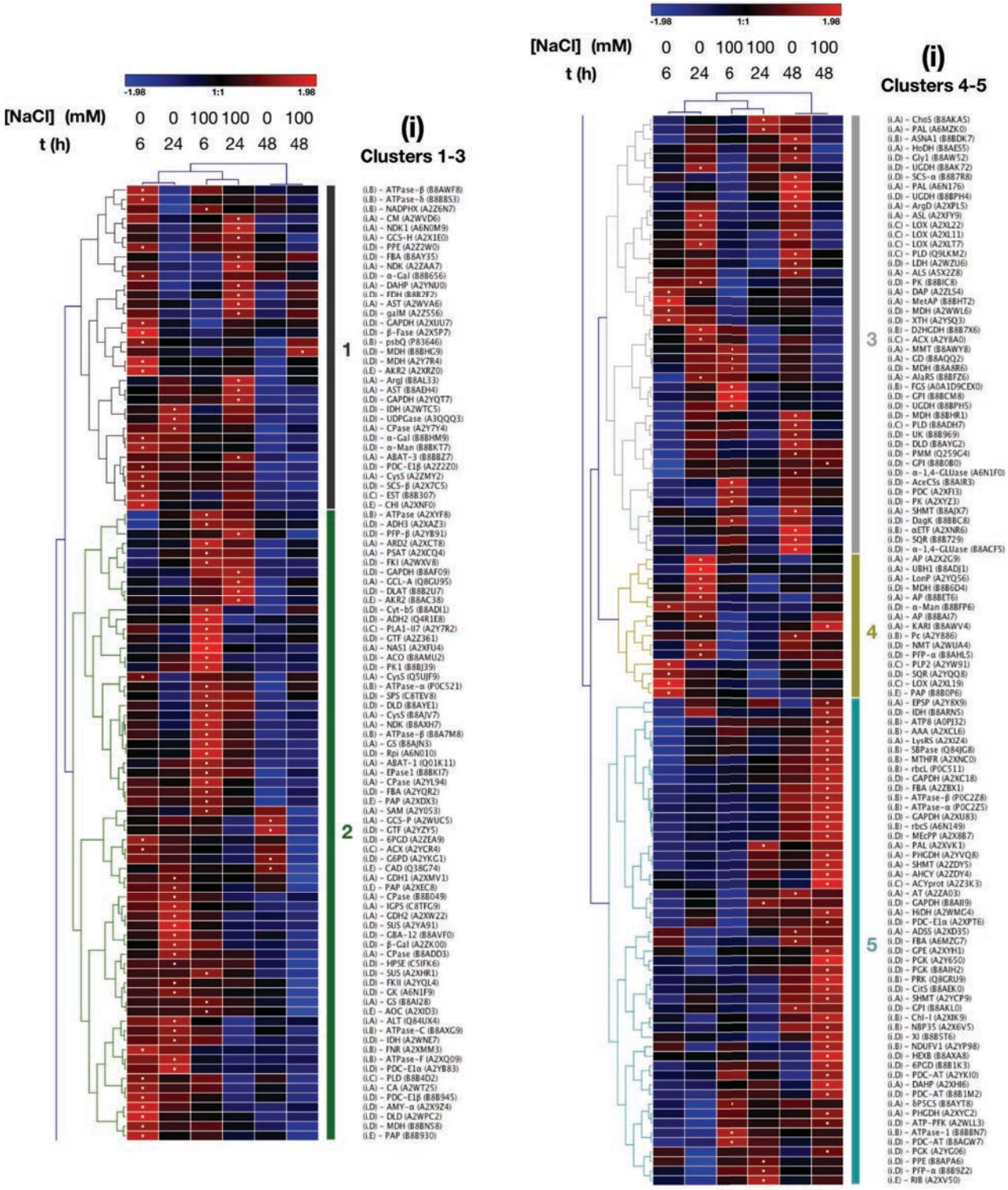
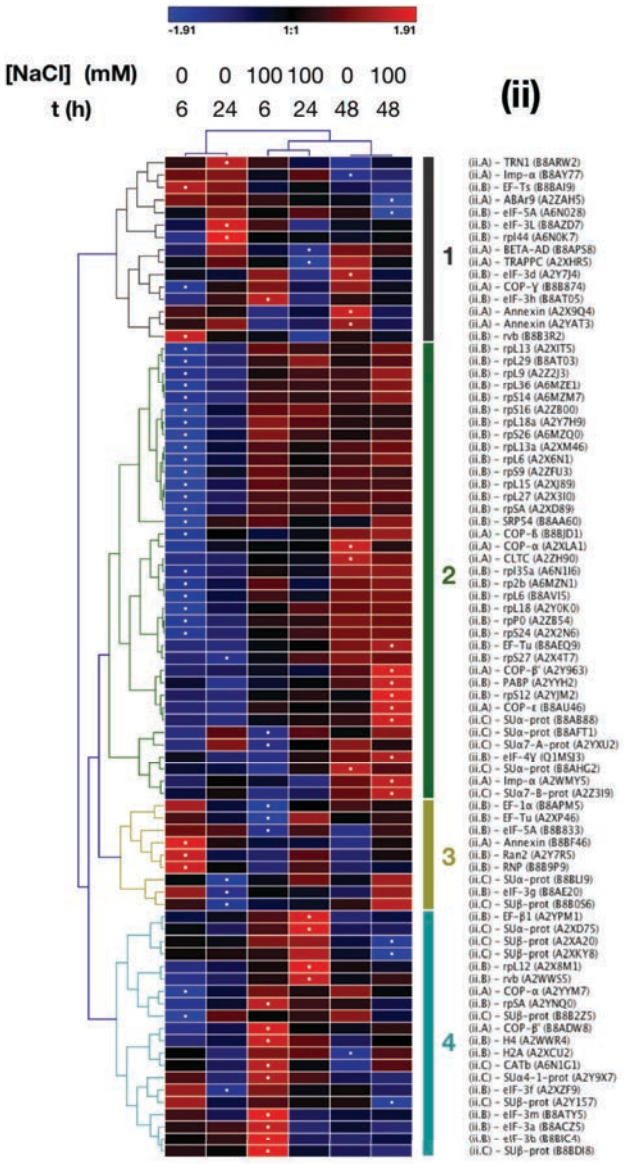

-2.04
1:1
2.04
[NaCl] (mM) 0 0 0 100 100 100
t (h) 6 24 48 6 24 48
0 mM
100 mM
(iii)
1
2
3
4
-1.83
1:1
1.83
[NaCl] (mM) 0 0 0 100 100 100
t (h) 24 6 48 6 24 48
0 mM
100 mM
(iv)
1
2
-2.03
1:1
2.03
[NaCl] (mM) 0 0 0 100 100 100
t (h) 6 24 48 6 24 48
0 mM
100 mM
(v)

Figure SM.1.3 (pages 292 and 293). Heatmaps of the identified root proteins. The full list can be consulted in Table SM.1.4. Heatmaps are shown for each functional category: (i) energy and biomolecule metabolism (page 292); (ii) genetic and environmental information processing (page 293); (iii) antioxidant and defense function (page 293); (iv) cytoskeleton-related (page 293); (v) unassigned function (apparently not involved in tolerance to salinity stress, page 293). Identifiers for proteins in heatmaps are as follows: category.sub-category – protein abbreviation (UniProt accession). Both proteins and treatments were clustered using Pearson correlations, and clusters for proteins are displayed with colored bars and an assigned number. The color of each square corresponds to the relative expression in a $\log_2$ scale, which is shown in the colored box at the top of each heatmap. White circles within the squares denote the most distant expression for each protein between the six conditions.


-1.98
1:1
1.98
[NaCl] (mM) 0 0 100 100 0 100
t (h) 6 24 6 24 48 48
(i)
Clusters 1-3
1
2
(i)
Clusters 4-5
3
4
5


-1.91
1:1
1.91
[NaCl] (mM) 0 0 100 100 0 100
t (h) 6 24 6 24 48 48
(ii)
1
2
3
4

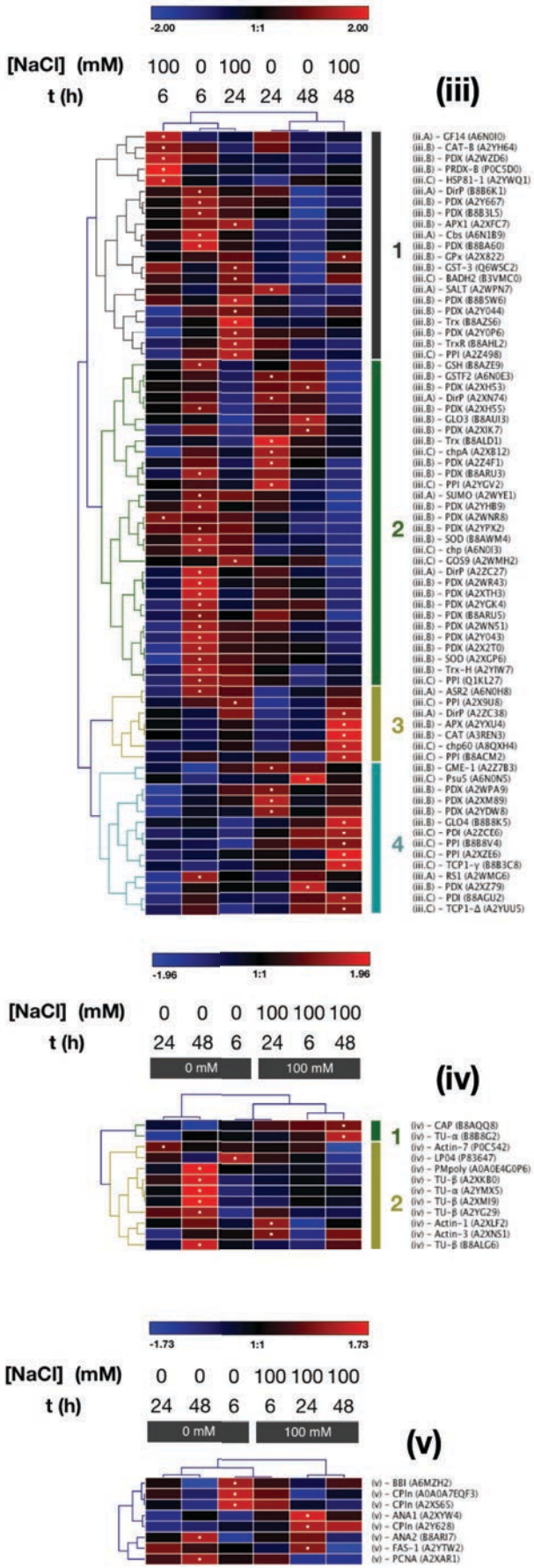

-2.00
1:1
2.00
[NaCl] (mM) 100 0 100 0 0 100
t (h) 6 6 24 24 48 48
(iii)
1
2
3
4
-1.96
1:1
1.96
[NaCl] (mM) 0 0 0 100 100 100
t (h) 24 48 6 24 6 48
0 mM
100 mM
(iv)
1
2
-1.73
1:1
1.73
[NaCl] (mM) 0 0 0 100 100 100
t (h) 24 48 6 6 24 48
0 mM
100 mM
(v)

Table SM.1.1. Modified Yoshida solution composition.

Element	Reagent	Final concentration
Macronutrients		
N	NH_4NO_3	1.428 mM
S	K_2SO_4	0.513 mM
P	KH_2PO_4	0.849 mM
	K_2HPO_4	0.123 mM
Ca	$CaCl_2$ x 2 H_2O	0.754 mM
Mg	$MgSO_4$ x 7 H_2O	1.644 mM
Micronutrients		
Fe	Na_2EDTA	37.75 μM
	$FeSO_4$ x 7 H_2O	
Mn	$MnCl_2$ x 4 H_2O	9.5 µM
Mo	$(NH_4)_6Mo_7O_{24}$ x 4 H_2O	0.075 µM
B	H_3BO_3	18.89 µM
Zn	$ZnSO_4$ x 7 H_2O	0.152 µM
Cu	$CuSO_4$ x 5 H_2O	0.156 µM

Table SM.1.2. Statistical results of the physiological parameters analyzed by a two-way ANOVA (F). *: Analyses was performed by a Kruskal-Wallis test (Chi^2) as data was non-homoscedastic.

		Plantlet		Shoot		Root	
		Statistic value (F/Chi^2)	p-value	Statistic value (F/Chi^2)	p-value	Statistic value (F/Chi^2)	p-value
Length	Salinity	8.67	0.0036	32.86	0.0000	12.56	0.0005
	Time	46.85	0.0000	41.13	0.0000	14.10	0.0000
	Salinity*Time	6.51	0.0018	7.88	0.0014	0.91	0.4059
Weight	Salinity	11.06	0.0017	16.84	0.0002	5.57	0.0224
	Time	13.30	0.0000	14.12	0.0000	10.11	0.0002
	Salinity*Time	4.74	0.0132	2.74	0.0750	5.25	0.0087
%H20	Salinity	-	-	146.31	0.0000	4.94	0.0462
	Time	-	-	18.10	0.0002	0.22	0.8071
	Salinity*Time	-	-	16.94	0.0003	0.23	0.7941
Na^+/K^+	Salinity	-	-	4.94*	0.0462	1110.47	0.0000
	Time	-	-	0.22*	0.8071	36.11	0.0000
	Salinity*Time	-	-	0.23*	0.7941	45.54	0.0000
SPAD	Salinity	-	-	0.021*	0.8857	-	-
	Time	-	-	17.94*	0.0001	-	-
	Salinity*Time	-	-	22.953*	0.0003	-	-

Table SM.1.3. List of the annotated proteins identified in shoots of FL478 through the shotgun proteomic approach. UniProt: uniprot accession number; Prot. name: protein name; Prot. abb.: protein abbreviation; Func. cat.: functional category; Cell. loc.: cellular localization.

UniProt	Prot. name	Prot. abb.	Func. cat.	Cell. loc.	Relative shoot protein expression					
					0 mM			100 mM		
					6h	24h	48h	6h	24h	48h
A6N1K8	Cysteine proteinase rd21a	CP	i.A	cyt	1.7833	1.6400	1.2900	2.3533	2.3100	2.7833
B8BP32	Carboxypeptidase	CPase	i.A	cyt	1.5067	1.4033	1.0533	1.4833	1.3000	1.2733
A2XYC2	D-3-phosphoglycerate dehydrogenase	PHGDH	i.A	cyt	1.2367	1.2567	1.0033	4.0133	2.1500	4.0667
C8TFG9	Indole-3-glycerol phosphate synthase-like	IGPS	i.A	cyt	1.4667	1.3500	0.9367	1.1500	1.2067	1.7000
A2WVA6	Aspartate aminotransferase	AST	i.A	cyt	1.1200	1.2067	1.0833	1.1867	1.2333	2.0833
A2Y8X9	3-phosphoshikimate 1-carboxyvinyltransferase	EPSP	i.A	cyt	0.9967	1.0900	0.8167	1.7200	1.7967	2.8767
Q25A68	Glutamyl-tRNA(Gln) amidotransferase subunit A	Glu-AdT	i.A	chl/mit	1.5133	0.9333	1.1933	0.7633	0.6333	0.7500
P83643	ACT domain-containing protein DS12	DS12	i.A	cyt	1.0133	1.0633	0.9233	2.2433	2.2967	2.4100

A2X2G9	Aminopeptidase	AP	i.A	cyt	1.2633	1.2233	1.1900	4.0900	2.2467	4.0500
B8BKI7	Probable bifunctional methylthioribulose-1-phosphate dehydratase/enolase-phosphatase E1	EPase1	i.A	cyt	1.8833	2.3033	1.4400	2.3733	2.4300	3.0767
A2Y053	S-adenosylmethionine synthase 1	SAM	i.A	cyt	2.0833	1.9033	1.7733	1.7967	0.7400	1.5800
B8AQ03	Adenylosuccinate synthetase	ADSS	i.A	chl	1.3767	2.5067	1.1233	1.1433	0.6433	1.0233
A2Y4Z1	Amidophosphoribosyltransferase	ATase	i.A	cyt	3.2000	2.5633	2.3600	0.8333	0.6800	0.8667
A2YR01	Tryptophan synthase	TS	i.A	cyt	1.3533	0.9200	0.9567	2.2033	2.2700	2.9400
A2ZA03	Branched-chain-amino-acid aminotransferase	BCAT	i.A	cyt	1.3033	1.3033	1.1200	1.6467	1.1033	1.2900
A6MZA5	Amine oxidase	AO	i.A	cyt	1.5867	1.6500	1.6133	1.0733	0.6033	1.1733
B8AJX7	Serine hydroxymethyltransferase	SHMT	i.A	cyt	2.0067	2.2133	2.1467	2.0133	1.4900	1.9567
A2YFS1	D-3-phosphoglycerate dehydrogenase	PHGDH	i.A	cyt	1.5533	1.6700	1.3933	1.0000	0.8600	1.2300
A2Z9B8	Glycine cleavage system H protein	GCS-H	i.A	mit	0.7767	0.8067	0.7867	2.3133	1.0400	1.7700
A2YVQ8	D-3-phosphoglycerate dehydrogenase	PHGDH	i.A	cyt	1.2400	1.1733	0.9667	1.2633	1.3433	1.5567

B8B4H5	Alanine--tRNA ligase	AlaRS	i.A	chl/mit	0.5867	0.5700	0.4367	0.7867	0.8900	1.1367
Q84UX4	Alanine aminotransferase	ALT	i.A	cyt	2.1533	2.0133	1.9933	1.8300	1.5667	2.0800
B8BAI7	Aminopeptidase	AP	i.A	cyt	0.9467	0.9233	1.0033	1.0000	0.7700	1.2667
B8AFT0	Carboxypeptidase	CPase	i.A	cyt	0.7033	0.8100	0.6300	0.9833	0.8833	1.3733
Q84XG9	IAA-amino acid hydrolase ILR1-like 1	ILR1	i.A	cyt	1.5367	1.4000	1.4100	1.3067	1.2700	1.6433
A5X300	Acetolactate synthase	ALS	i.A	cyt	1.4800	1.3533	1.2867	1.9967	2.0133	2.3600
B8BHT2	Methionine aminopeptidase	MetAP	i.A	cyt	1.7833	1.7367	1.7267	0.4967	0.5100	0.7000
A2XD35	Adenylosuccinate synthetase	AMPSase	i.A	chl	0.9867	0.9667	0.9200	0.8867	0.9500	1.3733
A2YNU0	Phospho-2-dehydro-3-deoxyheptonate aldolase	DAHP	i.A	cyt	0.6367	0.7133	0.7500	0.7100	0.8333	0.9733
A2ZMY2	Cysteine synthase	CysS	i.A	cyt	1.5900	1.5700	2.4433	1.1233	0.7267	0.9167
B8ATX6	Methionine aminopeptidase	MetAP	i.A	cyt	2.1767	1.1067	0.8167	1.8967	1.5967	1.9200
B8B3J3	Aspartate aminotransferase	AST	i.A	cyt	0.1400	0.3067	0.2467	1.2433	1.1467	1.2867
B8BFZ6	Alanine--tRNA ligase	AlaRS	i.A	cyt	1.0533	1.0800	1.0467	1.4800	0.9067	1.0700
B8AVN6	Glutamine synthetase	GS	i.A	cyt	0.9667	0.9900	1.0000	0.9233	0.9533	1.0567
A2Y7Y4	Carboxypeptidase	CPase	i.A	cyt	1.3267	1.1900	1.5633	1.3900	1.5567	1.4000

A2ZLS4	Putative diaminopimelate epimerase	DAP	i.A	chl	0.7267	0.7867	0.7967	1.1900	1.0467	1.2633
B8AI28	Glutamine synthetase	GS	i.A	cyt	1.4567	1.1033	1.2000	1.7000	1.5033	1.8100
B8B0E2	Stromal processing peptidase	SPP	i.A	chl	1.4333	1.5767	1.5300	2.6667	2.0200	2.5167
B8AWV4	Ketol-acid reductoisomerase	KARI	i.A	cyt	0.8500	0.9400	0.9300	0.8167	0.8500	1.0633
A2YCP9	Serine hydroxymethyltransferase	SHMT	i.A	cyt	1.2300	1.2333	1.4533	0.5700	0.5500	0.5500
A2X7K2	ATP-dependent Clp protease proteolytic subunit	clpP	i.A	cyt	1.1000	0.9867	1.2333	1.9200	1.3033	1.7933
Q5UJF9	Cysteine synthase	CysS	i.A	cyt	0.7733	0.8600	0.9167	1.4967	1.6667	1.8333
A2ZAA7	Nucleoside diphosphate kinase	NDK	i.A	cyt	0.9067	1.0133	1.0900	2.2233	1.8467	2.3267
B8AES5	Homoserine dehydrogenase	HDH	i.A	cyt	0.9867	0.9700	0.9367	4.0033	3.5267	3.3233
B8AXH7	Nucleoside diphosphate kinase	NDK	i.A	cyt	0.5867	0.6167	0.7100	2.6033	1.0900	1.6300
A2WMG4	Histidinol dehydrogenase	HDH	i.A	chl	0.9000	1.0267	1.2167	1.3567	1.2467	1.3367
B8AEH4	Aspartate aminotransferase	AST	i.A	cyt	1.1333	1.2100	1.4233	1.7633	0.6900	1.5767
B3VMC0	Betaine aldehyde dehydrogenase 2	BADH2	i.A	cyt	1.0433	1.0300	1.2900	1.2833	0.8833	0.9700

Q01K11	Gamma-aminobutyrate transaminase 1	ABAT-1	i.A	mit	0.8567	0.8500	1.0333	1.7733	1.2833	1.3800
A2ZLE4	Nucleoside diphosphate kinase	NDK	i.A	cyt	1.2067	1.1533	1.4967	1.5900	1.1833	1.2300
A2WNB9	ATP-dependent Clp protease proteolytic subunit	clpP	i.A	cyt	1.0100	0.9000	1.3200	1.3300	1.5733	1.4200
A2WT25	Carbonic anhydrase	CA	i.A	cyt	1.0700	1.0833	1.4267	3.6367	3.6633	3.8067
A2YWD9	Phospho-2-dehydro-3-deoxyheptonate aldolase	DAHP	i.A	cyt	1.0867	1.0800	1.2033	1.2400	0.9533	1.0633
A2XMV1	Glutamate dehydrogenase 1	GDH1	i.A	mit	1.1400	1.2867	1.4367	1.0433	1.0800	1.1500
A2XFY9	Adenylosuccinate lyase	ASL	i.A	cyt	0.8767	1.1333	1.2667	0.8133	0.8567	0.8767
A2ZDY5	Serine hydroxymethyltransferase	SHMT	i.A	cyt	1.4467	1.4467	2.1633	1.0067	0.8400	0.8833
A2XCQ4	Phosphoserine aminotransferase	PSAT	i.A	cyt	2.5367	1.2400	2.0267	1.3500	1.4100	1.3633
A6N0M9	Nucleoside diphosphate kinase 1	NDK1	i.A	chl	0.7633	0.8000	1.4267	1.6567	1.3733	1.5000
A2ZDY4	Adenosylhomocysteinase	AHCY	i.A	cyt	1.8500	1.7967	2.9200	0.5033	0.2700	0.4567
Q8GU95	Glutamate--cysteine ligase A	GCS-A	i.A	chl	0.3533	0.5300	0.6833	1.7833	2.1533	2.0200

A6N096	Chloroplast ornithine carbamoyltransferase	OTC	i.A	cyt	2.0133	1.9500	2.7633	0.8000	0.4400	0.7267
A2XVK1	Phenylalanine ammonia-lyase	PAL	i.A	cyt	1.0667	0.9300	1.5533	1.3567	1.3133	1.4833
A6MZK0	Phenyl ammonia lyase	PAL	i.A	cyt	0.6100	0.6433	1.1000	0.9400	0.9567	1.0667
A2XW22	Glutamate dehydrogenase 2	GDH-2	i.A	mit	0.7267	0.8333	1.5700	0.8633	0.5933	0.8133
B8A797	Ubiquitin ligase	UL	i.A	cyt	0.8500	0.9133	1.7633	0.9900	0.9267	1.1433
B8B9S4	Glutamine amidotransferase	GATase	i.A	cyt	1.4467	1.3700	1.9333	0.9033	0.8600	1.0400
B8AGU8	Hydroxypyruvate reductase	HPR	i.A	cyt	1.2967	1.1500	2.0167	0.7833	0.7800	0.8067
A6MZV5	Photosystem ii 10 kDa polypeptide	PsbB	i.B	cyt	0.9500	0.7833	0.4233	1.7233	1.7500	1.9167
P0C354	Photosystem I P700 chlorophyll a apoprotein A1	PSI-A	i.B	chl	1.2400	0.9900	0.8067	1.9200	1.8133	1.8200
P0C436	Photosystem II D2 protein	PsbD	i.B	chl	0.5767	0.5633	0.4567	1.9900	1.8433	1.6233
A2XYF8	Plasma membrane ATPase	ATPase	i.B	cyt	1.0400	1.1000	0.6267	0.4767	0.6700	0.6367
B8A7M8	ATP synthase subunit beta	ATPase-β	i.B	cyt	1.0800	1.0067	0.7500	1.3267	1.4200	1.4533
P0C521	ATP synthase subunit alpha	ATPase-α	i.B	mit	1.0900	0.9667	0.9033	2.6067	2.1867	2.6800

P0C2Z5	ATP synthase subunit alpha	ATPase-α	i.B	chl	2.7900	2.5100	1.8367	0.7300	0.6033	0.8133
P0C360	Photosystem I iron-sulfur center	PSI-C	i.B	chl	0.8733	0.7867	0.7767	1.3333	1.6933	1.5200
B8AWF8	ATP synthase subunit beta	ATPase-β	i.B	cyt	0.4933	0.4767	0.4033	1.0067	0.7933	0.9067
P0C363	Photosystem II CP47 reaction center protein	PsdB	i.B	chl	0.8433	0.7567	0.6567	0.5967	0.5867	0.7567
A0A1D9CEX0	Chloroplast ferredoxin-dependent glutamate synthase	NADPH	i.B	cyt	1.7900	1.8367	1.5533	2.7867	3.3300	3.9333
P0C366	Photosystem II CP43 reaction center protein	PsbC	i.B	chl	0.8533	0.7533	0.6667	1.5500	1.6200	1.6867
P0C2Z8	ATP synthase subunit beta	ATPase-β	i.B	chl	1.3133	1.2667	1.0433	1.6900	1.3367	1.3600
B8AKM4	Obg-like ATPase 1	ATPase1	i.B	cyt	0.9167	0.9467	0.7867	0.8900	0.8933	0.8967
A2XMM3	Ferredoxin-NADP reductase	FNR	i.B	cyt	1.2867	1.0433	1.2300	2.0433	1.4533	1.7367
A2YQD9	Ferredoxin-1	Fer-1	i.B	chl	1.7600	1.5567	1.5167	1.8600	2.0667	2.1367
B8AMB8	Magnesium-chelatase subunit ChlD	ChlD	i.B	chl	1.2233	1.1267	1.0467	1.1067	1.3800	1.5267
B8BBN7	Obg-like ATPase 1	ATPase-1	i.B	cyt	0.9767	0.8700	0.8967	1.4533	1.4567	1.3433
P0C511	Ribulose bisphosphate carboxylase large chain	rbcL	i.B	cyt	4.9467	4.9333	4.8300	0.9833	1.0633	1.1767

A2ZD01	Chlorophyll a-b binding protein	LHC	i.B	chl	0.7733	0.8967	0.7633	2.0800	2.3900	2.4100
A2WZT1	Ferredoxin--NADP reductase	FNR	i.B	cyt	1.5400	1.5167	1.1900	1.2400	1.2100	1.3333
A2Y886	Plastocyanin	Pc	i.B	chl	1.0833	0.9867	0.9733	1.1633	1.1100	1.2267
A2XIK9	Magnesium-chelatase subunit ChlI	Chl-I	i.B	chl	0.9900	1.0333	0.8667	1.1200	1.1833	1.3000
B8AXG9	V-type proton ATPase subunit C	ATPase-C	i.B	cyt	0.9667	1.0967	1.0000	0.6000	0.6200	0.6967
B8ANF1	Magnesium-chelatase subunit ChlH	Chl-H	i.B	chl	0.9633	0.8467	0.7900	2.6900	2.2100	2.7600
A2XNC0	Methylenetetrahydrofolate reductase	MTHFR	i.B	cyt	0.9167	0.9767	1.0200	1.0400	1.1000	1.0900
B8APK5	Ferredoxin	Fer	i.B	cyt	3.3533	2.4400	2.5300	1.2200	0.9567	1.1100
A6N154	Chlorophyll a-b binding protein	LHC	i.B	chl	0.9967	0.9133	1.0400	1.2833	1.2067	1.2900
Q84JG8	Sedoheptulose-1,7-bisphosphatase	SBPase	i.B	cyt	2.2333	2.3167	2.4800	1.1367	1.6767	1.3000
B8B7X6	Probable D-2-hydroxyglutarate dehydrogenase	D2HGDH	i.B	mit	1.3900	1.2267	1.3833	1.2100	1.2800	1.2900
Q8GRU9	Phosphoribulokinase	PRK	i.B	cyt	0.5933	0.6833	0.7700	1.2400	1.1433	1.2467
B8ABY1	Uroporphyrinogen decarboxylase	UROD	i.B	cyt	1.0233	0.7567	0.6300	3.6467	2.6500	3.0467

A2XC41	Chloroplast signal recognition particle 43	SRP43	i.B	chl	4.9833	iii.C867	4.0367	1.1533	0.9467	1.2167
A2XK95	Ferredoxin	Ferredoxin	i.B	cyt	1.3033	2.0233	1.8667	1.6267	1.5400	1.8367
A6N149	Ribulose bisphosphate carboxylase small chain	rbcS	i.B	chl	2.3167	2.5733	4.0867	1.8900	2.0100	1.8400
A2X8Q3	Soluble inorganic pyrophosphatase	PPase	i.B	cyt	0.8567	0.7900	ii.B5	2.9000	3.4967	2.9800
P83646	Oxygen-evolving enhancer protein 3	psbQ	i.B	chl	2.3200	2.2600	28.6267	0.9667	0.9667	0.8933
B8B2X2	Photosystem II stability assembly factor HCF136	HCF136	i.B	chl	1.3800	1.4433	0.7200	1.3067	1.2000	1.1100
B8AAX3	PsbP family	PsbP	i.B	chl	1.2433	1.1833	0.6600	0.9400	1.0100	0.9967
B8BNI3	photosystem I reaction center subunit N	PSI-N	i.B	chl	0.5467	0.5467	1.6900	1.6033	1.7933	1.7767
A2X7M2	Ferredoxin thioredoxin reductase variable alpha chain	FeThRed_A	i.B	cyt	1.7333	1.9833	5.5600	1.4933	1.4100	1.1567
B8ANZ3	photosystem II protein Psb27	Psb27	i.B	chl	1.0967	1.1833	3.0967	2.0767	2.2367	1.9333
A2YML2	photosystem II biogenesis protein	Psb29	i.B	chl	10.5567	13.9633	25.4500	1.0233	1.0700	1.1667
A2YJU3	Thylakoid soluble phosphoprotein	TSP9	i.B	chl	1.3733	1.9000	4.1033	0.9600	1.0000	1.1567

B8ADH7	Phospholipase D	PLD	i.C	cyt	1.0733	1.1033	1.0400	1.8633	2.2967	2.3933
B8AJB1	Lipoxygenase	LOX	i.C	cyt	0.9367	0.8000	0.9067	1.5033	1.5033	1.5533
A2XTX6	3-oxoacyl-[acyl-carrier-protein] synthase	KAS	i.C	cyt	2.3500	3.4000	2.9367	0.7267	0.8600	0.8767
B8BDB0	Acyl-[acyl-carrier-protein] hydrolase	ACYase	i.C	cyt	0.5933	0.6700	0.6067	1.2800	1.0000	1.0767
A2Y8A0	Acyl-coenzyme A oxidase	ACX	i.C	cyt	1.3333	1.1733	1.4500	0.7767	0.8100	0.8167
A2WYF4	Stearoyl-[acyl-carrier-protein] 9-desaturase 2	SSI2	i.C	chl	1.1067	0.9133	1.2200	0.7333	0.6633	0.7167
A6N1F8	Isopentenyl pyrophosphate: dimethyllallyl pyrophosphate isomerase	ipiAt1	i.C	cyt	0.9567	0.7700	1.8233	2.2067	1.5133	1.9067
A2Z3K3	Acyl carrier protein	ACYprot	i.C	cyt	0.8067	0.7300	1.5233	0.5700	0.4400	0.4800
A2YRI2	Acyl CoA binding protein	ACX	i.C	cyt	0.8400	0.4667	14.0700	1.5900	1.1200	1.2533
B8B7R8	Succinate--CoA ligase [ADP-forming] subunit alpha	SCS	i.D	cyt	1.5733	1.3800	0.2667	1.3400	1.3467	1.3467
B8AAV9	Phosphoglycerate kinase	PGK	i.D	cyt	0.6367	0.5133	0.3033	0.7800	0.9733	1.0000
A2WXL1	Pectinesterase	PE	i.D	cyt	1.2967	1.1833	0.9067	2.2400	1.9267	1.7400
A2YBW4	Glycosyltransferase	GT	i.D	cyt	1.0333	1.0867	0.6967	1.1033	0.8567	0.9300

A2Y650	Phosphoglycerate kinase	PGK	i.D	cyt	1.9533	2.0000	1.5133	1.6000	1.2800	1.2900
A2YQT7	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	i.D	cyt	0.7367	0.8267	0.6600	1.9600	2.1567	2.1733
B8BKT7	Alpha-mannosidase	α-Man	i.D	cyt	1.1867	1.1300	0.8967	1.1233	1.0967	1.1733
B8BFP6	Alpha-mannosidase	α-Man	i.D	cyt	1.0867	0.9667	0.8833	2.0267	1.4400	1.6133
B8A748	Pectinesterase	PE	i.D	cyt	1.2000	1.2100	1.0767	0.9867	0.7633	0.8067
B8AF09	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	i.D	cyt	1.3600	1.6600	1.6167	1.2567	1.3467	1.2433
A2YQQ8	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit	SQR	i.D	mit	0.6633	0.6467	0.5200	0.6900	0.8033	0.8600
B8AIH2	Phosphoglycerate kinase	PGK	i.D	cyt	2.6600	3.0767	3.2500	1.1767	1.1433	2.2333
B8AK72	UDP-glucose 6-dehydrogenase	UGDH	i.D	cyt	0.9933	1.1100	0.9900	0.9200	0.9167	0.9067
D7PPH8	Alcohol dehydrogenase	ADH1	i.D	cyt	2.4967	1.9767	2.0467	0.2933	0.3833	0.3033
D4AIA3	Glucose-1-phosphate adenylyltransferase	GPAt	i.D	cyt	1.2233	1.1533	1.1067	0.9867	1.1133	1.0733
B8AZJ5	Phosphoglycerate kinase	PGK	i.D	cyt	0.6233	0.6267	0.5933	1.6567	1.6633	1.4033
A2WPC2	Dihydrolipoyl dehydrogenase	DLD	i.D	cyt	1.2800	1.2900	1.1300	8.0567	7.3467	6.7400

A2XEN9	Beta-galactosidase	β-gal	i.D	cyt	1.1000	0.9433	1.0667	1.8233	1.7600	1.6133
A2XC18	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	i.D	cyt	0.9733	1.0100	0.8800	0.7467	0.7133	0.6700
A2XME9	Malate dehydrogenase	MDH	i.D	cyt	1.5467	1.6300	1.5700	1.0500	1.4300	2.0000
B8AII9	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	i.D	cyt	0.7433	0.7100	0.6500	0.8800	0.9200	0.9167
B8AYA3	Beta-hexosaminidase	HEXB	i.D	cyt	2.1567	1.7700	1.5900	0.8167	0.9033	0.9200
A2XEX2	Fructose-1,6-bisphosphatase	FBPase	i.D	chl	2.0367	1.4167	1.1800	1.2267	1.2133	1.1033
A2XAZ3	Alcohol dehydrogenase class-3	ADH3	i.D	cyt	1.2300	1.1933	1.0767	1.4333	1.5267	1.4333
B8AYE1	Dihydrolipoyl dehydrogenase	DLD	i.D	cyt	0.7000	0.7900	0.7967	0.8700	0.7133	0.8000
B8B0B0	Glucose-6-phosphate isomerase	GPI	i.D	cyt	0.2267	0.3267	0.2833	0.8767	0.8400	1.0100
B8AMU2	Aconitase	ACO	i.D	cyt	1.4500	1.5233	1.3133	1.0433	1.0533	0.9400
A2XU83	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	i.D	cyt	7.1100	6.2767	6.3267	4.5233	5.2333	5.0567
B8B9Z2	Pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit alpha	PFPα	i.D	cyt	0.8433	0.8533	0.8667	1.9233	1.5700	1.4333
A6N1F9	Glycerol kinase	GK	i.D	cyt	0.8033	0.9767	0.8700	1.6167	1.6200	1.5033

B8BHM9	Alpha-galactosidase	α-Gal	i.D	cyt	1.2333	1.5900	1.3600	0.9067	0.8400	0.7467
A2YBX1	Glycosyltransferase	GT	i.D	cyt	1.2367	1.1233	1.0967	0.6733	0.4600	0.6267
A2YKI0	Acetyltransferase component of pyruvate dehydrogenase complex	PDC	i.D	cyt	1.6633	1.4700	1.3233	1.3700	1.2467	1.1367
A2XUU7	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.-)	GAPDH	i.D	cyt	1.9533	2.5300	2.3467	1.3133	1.3833	1.4600
B8AIR3	Acetyl-coenzyme A synthetase	AceCSs	i.D	cyt	1.3267	1.0100	1.1867	1.5667	1.2300	1.3700
B8B4J4	Fructose-bisphosphate aldolase	FBA	i.D	cyt	1.5600	1.5133	1.5400	0.9533	0.8967	0.8833
A2XFI3	Pyruvate decarboxylase 2	PDC	i.D	cyt	1.2767	1.2033	1.1800	0.7367	0.8267	0.9367
B8AR31	Glucose-1-phosphate adenylyltransferase	GPAt	i.D	cyt	1.0933	0.9733	0.9233	0.7000	0.6833	0.7867
A2X5P7	Beta-fructofuranosidase	β-Fase	i.D	cyt	1.5367	1.6633	1.5733	2.1200	2.4167	2.4167
B8ADI1	NADH-cytochrome b5 reductase	Cyt-b5	i.D	cyt	1.4133	1.3733	1.4933	2.7367	3.5933	3.0233
B8BPH5	UDP-glucose 6-dehydrogenase	UGDH	i.D	cyt	1.0567	1.1033	1.1000	1.5133	1.2467	1.2233
B8AHL5	Pyrophosphate-fructose 6-phosphate 1-phosphotransferase subunit alpha	PFPα	i.D	cyt	1.1467	1.1867	1.1700	1.3933	1.1233	1.1300

A2YB91	Pyrophosphate-fructose 6-phosphate 1-phosphotransferase subunit beta	PFPβ	i.D	cyt	1.1367	1.0467	1.1300	1.0733	1.1900	1.1467
B8BIC8	Pyruvate kinase	PK	i.D	cyt	1.4367	1.2200	1.2667	0.9000	1.1467	0.9933
B8ACF5	Alpha-1,4 glucan phosphorylase	α-1,4-Glu	i.D	cyt	0.7033	0.9133	0.8433	1.7533	2.2500	1.7567
A2WNE7	Isocitrate dehydrogenase [NAD] subunit	IDH	i.D	mit	1.0000	1.0267	0.9633	1.2200	1.3000	1.2033
B8BHG9	Malate dehydrogenase	MDH	i.D	cyt	1.4067	1.3533	1.4033	0.6733	0.8400	0.7333
B8B7Z2	Pyruvate kinase	PK	i.D	cyt	0.9767	0.9700	0.9800	1.5800	1.1700	1.0733
B8APA6	Ribulose-phosphate 3-epimerase	PPE	i.D	cyt	0.7100	0.8833	0.8833	1.5500	1.5967	1.5500
B8ATH2	Acetyl-coenzyme A synthetase	AceCSs	i.D	cyt	0.6267	0.6733	0.7567	0.7300	0.5633	0.5867
B8BJ39	Pyruvate kinase 1	PK1	i.D	cyt	0.7100	0.7100	0.8100	1.1967	1.2967	1.1867
A2WXB2	Fructose-1,6-bisphosphatase	FBPase	i.D	cyt	1.2167	1.1667	1.3700	1.1533	1.0167	0.9267
B8B6F5	Glycosyltransferase	GT	i.D	cyt	1.3467	1.3767	1.4333	1.2733	1.3133	1.1000
A2XYZ3	Pyruvate kinase	PK	i.D	cyt	0.8767	0.8567	0.9900	1.4500	1.3800	1.2900
A2YBW7	Glycosyltransferase	GT	i.D	cyt	0.8233	1.0767	1.1500	1.2300	1.0267	1.0600
A2Y7R4	Malate dehydrogenase	MDH	i.D	cyt	2.4633	2.2067	2.2700	0.9400	1.0033	1.0267
B8A8R6	Malic enzyme	MDH	i.D	cyt	1.0867	1.1800	1.3033	1.2700	1.1800	1.3200

B8B729	Succinate dehydrogenase [ubiquinone] flavoprotein subuni	SQR	i.D	mit	0.9467	1.0000	1.0333	1.6533	1.3233	1.5567
A2YQL4	Fructokinase-2	FKII	i.D	cyt	1.2067	1.1633	1.3033	2.7100	3.2733	2.9500
A2ZF17	Citrate synthase	CitS	i.D	cyt	1.2167	1.3233	1.2267	1.3233	1.4100	1.4667
B8B6D4	Malic enzyme	MDH	i.D	cyt	0.8167	0.7300	0.9467	1.7867	1.7767	1.6900
A2YA91	Sucrose synthase	SUS	i.D	cyt	0.7700	0.8700	0.9933	0.9767	1.0533	1.0767
A3QQQ3	UDP-glucose pyrophosphorylase	UDPGase	i.D	cyt	1.1267	1.2500	1.5700	2.3767	2.3767	2.8900
B8AK73	Alpha-1,4 glucan phosphorylase	α-1,4-Glu	i.D	cyt	0.8133	0.9133	1.0800	0.9267	0.9367	0.8967
A2XHR1	Sucrose synthase	SUS	i.D	cyt	2.5733	2.5167	2.7467	1.2400	1.1233	1.0600
C5IFK6	Heparanase	HPSE	i.D	cyt	1.0867	1.0000	1.3833	1.4067	1.2533	1.2233
B8AY35	Fructose-bisphosphate aldolase	FBA	i.D	cyt	1.0700	1.1233	1.1867	1.9667	1.6833	2.3200
A2WTC5	Isocitrate dehydrogenase [NADP]	IDH	i.D	cyt	0.7333	0.7400	0.8700	1.1933	1.3567	1.1300
A2X7C5	Succinate--CoA ligase [ADP-forming] subunit beta	SCS	i.D	mit	1.4800	1.5900	1.9567	0.9433	0.9467	0.8367
B0EVM5	Glucanase I	Gase1	i.D	cyt	1.6800	1.0633	2.3700	1.1367	1.2167	1.0900
B8B7C5	Peroxisomal (S)-2-hydroxy-acid oxidase	GLO5	i.D	cyt	1.4100	1.6567	2.1233	0.7167	0.6400	0.7233
B8B2F2	Formate dehydrogenase	FDH	i.D	mit	1.3900	1.3900	1.8967	0.9900	0.9767	0.9633

A2ZEA9	6-phosphogluconate dehydrogenase	6PGD	i.D	cyt	1.1000	1.0367	1.2933	1.6167	2.5833	2.3500
A2WUA4	Glycylpeptide N-tetradecanoyltransferase	NMT	i.D	cyt	1.1167	0.9600	1.3200	1.3767	1.3300	1.2133
Q259G4	Phosphomannomutase	PMM	i.D	cyt	1.1867	1.2567	1.7567	0.6833	0.8033	0.8067
A2WUT5	Glycosyltransferase	GT	i.D	cyt	2.3233	1.5433	2.2333	0.9500	0.9633	0.9200
A2ZBX1	Fructose-bisphosphate aldolase	FBA	i.D	cyt	2.2333	2.0100	3.1533	0.7200	0.8167	0.7533
A2WXV8	Fructokinase-1	FKI	i.D	cyt	1.5167	1.4967	2.4833	1.3033	0.8367	0.7767
B8B1K3	6-phosphogluconate dehydrogenase	6PGD	i.D	cyt	0.6667	0.7633	1.3167	0.1400	0.1800	0.2333
A2YMB7	Beta-amylase	β-Amylase	i.D	cyt	0.7267	0.8600	2.1633	1.4467	1.4400	1.3267
B8B5T6	Xylose isomerase	XI	i.D	cyt	0.4967	0.5767	1.1133	0.6533	0.6467	0.6467
B8B7P6	Sugar Phosphate Isomerase family	SPiso	i.D	cyt	3.0133	3.9800	12.5033	0.3933	0.4833	0.4633
B8A8H2	Transaldolase	TAL	i.D	cyt	0.5433	0.5567	1.5467	1.1600	0.8433	0.9200
A2Z0L8	Enolase	Enolase	i.D	cyt	1.3633	1.2933	2.4600	1.0333	1.0200	0.9800
A2YRB6	Transaldolase	TAL	i.D	cyt	0.9733	0.8967	1.1400	0.8367	1.0433	0.9333
B8B930	Purple acid phosphatase	PAPy	i.E	cyt	1.1667	0.8267	0.8767	0.6967	0.8667	0.8200
A2XNF0	Chalcone-flavonone isomerase	CHI	i.E	cyt	0.6800	0.7833	0.6800	0.5333	0.6133	0.6967
B8ASB2	Probable aldo-keto reductase 1	AKR	i.E	cyt	1.3800	1.2833	1.2500	0.8400	0.6167	0.6333

A2ZEX7	Chalcone synthase 1	CHS	i.E	cyt	2.3767	2.6467	1.7300	0.6633	0.7867	0.7433
A2XU09	Zeaxanthin epoxidase	Zea-epo	i.E	chl	1.3200	1.0867	1.2600	0.7167	0.6200	0.6167
Q9FUS5	Violaxanthin de-epoxidase	VDE	i.E	cyt	1.1433	0.9733	0.9533	1.2567	1.2633	1.1733
A2ZMK2	Sulfurtransferase	SF	i.E	cyt	1.0200	1.0767	1.1333	0.9400	1.0333	0.9300
A2XA82	UMP-CMP kinase	UMP-CMP	i.E	cyt	0.5933	0.6300	0.6533	1.1567	1.2800	1.2467
Q9ZWM0	Plastidic ATP sulfurylase	APS	i.E	cyt	1.3600	1.1833	1.2167	0.9533	0.9367	0.8733
A2XID3	Allene oxide cyclase	AOC	i.E	chl	0.8267	0.6733	1.0333	1.1167	0.9367	0.9167
A2XRZ0	Probable aldo-keto reductase 2	AKR2	i.E	cyt	0.9133	1.1733	1.8600	1.4367	1.3800	1.3000
B8BIQ9	Chalcone-flavonone isomerase family protein	CHI	i.E	cyt	0.6433	0.8100	1.1400	0.9233	1.1100	0.8967
B8BBZ9	Inosine monophosphate cyclohydrolase	IMPCH	i.E	cyt	4.0000	1.0467	1.8767	0.9533	1.0467	0.8633
A2ZDQ6	Adenylate kinase	ADK	i.E	cyt	0.9967	1.1333	iii.B133	0.7400	0.8700	1.0400
B8AEU9	Aldo-keto reductase	AKR	i.E	cyt	0.7800	0.8467	1.5800	1.7667	0.9167	1.3833
A2Z5P1	Cinnamyl alcohol dehydrogenase	CAD	i.E	cyt	1.0133	0.9700	2.0900	0.7600	0.6600	0.6433
A2WLL0	Plant acid phosphatase	APase	i.E	cyt	1.0100	0.9967	1.8433	0.5433	0.2233	0.3800
A6N1G1	Cathepsin b-like cysteine proteinase 3	CATb	ii.A	cyt	1.5233	1.6533	1.4767	1.0067	1.1167	1.0167
B8BJD1	Coatomer subunit beta (Beta-coat protein)	COP-β	ii.A	cyt	1.6767	1.7167	1.3967	1.2800	1.3600	1.4000

B8APS8	Beta-adaptin-like protein (clathrin)	BETA-AD	ii.A	cyt	1.2833	1.1167	1.0900	1.4967	1.3433	1.5233
B8AQQ8	Adenylyl cyclase-associated protein	CAP	ii.A	cyt	1.5100	1.7000	1.6167	1.3133	1.3433	1.2133
A2ZAH5	ABA receptor 9	ABAr	ii.A	cyt	1.2333	1.2133	1.0233	0.7633	0.7600	0.7033
A2YYM7	Coatomer subunit alpha	COP-α	ii.A	cyt	0.9333	0.8200	0.7400	1.0267	1.2467	1.0567
B8B874	Coatomer subunit gamma	COP-ɣ	ii.A	cyt	1.0267	0.9167	0.8533	1.9400	2.0467	1.9933
A2Y2Z1	Guanosine nucleotide diphosphate dissociation inhibitor	GDI	ii.A	cyt	1.9000	1.7900	1.7267	1.1000	1.2600	1.1433
B8AY77	Importin subunit alpha	Imp-α	ii.A	cyt	1.4767	1.1900	1.2867	1.7433	1.6133	1.3633
A2WP63	Protein translocase subunit SecA	SecA	ii.A	cyt	1.6067	1.5500	1.7833	3.3933	2.3367	2.1967
A2WMY5	Importin subunit alpha	Imp-α	ii.A	cyt	0.7233	0.9733	0.9933	0.7533	0.9900	0.8967
B8BF46	Annexin	Annexin	ii.A	cyt	1.2100	1.1700	1.7933	1.2033	1.3800	1.1300
A2WYE1	Small ubiquitin-related modifier (SUMO)	SUMO	ii.A	cyt	0.3633	0.3333	1.0833	1.1467	1.2867	1.0633
A2WN93	Calmodulin-1	CaM-1	ii.A	cyt	1.1000	0.8533	5.2433	0.8600	0.7933	0.8200
A2WPH1	Sar1	Sar1	ii.A	cyt	1.1133	1.1267	2.0600	0.5867	0.6233	0.5233
A6N028	Eukaryotic translation initiation factor 5A	eIF-5A	ii.B	cyt	1.6767	2.0867	0.5433	1.1033	0.9533	0.8967
B8AEE7	40S ribosomal protein S30	rpS30	ii.B	cyt	2.8667	1.2867	0.9767	1.2833	1.1533	1.0300

A2Y7R3	Histone H2B.9	H2B.9	ii.B	cyt	1.3100	1.1733	0.8267	1.6600	1.5200	1.5300
A2YHI1	Eukaryotic translation initiation factor 5A	eIF-5A	ii.B	cyt	1.7967	2.0233	1.0200	0.8533	0.9767	0.8500
A2YNT9	40S ribosomal protein S6	rpS6	ii.B	cyt	3.2067	1.8067	1.7367	2.6867	1.2767	1.3067
B8B833	Eukaryotic translation initiation factor 5A	eIF-5A	ii.B	cyt	2.4400	3.1033	1.3433	1.9467	2.1800	2.1100
A2WPV4	40S ribosomal protein S4	rpS4	ii.B	cyt	1.4333	0.7500	0.7167	1.0500	0.9733	1.1333
A2X2N6	40S ribosomal protein S24	rpS24	ii.B	cyt	0.9767	0.6300	0.4900	0.8733	0.8433	0.7700
A2Z2J3	60S ribosomal protein l9	rpl9	ii.B	cyt	0.9500	0.7467	0.6500	0.5667	0.6167	0.5500
A2X4T7	40S ribosomal protein S27	rpS27	ii.B	cyt	1.7367	1.2400	1.0967	1.0133	1.2700	1.3200
P0C439	50S ribosomal protein L14	rpL14	ii.B	chl	1.5700	0.9833	1.0200	0.8400	0.9000	0.9067
A2ZLC1	Elongation factor Ts	mEF-Ts	ii.B	mit	2.0367	1.8100	1.3267	1.0800	1.1967	1.0167
P0C476	30S ribosomal protein S18	rpS18	ii.B	chl	1.0400	0.7900	0.7500	1.4033	1.3400	1.1967
A6MZM7	40S ribosomal protein S14	rpS14	ii.B	cyt	2.1700	1.7867	1.7167	0.8367	0.9600	0.8767
C8TFM3	Polyadenylate-binding protein	PABP	ii.B	cyt	2.9167	2.8133	2.4833	0.8533	0.8633	0.7667
A2XDL4	40S ribosomal protein S3a	rpS3a	ii.B	cyt	2.4933	1.3500	1.2233	0.7500	0.6300	0.5367

A2XD89	40S ribosomal protein SA	rpSA	ii.B	cyt	1.3400	0.7300	1.1867	2.5967	2.5667	2.2367
A2Y0T4	60S ribosomal protein L10-2	rpL10-2	ii.B	cyt	1.0900	0.9633	0.7333	0.8867	0.9000	0.8067
A6MZJ4	40S ribosomal protein S27a	rpS27a	ii.B	cyt	1.4667	1.1800	1.0467	1.0233	1.4533	1.1767
B8AEQ9	Elongation factor Tu	EF-Tu	ii.B	cyt	3.6433	3.7100	2.9133	1.1233	1.0667	0.9933
A6MZQ0	40S ribosomal protein S26	rpS26	ii.B	chl	0.9067	0.8233	0.6900	1.3467	1.5533	1.1100
B8AE20	Eukaryotic translation initiation factor 3 subunit G	eIF3g	ii.B	cyt	1.7067	1.5633	1.1900	0.5767	0.6633	0.6167
A2ZFU3	40S ribosomal protein S9	rpS9	ii.B	cyt	0.4267	0.3133	0.3633	1.2600	1.2567	1.1000
A6MZL6	50S ribosomal protein l19	rpl19	ii.B	cyt	0.5867	0.5300	0.5833	0.9467	1.0433	0.9367
A2X3J5	40S ribosomal protein S3a	rpS3a	ii.B	cyt	2.6267	2.0900	2.1967	1.0567	1.0767	1.0667
B8BIC4	Eukaryotic translation initiation factor 3 subunit B	eIF3b	ii.B	cyt	1.1167	1.0900	0.7467	0.8133	0.9067	0.7200
B8APM5	Elongation factor 1-alpha	EF-1α	ii.B	cyt	1.2033	1.2267	1.1333	0.6433	0.7167	0.6767
A0A165TZX1	Alba1	Alba1	ii.B	cyt	3.5900	iii.B1	2.6700	1.2567	1.1967	1.1267
A2YYH2	Polyadenylate-binding protein	PABP	ii.B	cyt	1.8500	1.8933	1.7200	0.8667	1.0267	0.8433
Q6XEB7	Ribosomal protein L3B	rpL3B	ii.B	cyt	1.0633	0.9000	1.0333	1.6133	1.8067	1.7533

P0C442	50S ribosomal protein L16	rpL16	ii.B	chl	1.0200	0.8600	0.8500	0.9100	0.8633	0.7133
A6N0J4	Nucleic acid binding protein (Fragment)	NABP	ii.B	cyt	2.2733	1.5833	1.4833	1.4500	1.6300	1.4900
B8AXA2	Histone H2A	H2A	ii.B	cyt	0.8067	0.9633	2.0700	0.5033	0.5800	0.5400
Q1MSJ3	Putative eukaryotic translation initiation factor 4 gamma	eIF4g	ii.B	cyt	1.0300	0.9233	0.9633	1.3233	1.5067	1.1600
A2XP46	Elongation factor Tu	EF-Tu	ii.B	cyt	0.7933	0.9133	0.7167	0.8633	1.0000	0.9000
P0C487	30S ribosomal protein S4	rpS4	ii.B	chl	0.8733	0.6067	0.6033	1.4767	1.6800	1.0500
A2ZCQ7	60S ribosomal protein L10-1	rpL10-1	ii.B	cyt	1.2867	1.1167	1.0633	1.0167	1.1667	0.9500
P0C484	30S ribosomal protein S3	rpS3	ii.B	chl	0.7300	0.6900	0.5833	16.1333	1.6900	1.6200
A1XFD1	AF-4 domain containing protein-like protein	AF-4p	ii.B	cyt	2.0067	2.0900	1.8433	0.7433	0.8900	0.7567
P0C481	30S ribosomal protein S2	rpS2	ii.B	chl	0.8000	0.6100	0.6900	0.6400	0.7567	0.7567
A2YPM1	Elongation factor beta-1	EF-β1	ii.B	cyt	0.6400	0.6900	0.6733	0.9767	1.0167	0.8867
A2XVY3	Elongation factor G	cEF-G	ii.B	chl	0.9267	1.0867	0.9600	1.1667	0.9067	0.9333
A6MZM9	40S ribosomal protein S13	rpS13	ii.B	cyt	1.0567	1.0133	1.5333	0.5000	0.5200	0.4567
A2ZB00	40S ribosomal protein S16	rpS16	ii.B	cyt	0.4900	0.3333	0.4233	0.8967	1.4800	1.7133

A2ZF53	Glutamyl-tRNA(Gln) amidotransferase subunit B	Gln-tRNA	ii.B	chl/mit	1.5567	1.7000	1.5633	1.1467	1.3167	1.0700
B8APG0	Nuclear pore protein	NPP	ii.B	cyt	0.8300	0.9200	0.9133	0.9167	0.8533	0.9433
A2WQF7	Tyrosine--tRNA ligase	TRase	ii.B	cyt	1.3133	1.5167	1.4333	1.0633	1.3467	1.0167
A2Y7R5	GTP-binding nuclear protein Ran-2	GTPRan-2	ii.B	cyt	0.9033	0.8667	1.0567	1.0100	0.9067	0.8500
D4N2J5	Eukaryotic translation initiation factor 4g	eIF4g	ii.B	cyt	0.6700	0.5933	0.6533	1.6167	4.5133	1.7200
A0A173CU41	30S ribosomal protein S16	rpS16	ii.B	chl	1.0767	1.0100	1.2867	1.0700	1.1967	1.0100
A2XIZ4	Lysine-tRNA ligase	LysRS	ii.B	cyt	1.0000	0.9633	1.3633	0.6533	0.7700	0.7767
A2YMU2	Ribosome-recycling factor	RRF	ii.B	chl	0.5233	0.7133	1.3467	0.6167	0.6267	0.6433
A2XN63	Universal ribosomal protein uS5 family	rpS5	ii.B	cyt	1.4467	0.7133	0.3633	1.4267	1.5333	1.2100
B8AS60	Ribosomal protein S17	rpS17	ii.B	cyt	0.8433	0.4367	0.2200	1.4333	1.7933	1.4533
B8AK27	Ribosomal protein S10	rpS10	ii.B	cyt	2.0367	1.4267	0.7067	0.8967	1.1500	0.8667
A2XCC4	60S ribosomal protein L21	rpL21	ii.B	cyt	6.8100	3.6700	3.0333	1.3633	1.6500	1.2733
A2Y7L1	ribosomal protein L12	rpL12	ii.B	cyt	0.8867	0.8900	1.6133	0.9933	1.2033	0.8667
A2Z2V9	20S proteasome	20S-prot	ii.C	cyt	0.8533	0.7233	0.5367	1.8433	1.7933	1.4133
A2Y992	Dirigent protein	DirP	iii.A	cyt	2.0467	1.6400	1.4700	0.9500	0.9633	0.8733

O24227	Water stress inducible protein	H1/H5	iii.A	cyt	1.1100	0.9000	0.7133	1.2233	1.5867	1.4100
A2Y986	Dirigent protein	DirP	iii.A	cyt	0.8433	0.8467	0.9100	1.7367	1.5333	1.2800
A2ZC27	Dirigent protein	DirP	iii.A	cyt	1.1767	0.9900	0.8600	1.1000	1.4500	1.1733
A6N1B9	Cbs domain protein	Cbs	iii.A	cyt	2.3067	1.9833	1.3200	4.7600	4.6167	2.4567
A6N0H8	Abscisic stress ripening protein 2	ASR2	iii.A	cyt	0.6700	0.7200	0.6233	0.7533	0.7933	0.9000
A2YNQ5	Dirigent protein	DirP	iii.A	cyt	1.2433	1.2633	1.3333	0.5733	0.8433	0.6667
A2WMG6	Salt stress root protein RS1	RS1	iii.A	cyt	0.6667	0.5533	2.4167	1.0400	1.4133	1.0067
A2X854	Plant dehydrin family	DHN	iii.A	cyt	0.8467	0.7467	0.1800	0.6567	0.8767	0.8167
A2X9Q0	PLAT_plant_stress	PLAT	iii.A	cyt	0.9167	0.8767	0.5633	0.8567	0.9467	0.7733
A2WPA9	Peroxidase	PDX	iii.B	cyt	1.2367	1.2033	0.6700	2.4300	1.6367	1.2633
A2WZD6	Peroxidase	PDX	iii.B	cyt	1.9000	2.0367	1.1433	1.6333	2.2000	1.6233
A2YY59	Superoxide dismutase [Cu-Zn]	Cu/Zn-SOD	iii.B	cyt	1.3300	1.4467	1.0000	1.5967	1.9167	1.8267
B8A753	Peroxidase	PDX	iii.B	cyt	1.6700	1.6267	1.3033	2.1100	1.1700	1.0200
B8B5I4	Thioredoxin reductase	TrxR	iii.B	cyt	1.9900	1.7400	1.3200	1.7567	2.4300	1.7167
A2YA34	Glutathione peroxidase	GPx	iii.B	cyt	0.8533	0.9767	0.8400	0.3233	0.4067	0.3700
B8APG3	Peroxidase	PDX	iii.B	cyt	1.0633	1.0467	1.1267	1.0533	1.1633	0.9667
A2X2T0	Peroxidase	PDX	iii.B	cyt	0.9333	0.8767	0.9367	1.7600	1.8833	1.4867
B8B3L5	Peroxidase	PDX	iii.B	cyt	1.4567	1.2000	1.3300	1.7067	1.1900	1.0633
A3REN3	Catalase	CAT	iii.B	cyt	1.2200	1.1567	1.0900	2.1733	2.3467	2.1633
A2YH64	Catalase isozyme B	CAT-B	iii.B	cyt	0.7733	0.6433	0.7167	1.2833	1.7600	1.2700

B8AKX6	Peroxisomal (S)-2-hydroxy-acid oxidase	GLO1	iii.B	cyt	1.3133	1.3133	1.3700	0.8400	1.2500	0.7867
B8AME2	Catalase	CAT	iii.B	cyt	1.1333	1.1600	1.2000	0.4400	0.4200	0.5033
B8AW52	Lactoylglutathione lyase	Gly1	iii.B	cyt	1.0567	1.0533	1.3567	0.5833	0.8833	0.6400
A2YPX2	Peroxidase	PDX	iii.B	cyt	1.3133	1.3400	2.4367	0.8200	0.7667	1.0800
A2XFC7	L-ascorbate peroxidase 1	APX1	iii.B	cyt	0.6500	0.6800	1.1100	0.6033	0.6067	0.6467
P0C5D4	Putative peroxiredoxin Q	PRDX-Q	iii.B	chl	0.5667	0.6867	0.9233	0.4700	0.7167	0.4933
A2Z7B3	GDP-mannose 3,5-epimerase 1	GME-1	iii.B	cyt	1.5500	1.5433	1.7400	1.4133	0.7900	0.6933
A2XGP6	Superoxide dismutase [Cu-Zn] 1	Cu/Zn-SOD	iii.B	cyt	1.7667	0.9833	2.1300	0.5900	0.9233	0.9033
A2Y043	Peroxidase	PDX	iii.B	cyt	1.0967	1.3100	1.4900	0.7900	0.7733	0.7433
A2YIW7	Thioredoxin H-type (Phloem sap)	TrxH	iii.B	cyt	1.5933	1.8600	iii.C167	0.7633	0.6633	0.6100
B8AWM4	Superoxide dismutase	SOD	iii.B	cyt	0.4733	0.6033	1.0033	0.7100	0.7633	0.6933
B8ARU5	Peroxidase	PDX	iii.B	cyt	0.7900	1.0633	ii.B4	0.3833	0.5400	0.4233
B8ARU3	Peroxidase	PDX	iii.B	cyt	0.7933	0.8233	1.8000	0.3933	0.3233	0.3867
A2ZIM1	Ascorbate peroxidase	APX	iii.B	cyt	5.2833	3.5800	2.1100	0.7600	0.9533	0.5800
B8B8V4	Peptidylprolyl isomerase	PPI	iii.C	cyt	1.5967	1.6467	1.3500	0.6833	0.5700	0.5467
A2X9U8	Peptidylprolyl isomerase	PPI	iii.C	cyt	1.4800	1.3867	1.2700	1.0400	0.7900	0.8367
A2WJU9	Peptidyl-prolyl cis-trans isomerase	PPI	iii.C	cyt	1.2167	1.2033	1.1233	2.9633	2.3533	2.5300

Q1KL27	Peptidyl-prolyl cis-trans isomerase	PPI	iii.C	cyt	2.9800	2.0900	2.6233	1.1267	1.0333	1.4967
A2YUU5	T-complex protein 1 subunit delta	TCP1-Δ	iii.C	cyt	1.0400	1.1500	1.1433	2.6100	1.0533	2.4600
A2YXM1	Peptidylprolyl isomerase	PPI	iii.C	cyt	0.7967	1.0733	0.9133	1.1900	0.5200	0.8367
A2YWQ1	Heat shock protein 81-1	HSP81-1	iii.C	cyt	1.1367	1.3267	1.1500	0.7833	0.8267	1.7267
B8B677	Peptidylprolyl isomerase	PPI	iii.C	cyt	0.8967	0.9300	0.8433	2.1000	1.4233	2.0300
A2YGV2	Peptidyl-prolyl cis-trans isomerase	PPI	iii.C	cyt	1.8267	1.5100	1.4400	0.8100	0.7433	0.8867
B8B3C8	T-complex protein 1 subunit gamma	TCP1-γ	iii.C	cyt	1.0567	1.1200	1.1000	7.5733	4.0700	7.9700
B8AGI3	Peptidylprolyl isomerase	PPI	iii.C	cyt	1.1633	1.1433	1.1233	1.2200	1.2600	1.7200
A2Z2T0	Peptidylprolyl isomerase	PPI	iii.C	cyt	0.7267	0.7767	0.7233	3.5767	3.7067	3.9467
A2XZE6	Peptidyl-prolyl cis-trans isomerase	PPI	iii.C	cyt	1.1033	1.1433	1.0967	1.3300	1.2267	1.4567
B8ACM2	Peptidyl-prolyl cis-trans isomerase	PPIase	iii.C	cyt	2.8900	2.7833	2.9900	0.9600	0.8833	1.1500
A8QXH4	Putative chaperonin 60 beta	Chap60	iii.C	cyt	3.0700	3.5967	2.9567	1.9233	0.9267	0.9467
B8B7Y1	Peptidylprolyl isomerase	PPI	iii.C	cyt	0.4167	0.4133	0.4933	1.4867	1.0700	1.1567
A2ZCE6	Protein disulfide-isomerase	PDI	iii.C	cyt	1.9633	1.9800	ii.B733	1.2233	0.9167	1.2933

A2Z498	Peptidyl-prolyl cis-trans isomerase	PPIase	iii.C	cyt	1.3200	1.2800	1.5633	4.1133	1.5567	1.5267
B8AWP4	chaperone protein DnaJ	DnaJ	iii.C	cyt	0.8900	0.8967	0.3333	0.9167	0.6600	0.6867
B8B3P0	GroES chaperonin family	chp-GroES	iii.C	cyt	4.1467	3.4833	1.6733	2.2300	2.6833	2.1433
A2Z2G1	heat shock protein 83 kDa	HSP83	iii.C	cyt	0.9600	1.1133	0.8033	0.6200	0.4533	0.4200
B8BIB2	Chaperonin 10	chp10	iii.C	cyt	1.7467	1.8467	2.5433	1.1767	2.0467	1.4267
A2XLF2	Actin-1	Actin-1	iv	cyt	0.8633	0.7933	0.6533	0.7933	0.9500	0.8633
P83647	Profilin LP04	LP04	iv	cyt	0.8033	0.8500	0.7433	1.1500	1.1733	0.9833
P0C539	Actin-2	Actin-2	iv	cyt	1.2067	1.1767	0.9800	0.4167	0.5767	0.5000
B8ALG6	Tubulin beta chain	TU-β	iv	cyt	0.9333	0.8067	0.7433	0.6467	0.8367	0.5500
A2WNJ7	Tubulin beta chain	TU-β	iv	cyt	0.8967	0.7933	0.7600	0.6600	0.7133	0.6633
A2XNS1	Actin-3	Actin-3	iv	cyt	1.9167	1.2267	1.3700	1.0200	1.4967	0.9533
A2YG29	Tubulin beta chain	Tu-β	iv	cyt	1.2833	1.0900	1.0633	12.3267	13.5567	10.1000
A2YMX5	Tubulin alpha chain	Tu-α	iv	cyt	1.0933	1.0133	1.0300	0.7933	0.6767	0.6467
A2ZD27	Tubulin alpha chain	Tu-α	iv	cyt	0.7400	0.8100	0.6867	2.6067	1.8600	1.6600
B8AAY0	Tubulin beta chain	TU-β	iv	cyt	1.1833	1.0467	1.0100	1.0900	0.9767	0.8600
A2XMI9	Tubulin beta chain	TU-β	iv	cyt	1.7200	1.6200	1.7133	1.4600	1.5767	1.0633
A2YTW2	Fasciclin-like arabinogalactan-protein-like	FAS1	v	cyt	1.0467	0.9767	0.7033	1.1033	1.0500	0.8900
B8BIN0	Ferritin	Fer	v	cyt	1.2000	1.2367	1.8867	0.9900	1.2167	0.8967

A2YM28	Thiamine thiazole synthase	THI	v	chl	11.9600	2.2700	2.2067	0.9700	1.0533	0.8933
A6MZE4	Senescence associated protein	SenAP	v	cyt	1.8200	1.6100	1.9867	0.7433	0.8800	0.5600
A2WJP2	Ricin-type beta-trefoil lectin domain-like	RIP-2	v	cyt	3.7167	1.0800	1.0667	0.9233	1.2733	0.9933

Table SM.1.4. List of the annotated proteins identified in roots of FL478 through the shotgun proteomic approach. UniProt: uniprot accession number; Prot. name: protein name; Prot. abb.: protein abbreviation; Func. cat.: functional category; Cell. loc.: cellular localization.

UniProt	Prot. name	Prot. abb.	Func. cat.	Cell. loc.	Relative root protein expression					
					0 mM			100 mM		
					6h	24h	48h	6h	24h	48h
A2WMG4	Histidinol dehydrogenase	HDH	i.A	chl	0.9931	1.0490	1.0546	0.9439	0.7805	0.8610
A2WT25	Carbonic anhydrase	CA	i.A	cyt	1.0545	1.0164	0.9735	0.9949	0.9756	0.8817
A2WUC5	Glycine cleavage system P protein	GCS-P	i.A	cyt	1.0363	1.1028	1.1553	1.0795	0.9603	0.9926
A2WVA6	Aspartate aminotransferase	AST	i.A	cyt	0.9886	0.9472	0.9101	1.1520	1.1409	1.2719
A2WVD6	Chorismate mutase	CM	i.A	cyt	1.0866	0.9472	0.9254	1.0775	1.0339	1.0937
A2WZD6	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase 2	MTPene	i.A	cyt	1.2901	0.8170	0.6679	0.9882	1.0618	1.0971
A2X1E0	Glycine cleavage system H protein	GCS-H	i.A	cyt	1.0441	0.9179	1.0110	0.8687	0.9508	0.9591
A2X2G9	Aminopeptidase	AP	i.A	cyt	0.9833	1.0612	0.9629	0.8589	0.9204	0.7666
A2XCQ4	Phosphoserine aminotransferase	PSAT	i.A	cyt	0.9899	1.0310	0.9713	1.0243	0.9734	1.1594

A2XCT8	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase 2	MTPene	i.A	cyt	1.0259	1.0319	0.8106	0.8534	0.9800	0.8851
A2XD35	Adenylosuccinate synthetase	ADSS	i.A	cyt	0.9636	0.9123	0.9704	1.0094	0.9255	0.9418
A2XFU4	Nicotianamine synthase 1	NAS1	i.A	cyt	1.0244	0.9864	0.7652	1.2248	1.1833	0.9720
A2XFY9	Adenylosuccinate lyase	ASL	i.A	cyt	1.0940	1.1524	1.1360	0.9920	1.0971	1.0916
A2XHI6	Phospho-2-dehydro-3-deoxyheptonate aldolase	DAHP	i.A	cyt	1.0095	1.0151	1.0229	0.9767	0.9688	0.9430
A2XIZ4	Lysine--tRNA ligase	LysRS	i.A	cyt	0.9466	0.9756	0.9985	0.9523	1.0825	0.6547
A2XMV1	Glutamate dehydrogenase 1	GDH1	i.A	cyt	1.0353	1.0501	1.0247	0.8240	0.8806	0.7399
A2XPL5	Arginine decarboxylase	ArgD	i.A	cyt	0.9786	1.1599	1.1910	1.0280	0.9195	0.9147
A2XVK1	Phenylalanine ammonia-lyase	PAL	i.A	cyt	0.8700	0.8913	1.0374	0.8851	0.9538	0.8631
A2XW22	Glutamate dehydrogenase 2	GDH2	i.A	cyt	1.0800	1.1570	0.9732	0.9363	0.9857	1.0347
A2XYC2	D-3-phosphoglycerate dehydrogenase	PHGDH	i.A	cyt	1.0009	0.9394	1.0534	1.0474	0.9284	0.8883
A2Y053	S-adenosylmethionine synthase 1	SAM	i.A	cyt	1.0041	0.9785	1.0370	0.8870	1.0423	0.9778
A2Y7Y4	Carboxypeptidase	CPase	i.A	cyt	1.0783	1.1081	0.9159	0.9981	1.0926	0.9272
A2Y8X9	3-phosphoshikimate 1-carboxyvinyltransferase	EPSP	i.A	cyt	0.9421	1.0115	0.9387	0.9146	0.9435	0.9105

A2YCP9	Serine hydroxymethyltransferase	SHMT	i.A	cyt	0.9286	0.9872	1.0142	1.6468	1.3157	1.0178
A2YL94	Carboxypeptidase	CPase	i.A	cyt	1.0084	0.9734	0.8875	1.0875	1.2137	1.1070
A2YNU0	Phospho-2-dehydro-3-deoxyheptonate aldolase	DAHP	i.A	cyt	1.0375	0.9892	1.0025	0.9845	0.9809	0.9415
A2YQ56	Lon protease homolog	LONP	i.A	mit	0.9770	1.0822	0.9858	0.9515	1.0080	0.8861
A2YVQ8	D-3-phosphoglycerate dehydrogenase	PHGDH	i.A	cyt	1.0450	1.0561	1.0680	1.5230	0.8088	0.7794
A2ZA03	Branched-chain-amino-acid aminotransferase	AT	i.A	cyt	0.9062	0.9447	0.9913	0.9819	0.8684	1.0157
A2ZAA7	Nucleoside diphosphate kinase	NDK	i.A	cyt	1.0968	0.9755	1.0648	1.0770	1.1147	0.8828
A2ZDY4	Adenosylhomocysteinase	AHCY	i.A	cyt	0.9596	0.9432	0.9505	1.0158	0.9928	1.0014
A2ZDY5	Serine hydroxymethyltransferase	SHMT	i.A	cyt	0.8686	0.9196	0.9833	0.9665	1.0449	1.1169
A2ZLS4	Putative diaminopimelate epimerase	DAP	i.A	chl	1.0322	0.9951	1.0051	0.8386	0.9582	0.8766
A2ZMY2	Cysteine synthase	CysS	i.A	cyt	1.1599	1.0932	0.9866	1.1749	1.1647	1.1948
A5X2Z8	Acetolactate synthase	ALS	i.A	cyt	1.0292	1.0442	1.0564	1.0645	1.1464	1.1744
A6MZK0	Phenyl ammonia lyase	PAL	i.A	cyt	0.7583	0.8950	0.9140	0.9706	1.0229	0.9968

A6N0M9	Nucleoside diphosphate kinase 1	NDPK1	i.A	cyt	0.9872	0.8911	0.8973	1.1311	1.0306	1.1829
A6N176	Phenylalanine ammonia-lyase	PAL	i.A	cyt	0.8752	0.9419	1.0896	1.0387	0.9507	1.1262
B3VMC0	Betaine aldehyde dehydrogenase 2	BADH2	i.A	cyt	0.9887	0.9758	0.9517	1.0831	1.0954	1.0262
B8ADD3	Carboxypeptidase	CPase	i.A	cyt	1.0464	1.1344	0.9631	0.9605	1.0088	1.0224
B8ADJ1	Ubiquitinyl hydrolase 1	UBHase1	i.A	cyt	0.8833	1.1755	0.9258	0.8653	0.8945	1.0974
B8AEH4	Aspartate aminotransferase	AST	i.A	cyt	0.9491	0.9724	0.9096	0.9301	0.9518	0.9456
B8AES5	Homoserine dehydrogenase	HDH	i.A	cyt	0.9320	0.9643	0.9646	0.9487	1.0885	1.0682
B8AI28	Glutamine synthetase	GS	i.A	cyt	0.9970	1.0044	0.9461	1.0077	0.8183	0.8593
B8AJN3	Glutamine synthetase	GS	i.A	cyt	0.9453	0.9345	0.9024	1.0790	1.0879	0.9345
B8AJV7	Cysteine synthase	CysS	i.A	cyt	1.0029	0.9482	0.8923	1.0480	0.9389	0.9389
B8AJX7	Serine hydroxymethyltransferase	SHMT	i.A	cyt	1.0005	0.9771	1.0299	0.9473	0.9451	0.9022
B8AKA5	Chorismate synthase	ChS	i.A	cyt	0.9443	0.9796	1.0262	1.0292	1.1086	1.1189
B8AL33	Arginine biosynthesis bifunctional protein ArgJ	ArgJ	i.A	chl	0.9260	0.9585	0.8939	0.9663	1.0133	1.1223
B8AQQ2	Glutamate decarboxylase	GD	i.A	cyt	0.8954	1.0000	0.9501	1.0409	0.9798	1.0766
B8AWV4	Ketol-acid reductoisomerase	KARI	i.A	cyt	0.9751	1.0275	1.0157	1.0599	1.0565	1.0169

B8AWY8	Methionine S-methyltransferase	MMT	i.A	cyt	0.9830	1.0913	1.0502	1.4182	1.2298	1.0205
B8AXH7	Nucleoside diphosphate kinase	NDK	i.A	cyt	1.0095	0.9440	0.9192	0.9404	0.9083	0.8932
B8AYT8	Delta-1-pyrroline-5-carboxylate synthase	δP5CS	i.A	cyt	0.9054	0.8356	1.0108	0.9132	0.9409	0.9683
B8B049	Carboxypeptidase	CPase	i.A	cyt	1.1368	1.2015	0.5137	1.0327	1.0715	0.9838
B8BAI7	Aminopeptidase	AP	i.A	cyt	0.9826	1.0053	0.9664	1.0243	1.0056	1.0125
B8BBZ7	Probable gamma-aminobutyrate transaminase 3	ABAT-3	i.A	mit	0.9991	0.9862	0.8818	1.0592	1.0081	0.9273
B8BET6	Aminopeptidase	AP	i.A	cyt	1.0689	1.1400	0.8829	1.0307	0.9853	0.9077
B8BFZ6	Alanine-tRNA ligase	AlaRS	i.A	mit	0.9599	1.0398	1.0201	0.8837	0.9771	0.8564
B8BHT2	Methionine aminopeptidase	MetAP	i.A	cyt	1.1243	1.0559	1.0781	1.0171	0.9080	0.9734
B8BKI7	Probable bifunctional methylthioribulose-1-phosphate dehydratase/enolase-phosphatase E1	MTRu-1-P	i.A	cyt	1.0720	1.1127	0.7761	2.3716	1.1573	0.6644
C8TFG9	Indole-3-glycerol phosphate synthase-like	IGPS	i.A	cyt	1.2319	1.3299	1.0082	1.0700	1.0710	1.0794
Q01K11	Gamma-aminobutyrate transaminase 1	ABAT-1	i.A	mit	1.0083	1.0442	0.9756	0.9300	1.0849	0.9956
Q5UJF9	Cysteine synthase	CysS	i.A	cyt	1.1157	0.9197	0.8701	0.9945	0.7505	0.6088

Q84UX4	Alanine aminotransferase	ALT	i.A	cyt	1.0227	1.0373	0.9644	1.0245	0.8452	0.8513
Q8GU95	Glutamate-cysteine ligase A	GCL-A	i.A	cyt	1.0231	1.0122	1.0048	1.0470	1.0736	1.1170
A0A1D9CEX0	Chloroplast ferredoxin-dependent glutamate synthase	GLU	i.B	chl	0.9614	1.0231	1.0081	1.0006	0.9503	0.8573
A0PJ32	ATP8	ATP8	i.B	chl	0.6134	0.8328	1.1089	1.1184	0.9508	1.1157
A2X6V5	Cytosolic Fe-S cluster assembly factor NBP35	NBP35	i.B	cyt	1.0022	0.9768	1.1199	1.0596	1.0378	0.9183
A2XCL6	AAA-ATPase	AAA	i.B	chl	0.9007	0.9866	1.0072	0.8763	0.8581	0.9716
A2XIK9	Magnesium-chelatase subunit ChlI	Chl-I	i.B	chl	1.0852	0.9476	1.2302	1.1416	1.0194	1.3581
A2XMM3	Ferredoxin-NADP reductase	FNR	i.B	cyt	1.1938	1.1460	0.9994	1.1702	1.1006	1.1934
A2XNC0	Methylenetetrahydrofolate reductase	MTHFR	i.B	cyt	0.9443	0.9754	0.9860	0.9945	0.9689	1.0258
A2XNR6	Electron transfer flavoprotein subunit alpha	αETF	i.B	cyt	0.8964	0.9429	1.1181	1.1994	1.1476	1.1635
A2XQ09	V-type proton ATPase subunit F	ATPase-F	i.B	cyt	1.0548	1.0775	0.9539	1.1027	1.0792	1.0569
A2XYF8	Plasma membrane ATPase	ATPase	i.B	cyt	0.8090	0.9573	0.9213	1.0037	1.0332	0.9482
A2Y886	Plastocyanin	Pc	i.B	chl	1.1376	1.0973	1.2149	0.9187	0.7656	0.8467

A2YP98	NADH dehydrogenase [ubiquinone] flavoprotein 1	NDUFV1	i.B	mit	1.0489	0.8885	0.9344	0.9668	0.9141	0.9946
A2Z6N7	NAD(P)H-hydrate epimerase	NADPHX	i.B	cyt	1.2444	0.9215	1.1857	0.9764	0.9996	0.9421
A6N149	Ribulose bisphosphate carboxylase small chain	rbcS	i.B	chl	0.7064	0.8491	1.1116	1.0023	1.0052	1.0369
B8A7M8	ATP synthase subunit beta	ATPase-β	i.B	cyt	1.0799	0.9665	0.8362	0.9604	1.1040	1.0456
B8AWF8	ATP synthase subunit beta	ATPase-β	i.B	cyt	0.9843	0.7303	0.7952	0.8750	0.8047	0.7971
B8AXG9	V-type proton ATPase subunit C	ATPase-C	i.B	cyt	1.0865	1.1068	1.0575	1.0868	1.2213	1.2350
B8B7X6	Probable D-2-hydroxyglutarate dehydrogenase, mitochondrial	D2HGDH	i.B	mit	0.9521	1.0645	0.9575	1.0447	0.9937	1.0191
B8BBN7	Obg-like ATPase 1	ATPase-1	i.B	cyt	0.9732	0.9556	1.0198	0.9968	0.9874	0.9428
B8BBS3	ATP synthase subunit d	ATPase-D	i.B	mit	1.0648	0.9511	1.0336	1.0086	1.0049	0.9052
B8BDK7	ATPase ASNA1 homolog	ASNA1	i.B	cyt	0.8938	0.9832	1.0265	1.1042	0.9474	0.9114
P0C2Z5	ATP synthase subunit alpha	ATPase-α	i.B	chl	0.9309	0.9762	1.1366	0.9588	0.9721	0.9956
P0C2Z8	ATP synthase subunit beta	ATPase-ß	i.B	chl	0.8997	0.9772	1.1659	1.0135	1.0890	0.9701

P0C511	Ribulose bisphosphate carboxylase large chain	rbcL	i.B	chl	0.7425	0.8789	1.1357	0.9980	0.8969	0.9350
P0C521	ATP synthase subunit alpha	ATPase-α	i.B	mit	0.9493	0.9171	0.8863	1.0333	1.1889	1.1836
P83646	Oxygen-evolving enhancer protein 3	psbQ	i.B	chl	1.1372	1.0513	0.9270	0.9601	1.0298	1.0111
Q84JG8	Sedoheptulose-1,7-bisphosphatase	SBPase	i.B	chl	0.8662	0.9818	1.0463	0.9213	0.9815	0.9679
Q8GRU9	Phosphoribulokinase	PRK	i.B	cyt	0.9746	1.0265	1.2097	0.9134	1.1704	1.2437
A2XL11	Lipoxygenase	LOX	i.C	cyt	1.0356	1.2285	1.3010	0.9797	0.9443	0.9536
A2XL19	Lipoxygenase	LOX	i.C	cyt	1.2316	1.0562	0.9924	1.0904	1.1439	1.1022
A2XL22	Lipoxygenase	LOX	i.C	cyt	0.9944	1.0850	1.0677	1.1819	0.9458	0.8658
A2XLT7	Lipoxygenase	LOX	i.C	cyt	0.9944	1.1687	1.0772	0.8727	0.8745	0.8098
A2Y7R2	Phospholipase A1-II 7	PLA1-II7	i.C	cyt	1.0835	0.9946	0.9738	0.8814	0.9311	1.2829
A2Y8A0	Acyl-coenzyme A oxidase	ACX	i.C	cyt	1.0365	1.1036	1.0727	1.0268	1.1739	1.1605
A2YCR4	Acyl-coenzyme A oxidase	ACX	i.C	cyt	1.0076	0.9930	1.0030	1.0756	1.1430	1.0916
A2YW91	Patatin-like protein 2	PLP2	i.C	cyt	1.0734	0.8875	0.9280	0.9270	1.2893	1.2395
A2Z3K3	Acyl carrier protein	ACYprot	i.C	cyt	1.1856	1.0906	1.0194	1.0621	0.9428	0.9336
B8ADH7	Phospholipase D	PLD	i.C	cyt	0.9630	1.0152	1.0446	1.0866	1.0454	1.1083
B8B307	Putative esterase	EST	i.C	cyt	1.2384	1.0851	0.9366	1.0493	1.0028	0.8889
B8B4D2	Phospholipase D	PLD	i.C	cyt	1.1100	1.0696	1.0721	1.0520	1.0644	1.1431
Q9LKM2	Phospholipase D	PLD	i.C	cyt	1.1313	1.1615	1.3180	1.0475	1.1513	1.0579

A2WLL3	ATP-dependent 6-phosphofructokinase	ATP-PFK	i.D	chl	0.9304	0.8674	1.0706	0.9957	0.9537	0.9695
A2WNE7	Isocitrate dehydrogenase [NAD] subunit	IDH	i.D	mit	1.0076	1.0191	0.9477	0.9416	0.9996	0.9944
A2WPC2	Dihydrolipoyl dehydrogenase	DLD	i.D	cyt	1.0329	0.9821	0.9356	0.9818	1.0182	1.1181
A2WTC5	Isocitrate dehydrogenase [NADP]	IDH	i.D	cyt	0.9226	0.9660	0.8790	1.0145	0.9778	0.9561
A2WUA4	Glycylpeptide N-tetradecanoyltransferase	NMT	i.D	cyt	0.9893	1.1109	1.0877	0.9338	1.2378	0.9159
A2WWL6	Malate dehydrogenase	MDH	i.D	cyt	0.9720	0.9624	0.9476	0.9052	1.1729	1.1253
A2WXV8	Fructokinase-1	FKI	i.D	cyt	0.9427	0.9595	0.9407	1.0406	0.9929	0.9230
A2WZU6	L-lactate dehydrogenase	LDH	i.D	cyt	1.0215	1.1115	1.1254	1.1662	1.3457	1.3920
A2X5P7	Beta-fructofuranosidase, insoluble isoenzyme 1	β-Fase	i.D	cyt	1.0931	0.9938	0.9885	0.9336	1.0658	0.9893
A2X7C5	Succinate--CoA ligase [ADP-forming] subunit beta, mitochondrial	SCS	i.D	cyt	1.1086	1.0864	1.0384	1.0485	1.0283	0.9615
A2X8B7	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	MEcPP	i.D	cyt	0.8272	0.8937	1.0331	1.0695	1.3567	1.3497
A2X9Z4	Alpha-amylase	AMY-α	i.D	cyt	1.3459	1.1339	0.7870	0.9681	1.0496	1.2806
A2XAZ3	Alcohol dehydrogenase class-3	ADH3	i.D	cyt	0.8650	0.9086	0.9022	1.1624	1.2661	0.9715

A2XC18	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	i.D	cyt	0.8771	0.9478	1.0493	1.1889	1.1302	1.0629
A2XFI3	Pyruvate decarboxylase 2	PDC	i.D	cyt	0.9069	0.9078	1.0042	1.1154	1.0619	1.0788
A2XHR1	Sucrose synthase	SUS	i.D	cyt	0.9638	0.9671	0.8897	1.1426	1.0109	1.0331
A2XPT6	Pyruvate dehydrogenase E1 component subunit alpha	PDC-E1α	i.D	cyt	0.9703	1.1937	1.2027	0.9756	0.9790	0.9757
A2XU83	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	i.D	cyt	0.8657	0.9457	1.1832	0.9363	1.0031	0.9809
A2XUU7	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	i.D	cyt	1.2347	1.1205	1.1321	1.0143	1.0445	0.9896
A2XYH1	Glucose-6-phosphate 1-epimerase	GPE	i.D	cyt	1.0727	0.9406	1.0672	0.8336	0.9725	1.1458
A2XYZ3	Pyruvate kinase	PK	i.D	cyt	0.9509	0.9365	0.9903	1.0644	0.9407	0.9746
A2Y650	Phosphoglycerate kinase	PGK	i.D	cyt	0.9760	0.9055	1.0523	0.9828	1.0197	1.0267
A2Y7R4	Malate dehydrogenase	MDH	i.D	cyt	1.0726	0.9752	0.8930	1.0315	1.0073	1.3063
A2YA91	Sucrose synthase	SUS	i.D	cyt	0.9541	1.0106	0.9096	1.1038	1.0632	0.9828
A2YB83	Pyruvate dehydrogenase E1 component subunit alpha	PDC-E1α	i.D	cyt	1.2585	1.4032	1.0212	0.9450	0.9143	0.9002

A2YB91	Pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit beta	PFPβ	i.D	cyt	0.9772	0.9968	0.9850	0.9470	0.9826	0.9887
A2YG06	Phosphoglycerate kinase	PGK	i.D	cyt	1.0949	0.8516	0.9630	1.0281	0.9819	0.9110
A2YKG1	Glucose-6-phosphate 1-dehydrogenase	G6PD	i.D	cyt	1.1201	1.0634	1.1244	1.0069	1.0460	0.9283
A2YKI0	Acetyltransferase component of pyruvate dehydrogenase complex	PDC-AT	i.D	cyt	0.9818	0.9876	0.9563	0.9414	0.9384	1.0269
A2YQL4	Fructokinase-2	FKII	i.D	cyt	0.9937	1.0187	0.9427	0.9701	0.9413	0.9075
A2YQQ8	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit	SQR	i.D	mit	1.0249	0.9871	0.9771	0.8760	0.9689	1.0849
A2YQR2	Fructose-bisphosphate aldolase	FBA	i.D	cyt	1.0892	1.0840	0.8832	1.0818	1.1077	1.1287
A2YQT7	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	i.D	cyt	1.0264	1.0764	0.9197	0.9105	0.9626	0.9073
A2YSQ3	Xyloglucan endotransglucosylase/hydrolase	XTH	i.D	cyt	1.0678	1.0257	0.9516	0.7821	0.8826	0.7571
A2YZY5	Glycosyltransferase	GTF	i.D	cyt	0.9916	0.9934	1.0474	1.0502	1.0357	1.0048
A2Z2W0	Ribulose-phosphate 3-epimerase	PPE	i.D	cyt	1.0601	0.8793	0.9536	1.0690	0.9428	0.9323

A2Z2Z0	Pyruvate dehydrogenase E1 component subunit beta	PDC-E1β	i.D	cyt	1.0232	1.0010	0.8062	0.9916	0.9282	0.7124
A2Z361	Glycosyltransferase	GTF	i.D	cyt	1.0677	0.8685	0.8831	0.9140	1.0065	0.9358
A2Z556	Aldose 1-epimerase	galM	i.D	cyt	1.1093	1.0019	1.0056	1.0381	1.0983	1.3423
A2ZBX1	Fructose-bisphosphate aldolase	FBA	i.D	cyt	0.9007	0.9269	1.0634	0.9607	0.9133	0.8306
A2ZEA9	6-phosphogluconate dehydrogenase	6PGD	i.D	cyt	1.1193	1.0465	1.0358	1.0410	1.0000	1.0470
A2ZK00	Beta-galactosidase	β-Gal	i.D	cyt	0.9622	1.0475	0.9196	1.0643	0.9715	0.8992
A3QQQ3	UDP-glucose pyrophosphorylase	UDPGase	i.D	cyt	0.9940	1.0204	0.9628	0.9873	0.8880	0.9844
A6MZG7	Fructose bisphosphate aldolase	FBA	i.D	cyt	0.9244	0.8546	0.9392	1.0513	0.9937	0.9250
A6N010	Ribose 5 phosphate isomerase	Rpi	i.D	cyt	1.0156	0.9280	0.6224	1.0102	1.0488	1.0353
A6N1F0	Alpha-1,4-glucan-protein synthase	α-1,4-GLUase	i.D	cyt	0.7725	0.8463	0.8549	1.0430	0.9541	1.1167
A6N1F9	Glycerol kinase	GK	i.D	cyt	1.1041	1.1411	1.0529	1.0435	1.1190	0.9578
B8A8R6	Malic enzyme	MDH	i.D	cyt	0.9291	1.0020	0.9786	1.0969	1.1080	0.8809
B8ACF5	Alpha-1,4 glucan phosphorylase	α-1,4-GLUase	i.D	cyt	0.9719	0.9269	1.1069	1.0157	0.9230	1.0197
B8ADI1	NADH-cytochrome b5 reductase	Cyt-b5	i.D	cyt	1.0021	1.0116	0.9769	0.9688	0.9880	0.7947
B8AEK0	Citrate synthase	CitS	i.D	cyt	0.9235	0.9695	1.0982	0.9689	0.8777	0.9635

B8AF09	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	i.D	cyt	0.9044	0.8954	0.8582	1.1376	1.0224	0.9606
B8AGW7	Acetyltransferase component of pyruvate dehydrogenase complex	PDC-AT	i.D	cyt	0.8569	0.8760	0.9197	1.0723	1.1493	0.9309
B8AHL5	Pyrophosphate-fructose 6-phosphate 1-phosphotransferase subunit alpha	PFP-α	i.D	cyt	0.8932	1.0261	0.9953	0.8643	0.9166	0.8386
B8AIH2	Phosphoglycerate kinase	PGK	i.D	cyt	0.9874	0.9241	1.0829	0.9657	1.0476	0.9010
B8AII9	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	i.D	cyt	0.9490	0.9942	1.0261	0.9677	0.9346	1.0896
B8AIR3	Acetyl-coenzyme A synthetase	AceCSs	i.D	cyt	0.9409	0.9558	0.9975	1.0458	1.0770	1.0891
B8AK72	UDP-glucose 6-dehydrogenase	UGDH	i.D	cyt	0.8900	1.0047	0.9492	0.9075	0.8558	1.0195
B8AKL0	Glucose-6-phosphate isomerase	GPI	i.D	cyt	0.8333	0.8672	0.9177	1.0683	0.9123	0.9575
B8AMU2	Aconitase	ACO	i.D	cyt	0.9904	1.0069	0.9697	1.0097	1.0642	1.0740
B8APA6	Ribulose-phosphate 3-epimerase	PPE	i.D	chl	0.9630	0.8705	0.9328	1.0940	1.1044	1.1571
B8ARN5	Isocitrate dehydrogenase	IDH	i.D	cyt	0.8261	0.9745	0.8739	1.0536	1.0109	0.9904
B8AVF0	Beta-glucosidase 12	GBA-12	i.D	cyt	0.9514	1.0276	0.9135	1.0058	0.9452	0.9290

B8AW52	Lactoylglutathione lyase (Glyoxalase I)	Gly1	i.D	cyt	1.0020	1.0549	1.0843	1.1704	1.2277	1.3183
B8AXA8	Beta-hexosaminidase	HEXB	i.D	cyt	0.8987	0.8954	0.8669	1.0100	1.0469	0.9071
B8AY35	Fructose-bisphosphate aldolase	FBA	i.D	cyt	0.9597	0.8963	0.9641	0.9520	1.0131	0.8384
B8AYE1	Dihydrolipoyl dehydrogenase	DLD	i.D	cyt	1.0556	0.9688	0.9500	1.4205	0.7857	1.0362
B8AYG2	Dihydrolipoyl dehydrogenase	DLD	i.D	cyt	0.8403	0.9318	0.9740	1.0303	1.0786	1.3203
B8B0B0	Glucose-6-phosphate isomerase	GPI	i.D	cyt	1.0200	1.0423	1.0460	0.9129	1.2250	1.7609
B8B1K3	6-phosphogluconate dehydrogenase	6PGD	i.D	cyt	0.9691	0.9864	0.9682	0.8580	1.0533	0.9042
B8B1M2	Acetyltransferase component of pyruvate dehydrogenase complex	PDC-AT	i.D	cyt	1.0804	1.0418	1.1463	1.1171	1.0646	1.0009
B8B2F2	Formate dehydrogenase	FDH	i.D	mit	1.0551	0.9827	0.9660	0.9852	1.1266	1.1019
B8B2U7	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	DLAT	i.D	cyt	1.1315	1.1397	1.0257	1.2591	1.1455	1.1117
B8B5T6	Xylose isomerase	XI	i.D	cyt	0.9809	0.9606	0.9852	0.9754	1.0561	1.0116
B8B656	Alpha-galactosidase	α-Gal	i.D	cyt	0.8731	0.7931	0.8608	0.8921	0.9665	0.9721
B8B6D4	Malic enzyme	MDH	i.D	cyt	1.0257	1.1092	1.0268	0.9509	1.1779	1.0231
B8B729	Succinate dehydrogenase	SQR	i.D	mit	0.9155	0.9207	0.9983	1.0179	0.9967	0.9299

B8B7R8	Succinate-CoA ligase [ADP-forming] subunit alpha	SCS-α	i.D	mit	0.9597	0.9019	1.0213	1.1472	1.1686	1.0754
B8B945	Pyruvate dehydrogenase E1 component subunit beta	PDC-E1β	i.D	cyt	1.3436	1.2719	1.1649	1.0407	1.0409	1.1244
B8B969	Uridine kinase	UK	i.D	cyt	0.8770	0.9975	1.0165	0.9670	0.9287	1.1076
B8B9Z2	Pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit alpha	PFP-α	i.D	cyt	0.9439	0.8407	0.9702	0.9353	0.9594	0.9088
B8BBC8	Diacylglycerol kinase	DagK	i.D	cyt	0.9930	0.9993	1.0836	1.0718	1.2859	1.3369
B8BCM8	Glucose-6-phosphate isomerase	GPI	i.D	cyt	1.0452	1.0995	1.0696	0.8986	0.8906	0.9954
B8BFP6	Alpha-mannosidase	α-Man	i.D	cyt	0.9997	0.9981	0.9251	0.9356	1.0095	1.0520
B8BHG9	Malate dehydrogenase	MDH	i.D	cyt	0.9962	0.9725	0.9508	0.9029	1.0092	1.0864
B8BHM9	Alpha-galactosidase	α-Gal	i.D	cyt	1.0847	1.0632	0.8293	0.9806	0.9578	0.8257
B8BHR1	Malic enzyme	MDH	i.D	cyt	1.0632	1.1479	1.1742	1.0579	0.9577	1.0061
B8BIC8	Pyruvate kinase	PK	i.D	cyt	1.0022	1.0095	0.9840	1.0262	1.0195	1.0629
B8BJ39	Pyruvate kinase 1	PK1	i.D	cyt	0.9836	1.0074	0.9725	1.0188	0.9170	0.8832
B8BKT7	Alpha-mannosidase	α-Man	i.D	cyt	1.0730	1.0478	0.8970	0.9167	0.9819	0.9597
B8BN58	Malate dehydrogenase	MDH	i.D	cyt	1.2428	1.1628	1.0320	1.1074	1.1181	1.0008
B8BPH4	UDP-glucose 6-dehydrogenase	UGDH	i.D	cyt	0.7434	0.8107	0.9480	0.9839	1.0085	0.9660

B8BPH5	UDP-glucose 6-dehydrogenase	UGDH	i.D	cyt	0.9072	1.3135	1.1033	0.9669	0.9798	1.1632
C5IFK6	Heparanase	HPSE	i.D	cyt	1.1387	1.1417	1.0444	0.9315	0.9783	0.9485
C8TEV8	Putative sucrose-phosphate synthase	SPS	i.D	cyt	1.0721	0.9975	0.9707	1.1435	1.1481	1.2327
Q259G4	Phosphomannomutase	PMM	i.D	cyt	0.9342	1.0555	1.0660	0.8774	0.8611	0.9315
Q4R1E8	Alcohol dehydrogenase 2	ADH2	i.D	cyt	0.9444	0.9387	0.9103	1.0632	1.0206	1.0684
A2XDX3	Purple acid phosphatase	PAP	i.E	cyt	1.0400	1.0094	0.8738	0.7912	0.9473	0.7513
A2XEC8	Purple acid phosphatase	PAP	i.E	cyt	1.0824	1.1097	1.0563	1.0869	1.0729	1.1292
A2XID3	Allene oxide cyclase	AOC	i.E	chl	1.0429	1.0485	1.0207	0.9081	0.8109	0.9322
A2XNF0	Chalcone-flavonone isomerase	CHI	i.E	cyt	1.1495	1.0263	0.9603	1.1208	1.0640	1.0738
A2XRZ0	Probable aldo-keto reductase 2	AKR2	i.E	cyt	1.2536	1.0856	0.9877	1.6419	1.1245	0.5930
A2XV50	6,7-dimethyl-8-ribityllumazine synthase	RIB	i.E	cyt	1.0241	0.9670	1.0373	0.9080	0.9335	0.8245
B8AC38	Aldo-keto reductase	AKR2	i.E	cyt	0.9683	0.9115	0.8600	0.9315	0.8387	0.7645
B8B0P6	Purple acid phosphatase	PAP	i.E	cyt	1.1454	1.0594	0.9852	1.0300	1.3167	1.2892
B8B930	Purple acid phosphatase	PAP	i.E	cyt	1.0568	0.9594	0.9437	1.0613	0.9787	0.9841
Q38G74	Cinnamyl-alcohol dehydrogenase	CAD	i.E	cyt	0.9755	1.0370	1.0479	1.1098	1.1346	0.9838
A2WMY5	Importin subunit alpha	Imp-α	ii.A	cyt	0.9929	1.0566	1.1120	1.0834	1.0644	1.0707

A2WYE1	Small ubiquitin-related modifier (SUMO)	SUMO	ii.A	cyt	1.0101	0.9828	0.9370	0.9842	1.0565	0.9276
A2X9Q4	Annexin	Annexin	ii.A	cyt	0.9910	0.9767	1.0243	0.9195	0.9117	0.9242
A2XHR5	Trafficking protein particle complex subunit	TRAPPC	ii.A	cyt	1.1078	1.2138	1.2740	0.8156	0.8442	1.2885
A2XLA1	Coatomer subunit alpha	COP-α	ii.A	cyt	0.8807	0.9811	1.1441	0.8571	0.9817	0.9429
A2Y963	Coatomer subunit beta'	COP-β'	ii.A	cyt	0.9438	0.9667	1.1613	1.0192	1.0047	0.9833
A2YAT3	Annexin	Annexin	ii.A	cyt	1.0772	1.1333	1.1471	0.8427	0.8254	0.8482
A2YYM7	Coatomer subunit alpha	COP-α	ii.A	cyt	0.8894	0.9634	1.0637	1.1006	1.0557	0.9318
A2ZAH5	ABA receptor 9	ABAr9	ii.A	cyt	1.0372	1.0312	0.9918	1.0455	0.9307	0.9893
A2ZH90	Clathrin heavy chain	CLTC	ii.A	cyt	0.9795	0.9935	1.0839	0.9445	0.8803	1.0923
A6N0I0	14-3-3-like protein	GF14	ii.A	cyt	0.9658	1.0396	0.9690	0.9403	1.0642	1.2322
A6N1G1	Cathepsin b-like cysteine proteinase 3	CATb	ii.A	cyt	0.9432	0.8843	0.8406	1.0172	1.0288	1.0251
B8ADW8	Coatomer subunit beta'	COP-β'	ii.A	cyt	0.8839	0.9361	0.9685	1.2052	1.0657	0.8955
B8APS8	Beta-adaptin-like protein (clathrin)	BETA-AD	ii.A	cyt	0.9875	1.0862	1.0719	1.0154	0.9001	0.9057
B8ARW2	Transportin-1	TRN1	ii.A	cyt	1.0973	1.1727	0.9715	0.9995	0.9747	1.0102
B8AU46	Coatomer subunit epsilon	COP-ε	ii.A	cyt	0.7264	0.7181	0.7971	0.9950	1.0217	1.1180
B8AY77	Importin subunit alpha	Imp-α	ii.A	cyt	1.0845	1.0966	0.9318	1.0016	1.0623	0.9188
B8B874	Coatomer subunit gamma	COP-ɣ	ii.A	cyt	0.9195	1.0051	1.0482	1.0041	0.9270	0.9904

B8BF46	Annexin	Annexin	ii.A	cyt	1.1248	1.0404	0.9597	0.9360	0.9443	1.0424
B8BJD1	Coatomer subunit beta (Beta-coat protein)	COP-ß	ii.A	cyt	0.8829	1.0179	1.1063	0.9852	0.8248	0.8230
A2WWR4	Histone H4	H4	ii.B	nuc	0.6007	0.7926	0.7109	1.0630	1.0554	0.8784
A2WWS5	RuvB-like helicase	rvb	ii.B	cyt	0.9410	0.9861	1.0424	1.0274	0.9913	0.9890
A2X2N6	40S ribosomal protein S24	rpS24	ii.B	cyt	0.6985	0.7984	1.1169	1.0417	0.9922	1.0191
A2X3I0	60S ribosomal protein L27	rpL27	ii.B	cyt	0.7032	0.8863	1.1663	0.9026	0.9532	0.9544
A2X4T7	40S ribosomal protein S27	rpS27	ii.B	cyt	0.9767	0.9432	1.1909	1.1214	0.9891	1.0160
A2X6N1	60S ribosomal protein L6	rpL6	ii.B	cyt	0.7257	0.9223	1.1127	1.2136	1.1954	1.3223
A2XCU2	Histone H2A	H2A	ii.B	cyt	0.8164	0.6877	0.6269	0.9528	0.9923	0.9183
A2XD89	40S ribosomal protein SA	rpSA	ii.B	cyt	0.7329	0.8638	1.0651	0.9768	0.8670	1.1657
A2XIT5	60S ribosomal protein L13	rpL13	ii.B	cyt	0.6115	0.7700	1.0503	1.0171	1.0441	1.2153
A2XJ89	Ribosomal protein L15	rpL15	ii.B	cyt	0.6430	0.8887	1.1631	0.9152	0.9626	0.8887
A2XP46	Elongation factor Tu	EF-Tu	ii.B	cyt	1.0162	0.9906	0.9999	1.0444	1.0624	0.8692
A2XZF9	Eukaryotic translation initiation factor 3 subunit F	eIF3f	ii.B	cyt	1.0201	0.9383	0.9856	1.2267	1.4130	1.4477
A2Y0K0	60S ribosomal protein l18	rpL18	ii.B	cyt	0.7709	1.0244	1.3779	0.9251	0.9572	1.0787
A2Y7H9	60S ribosomal protein L18a	rpL18a	ii.B	cyt	0.7378	0.8779	1.0598	1.0392	0.9896	0.9759

A2Y7J4	Eukaryotic translation initiation factor 3 subunit D	eIF3d	ii.B	cyt	1.0849	1.0810	1.1218	0.9515	1.0132	1.0299
A2Y7R5	GTP-binding nuclear protein Ran-2	Ran2	ii.B	cyt	1.0383	0.9491	0.9343	0.8443	0.9911	0.8372
A2YJM2	40S ribosomal protein S12	rpS12	ii.B	cyt	0.9178	0.8822	1.0311	0.8215	0.9149	0.9943
A2YNQ0	40S ribosomal protein SA	rpSA	ii.B	cyt	0.8796	0.9394	0.9820	1.0281	0.9896	0.8436
A2YPM1	Elongation factor beta-1	EF-β1	ii.B	cyt	0.9583	0.9803	0.9285	0.9768	1.0557	1.1234
A2YYH2	Polyadenylate-binding protein	PABP	ii.B	cyt	1.0927	1.0641	1.1266	0.9518	1.0380	1.0267
A2Z2J3	60S ribosomal protein l9	rpl9	ii.B	cyt	0.9452	0.9835	1.2233	1.0202	0.9501	0.9648
A2ZB00	40S ribosomal protein S16	rpS16	ii.B	cyt	0.6975	0.9264	1.0555	1.0134	0.9718	0.8935
A2ZB54	60S acidic ribosomal protein P0	rbP0	ii.B	cyt	0.7699	0.8585	1.1378	1.0961	1.0367	0.9449
A2ZFU3	40S ribosomal protein S9	rpS9	ii.B	cyt	0.6299	0.8768	1.0415	1.0089	0.9508	0.9782
A6MZE1	60S ribosomal protein L36	rpL36	ii.B	cyt	0.8106	0.9306	1.1523	0.8477	0.9863	0.8860
A6MZM7	40S ribosomal protein S14	rpS14	ii.B	cyt	0.9298	0.9559	1.0875	0.9619	1.0434	0.9466
A6MZN1	60S acidic ribosomal protein p2b	rbp2b	ii.B	cyt	0.7375	0.8057	0.9199	0.8294	0.7934	0.9107
A6MZQ0	40S ribosomal protein S26	rpS26	ii.B	cyt	0.8079	0.9412	1.0263	0.9261	0.9847	0.9329

A6N028	Eukaryotic translation initiation factor 5A	eIF-5A	ii.B	cyt	0.8954	0.9499	0.9166	0.9721	0.9206	0.9700
A6N0K7	60S ribosomal protein l44	rpl44	ii.B	cyt	0.7824	1.5408	1.0121	0.8574	0.8994	1.0758
A6N1I6	60S ribosomal protein l35a	rpl35a	ii.B	cyt	0.7110	0.7971	1.0970	1.0350	0.9739	0.9802
B8AA60	Signal recognition particle 54 kDa protein	SRP54	ii.B	cyt	0.9203	0.9905	0.9690	0.9583	0.9736	0.9561
B8ACZ5	Eukaryotic translation initiation factor 3 subunit A	eIF3a	ii.B	cyt	0.9075	0.9212	0.8612	0.8906	0.9330	0.9380
B8AE20	Eukaryotic translation initiation factor 3 subunit G	eIF3g	ii.B	cyt	1.3205	1.1085	1.1546	1.0031	0.9634	1.0519
B8AEQ9	Elongation factor Tu	EF-Tu	ii.B	cyt	0.8852	0.8834	1.1658	1.0155	0.8158	0.8253
B8APM5	Elongation factor 1-alpha	EF-1α	ii.B	cyt	0.9657	0.9200	0.9473	0.9722	0.9702	0.9746
B8AT05	Eukaryotic translation initiation factor 3 subunit H	eIF3h	ii.B	cyt	0.9393	1.0618	1.0536	1.0914	1.1476	1.0079
B8ATY5	Eukaryotic translation initiation factor 3 subunit M	eIF3m	ii.B	cyt	0.9670	0.8787	0.8933	0.8723	0.8713	0.9782
B8AVI5	60S ribosomal protein L6	rpL6	ii.B	cyt	0.7169	0.9302	1.2033	0.8677	0.8863	0.8355
B8AZD7	Eukaryotic translation initiation factor 3 subunit L	eIF3L	ii.B	cyt	1.0602	1.1889	1.0770	0.9894	1.0692	0.8872

B8B3R2	RuvB-like helicase	rvb	ii.B	cyt	1.0992	1.0341	1.0392	1.1035	1.0278	1.0407
B8B833	Eukaryotic translation initiation factor 5A	eIF-5A	ii.B	cyt	1.0308	1.0219	0.9366	1.1320	0.9498	1.0120
B8B9P9	Ribonucleoprotein-like	RNP	ii.B	cyt	1.0674	0.9640	0.9018	1.0558	0.8745	0.8964
B8BAI9	Elongation factor Ts	EF-Ts	ii.B	mit	1.0566	1.0326	0.9325	0.8033	0.7870	0.8745
B8BIC4	Eukaryotic translation initiation factor 3 subunit B	eIF-3b	ii.B	cyt	1.0452	1.0719	0.9768	0.8645	0.6964	0.8329
Q1MSJ3	Putative eukaryotic translation initiation factor 4 gamma	eIF-4ɣ	ii.B	cyt	0.9301	0.8995	0.9810	0.9639	0.8869	0.8438
B8AT03	23S ribosomal protein L29	rpL29	ii.B	cyt	0.7330	0.8385	1.0759	1.1309	0.9704	1.2073
A2XM46	60S ribosomal protein L13a	rpL13a	ii.B	cyt	0.6698	0.9240	1.1368	0.9481	1.0416	0.9491
A2X8M1	60S ribosomal protein L12	rpL12	ii.B	cyt	0.8801	1.0196	1.2356	0.9444	0.8387	0.8272
A2XA20	Proteasome subunit beta type	SUβ-prot	ii.C	cyt	1.0285	1.0140	0.9933	0.9749	1.0149	0.8669
A2XD75	Proteasome subunit alpha type	SUα-prot	ii.C	cyt	1.0080	0.9844	0.9864	0.9362	0.9627	1.0945
A2XKY8	Proteasome subunit beta type	SUβ-prot	ii.C	cyt	0.9979	1.0013	0.9856	1.1400	0.8838	0.8513
A2Y157	Proteasome subunit beta type	SUβ-prot	ii.C	cyt	1.0710	1.0034	1.0320	0.9009	0.8680	0.9542

A2Y9X7	Proteasome subunit alpha type-4-1	SUα4-1-prot	ii.C	cyt	1.0655	0.9835	0.9984	1.0846	1.0095	1.0104
A2YXU2	Proteasome subunit alpha type-7-A	SUα7-A-prot	ii.C	cyt	1.0139	1.1062	1.1085	1.0773	1.0110	0.9297
A2Z3I9	Proteasome subunit alpha type-7-B	SUα7-B-prot	ii.C	cyt	1.1032	1.1753	1.2433	0.9071	1.0020	0.9890
B8AB88	Proteasome subunit alpha type	SUα-prot	ii.C	cyt	0.9493	0.9338	1.0383	1.0083	1.0749	0.9599
B8AFT1	Proteasome subunit alpha type	SUα-prot	ii.C	cyt	1.2390	1.4158	1.3397	1.1072	1.0865	0.9354
B8AHG2	Proteasome subunit alpha type	SUα-prot	ii.C	cyt	0.9879	0.9832	1.0600	0.8745	0.8709	0.9415
B8B0S6	Proteasome subunit beta type	SUβ-prot	ii.C	cyt	0.9723	0.8472	0.9670	1.0280	0.9701	0.9872
B8B2Z5	Proteasome subunit beta type	SUβ-prot	ii.C	cyt	0.8876	0.9784	0.9917	1.1258	1.0267	1.0872
B8BDI8	Proteasome subunit beta	SUβ-prot	ii.C	cyt	0.9600	0.9522	0.9282	1.0310	0.9926	0.9436
B8BLI9	Proteasome subunit alpha type	SUα-prot	ii.C	cyt	1.0033	0.9514	0.9985	1.0142	1.2861	1.1158
A2WMG6	Salt stress root protein RS1	RS1	iii.A	cyt	1.0192	0.9066	0.9932	1.0112	0.6420	0.6670
A2WPN7	Salt stress-induced protein (SalT protein)	SALT	iii.A	cyt	0.7664	1.1933	0.7243	1.0445	1.0378	1.0473
A2XN74	Dirigent protein	DirP	iii.A	cyt	1.1939	1.2451	1.1715	1.0409	0.9628	1.0640
A2ZC27	Dirigent protein	DirP	iii.A	cyt	1.0354	0.9906	0.9368	0.9100	0.9223	1.0372

A2ZC38	Dirigent protein	DirP	iii.A	cyt	0.9874	0.8678	0.9744	1.0296	1.0236	1.0557
A6N0H8	Abscisic stress ripening protein 2	ASR2	iii.A	cyt	1.3996	0.9679	1.1373	1.2790	1.2585	1.3629
A6N1B9	Cbs domain protein	Cbs	iii.A	cyt	1.0788	0.9548	0.9367	1.0285	1.1045	1.0680
B8B6K1	Dirigent protein	DirP	iii.A	cyt	1.1274	1.0882	0.8754	1.2116	1.3002	1.1165
A2WN51	Peroxidase	PDX	iii.B	cyt	1.0418	0.9871	0.8969	0.9533	0.9654	0.9317
A2WNR8	Peroxidase	PDX	iii.B	cyt	1.1973	1.0780	1.0148	0.9604	1.1717	0.9583
A2WPA9	Peroxidase	PDX	iii.B	cyt	1.0479	1.1124	1.0640	0.9464	0.9395	1.0628
A2WR43	Peroxidase	PDX	iii.B	cyt	1.1668	0.9679	0.9012	1.0573	1.1130	1.0168
A2X2T0	Peroxidase	PDX	iii.B	cyt	1.0185	0.9455	0.9350	1.0171	0.9577	1.0130
A2X822	Glutathione peroxidase	GPx	iii.B	cyt	0.9899	0.9268	0.8647	1.1012	0.9874	0.9768
A2XFC7	L-ascorbate peroxidase 1	APX1	iii.B	cyt	0.9529	0.8132	0.7916	0.9461	0.9118	1.0232
A2XGP6	Superoxide dismutase [Cu-Zn] 1	SOD	iii.B	cyt	1.1247	1.0582	1.0379	1.2601	1.4163	0.9993
A2XH53	Peroxidase	PDX	iii.B	cyt	1.0323	1.1593	1.2313	0.7991	0.8662	0.8250
A2XH55	Peroxidase	PDX	iii.B	cyt	1.1614	1.1137	1.1089	1.0071	0.9381	1.0023
A2XIK7	Peroxidase	PDX	iii.B	cyt	1.1517	1.0736	1.1736	1.0596	1.0428	0.9575
A2XM89	Peroxidase	PDX	iii.B	cyt	1.1746	1.3186	1.1810	0.9725	0.9247	0.9229
A2XTH3	Peroxidase	PDX	iii.B	cyt	1.1518	1.0271	0.9181	0.8788	0.9609	0.9226
A2XZ79	Peroxidase	PDX	iii.B	cyt	1.0096	0.9974	1.0694	1.0157	0.8752	0.9075
A2Y043	Peroxidase	PDX	iii.B	cyt	1.4090	1.1702	1.0501	0.9341	1.0183	1.0154
A2Y044	Peroxidase	PDX	iii.B	cyt	1.1334	1.1015	0.9657	1.0407	0.9595	0.9801

A2Y0P6	Peroxidase	PDX	iii.B	cyt	1.0023	1.0271	1.0268	1.1092	1.0589	1.1804
A2Y667	Peroxidase	PDX	iii.B	cyt	1.3955	1.1906	0.8379	0.9802	0.9260	1.0971
A2YDW8	Peroxidase	PDX	iii.B	cyt	0.9730	1.0914	0.9913	0.9904	0.9281	1.0852
A2YGK4	Peroxidase	PDX	iii.B	cyt	1.2487	1.0708	1.0955	0.9889	0.9243	0.8535
A2YH64	Catalase isozyme B	CAT-B	iii.B	cyt	1.0358	1.0489	0.9537	1.2039	1.0573	1.0302
A2YHB9	Peroxidase	PDX	iii.B	cyt	1.1240	1.0153	0.8562	0.9840	0.8777	0.9196
A2YIW7	Thioredoxin H-type	Trx-H	iii.B	cyt	1.0419	0.9523	0.8901	0.8925	0.9997	0.9296
A2YPX2	Peroxidase	PDX	iii.B	cyt	1.1091	1.0002	0.8962	1.0318	1.0546	0.9919
A2Z4F1	Peroxidase	PDX	iii.B	cyt	1.2499	1.3207	1.1490	0.9492	0.9920	0.9622
A2Z7B3	GDP-mannose 3,5-epimerase 1	GME-1	iii.B	cyt	0.8843	1.0934	1.0675	0.9396	0.9618	1.0052
A3REN3	Catalase	CAT	iii.B	cyt	0.8987	0.8923	0.9064	0.9428	0.9657	0.9008
A6N0E3	Glutathione s-transferase gstf2	GSTF2	iii.B	cyt	0.9953	1.0336	1.0258	1.0862	0.9226	0.9519
B8AHL2	Thioredoxin reductase	TrxR	iii.B	cyt	0.9122	0.9474	0.8560	1.0913	1.0451	1.0419
B8ALD1	Thioredoxin	Trx	iii.B	cyt	0.9791	1.1370	1.0240	1.0169	1.0224	0.9825
B8ARU3	Peroxidase	PDX	iii.B	cyt	1.0242	1.0106	0.8978	0.9469	1.0246	0.8349
B8ARU5	Peroxidase	PDX	iii.B	cyt	1.2004	1.1910	1.0984	1.1784	1.0485	1.0461
B8AUI3	Peroxisomal (S)-2-hydroxy-acid oxidase	GLO3	iii.B	cyt	0.8534	0.9496	1.0339	1.0062	0.9379	0.9189
B8AWM4	Superoxide dismutase	SOD	iii.B	cyt	1.0676	0.9115	0.9089	0.9529	0.9715	0.9181
B8AZE9	Glutathione synthetase	GSH	iii.B	cyt	1.1394	1.0415	1.1177	0.9534	0.9821	1.0693
B8AZS6	Thioredoxin	TRX	iii.B	cyt	1.1229	1.0733	1.0671	0.9977	1.0007	1.0365

B8B3L5	Peroxidase	PDX	iii.B	cyt	1.1960	1.0496	0.8354	0.9291	0.9225	0.9527
B8B5W6	Peroxidase	PDX	iii.B	cyt	1.0918	1.0745	0.9132	1.0355	1.0074	0.9804
B8B8K5	Peroxisomal (S)-2-hydroxy-acid oxidase	GLO4	iii.B	cyt	0.9906	0.9763	1.0290	0.9531	0.9908	1.0126
B8BA60	Peroxidase	PDX	iii.B	cyt	1.0947	0.9312	0.8814	0.9863	0.9750	0.8672
P0C5D0	1-Cys peroxiredoxin B	PRDX-B	iii.B	cyt	0.8352	0.7730	0.6252	1.1105	1.0854	1.1127
Q6WSC2	Glutathione S-transferase	GST-3	iii.B	cyt	0.8639	0.8116	0.6027	1.0224	1.0566	1.0410
A2YXU4	Ascorbate peroxidase	APX	iii.B	cyt	0.6733	0.6455	0.6960	1.1678	0.9862	1.0009
A2WMH2	Protein GOS9	GOS9	iii.C	cyt	0.8645	0.8544	0.8814	0.8858	0.9337	0.9417
A2X9U8	Peptidylprolyl isomerase	PPI	iii.C	cyt	1.0491	0.8603	0.9325	1.0285	0.9856	0.9765
A2XB12	Tubulin-specific chaperone A	chpA	iii.C	cyt	1.1798	1.2232	1.1507	0.9725	1.0027	1.1233
A2XZE6	Peptidyl-prolyl cis-trans isomerase	PPI	iii.C	cyt	0.9988	1.0316	1.0013	1.2473	1.1779	0.9409
A2YGV2	Peptidyl-prolyl cis-trans isomerase	PPI	iii.C	cyt	1.0724	1.1084	1.0217	0.9752	0.9800	0.9404
A2YUU5	T-complex protein 1 subunit delta	TCP1-Δ	iii.C	cyt	1.0632	0.9831	1.0839	0.9981	1.0351	1.0487
A2YWQ1	Heat shock protein 81-1	HSP81-1	iii.C	cyt	0.9442	0.8712	0.9692	1.1881	1.0983	0.9575
A2Z498	Peptidyl-prolyl cis-trans isomerase	PPI	iii.C	cyt	0.9914	0.9566	0.8900	0.6730	0.7813	0.7771
A2ZCE6	Protein disulfide-isomerase	PDI	iii.C	cyt	0.9168	0.9411	0.9811	1.4659	1.0635	1.0620

A6N0I3	Chaperonin	chp	iii.C	cyt	1.1813	1.0068	1.0472	1.1411	1.1335	0.9616
A6N0N5	Prefoldin subunit 5	Psu5	iii.C	cyt	0.9021	0.9151	0.9508	1.1847	1.1542	1.0711
A8QXH4	Putative chaperonin 60 beta	chp60	iii.C	cyt	0.9659	0.9138	1.0831	1.1323	1.1103	0.9650
B8ACM2	Peptidyl-prolyl cis-trans isomerase	PPI	iii.C	cyt	0.9889	0.9798	0.9023	0.9382	0.9515	1.2090
B8AGU2	Protein disulfide-isomerase	PDI	iii.C	cyt	1.0258	0.9428	1.0424	0.9411	0.9872	1.1968
B8B3C8	T-complex protein 1 subunit gamma	TCP1-ɣ	iii.C	cyt	0.9192	0.9799	0.9871	0.9274	1.0107	1.2876
B8B8V4	Peptidylprolyl isomerase	PPI	iii.C	cyt	0.9766	1.0505	1.0783	0.9860	0.9640	0.9545
Q1KL27	Peptidyl-prolyl cis-trans isomerase	PPI	iii.C	cyt	1.0999	0.9927	0.8535	1.0321	1.0087	0.8644
A0A0E4G0P6	Developmentally regulated plasma membrane polypeptide	PMpoly	iv	cyt	1.0239	0.9804	1.2680	2.0741	0.8422	0.9593
A2XKB0	Tubulin beta chain	TU-β	iv	cyt	1.0510	1.1386	1.2269	1.0244	1.0385	1.1263
A2XLF2	Actin-1	Actin-1	iv	cyt	1.0184	1.0388	1.0886	1.0260	1.0702	0.9144
A2XMI9	Tubulin beta chain	TU-β	iv	cyt	0.9944	1.0292	1.1613	1.0673	1.0411	0.9810
A2XNS1	Actin-3	Actin-3	iv	cyt	1.1286	1.0412	1.1413	0.8651	1.0787	0.9283
A2YG29	Tubulin beta chain	TU-β	iv	cyt	0.8791	0.9727	0.9984	0.8867	0.9533	1.0044
A2YMX5	Tubulin alpha chain	TU-α	iv	cyt	0.7905	0.9850	1.4141	0.9901	0.9913	1.0615
B8ALG6	Tubulin beta chain	TU-β	iv	cyt	0.9555	0.9154	1.2014	0.9939	0.9888	0.8531
B8AQQ8	Adenylyl cyclase-associated protein	CAP	iv	cyt	0.9601	0.9572	0.9430	1.0464	0.8475	0.9126

B8B8G2	Tubulin alpha chain	TU-α	iv	cyt	1.0744	0.9807	1.0845	1.1134	1.0633	1.0562
P0C542	Actin-7	Actin-7	iv	cyt	1.0240	1.0603	1.0076	1.2159	1.2209	1.0085
P83647	Profilin LP04	LP04	iv	cyt	1.1609	1.0764	0.9701	1.0278	0.9506	1.1240
A0A0A7EQF3	Cysteine proteinase inhibitor	CPIn	v	cyt	0.9990	0.9171	0.8234	0.9801	0.9214	0.9582
A2XAR1	Proliferating cell nuclear antigen	PCNA	v	cyt	1.0249	1.0438	1.0812	0.9730	0.9574	1.3497
A2XS65	Cysteine proteinase inhibitor 10	CPIn	v	cyt	1.2658	0.9808	0.8198	1.0384	1.0490	1.0278
A2XYW4	Anamorsin homolog 1	ANA1	v	cyt	0.9408	0.9375	1.0306	0.9773	0.8965	1.1078
A2Y628	Cysteine proteinase inhibitor	CPIn	v	cyt	1.0538	0.9127	0.9556	1.1158	1.2408	1.1584
A2YTW2	Fasciclin-like arabinogalactan-protein-like	FAS-1	v	cyt	0.9943	1.0412	1.0180	1.1387	1.0432	1.1727
A6MZH2	Bowman-Birk type bran trypsin inhibitor	BBI	v	cyt	1.0947	1.0529	0.9709	1.2266	1.6619	1.0908
B8ARI7	Anamorsin homolog 2	ANA2	v	cyt	0.9543	1.0852	1.1843	0.7906	0.8705	1.1974

Table SM.1.5. List of the proteins with significant abundance changes (p-value<0.05) and low false discovery rate (q-value<0.15) in shoots and roots of FL478. 100/0 mM NaCl: treatment/mock ratio.

Shoot or Root	Protein Accession	Abbreviation	Protein name	100/0 mM NaCl	p-value	q-value
Shoot	B8B1K3	6PGD	6-phosphogluconate dehydrogenase	0.767	0.000	0.014
Shoot	A2YRI2	ACX	Acyl CoA binding protein	0.147	0.013	0.147
Shoot	A2Z3K3	ACYprot	Acyl carrier protein	0.666	0.013	0.147
Shoot	A2ZDQ6	ADK	Adenylate kinase	0.488	0.003	0.056
Shoot	B8AEU9	AKR	Aldo-keto reductase	0.634	0.002	0.043
Shoot	B8BKT7	α-Man	Alpha-mannosidase	1.144	0.002	0.043
Shoot	B8BF46	Annexin	Annexin	0.763	0.000	0.009
Shoot	A2WLL0	Apase	Plant acid phosphatase	0.758	0.007	0.090
Shoot	A2YXU4	APX	Ascorbate peroxidase	0.850	0.004	0.067
Shoot	A2WVA6	AST	Aspartate aminotransferase	1.268	0.006	0.087
Shoot	A2YMB7	β-Amy	Beta-amylase	0.644	0.005	0.085
Shoot	B3VMC0	BADH2	Betaine aldehyde dehydrogenase 2	0.932	0.013	0.147
Shoot	A2Z5P1	CAD	Cinnamyl alcohol dehydrogenase	0.718	0.001	0.030
Shoot	A2X854	DHN	Plant dehydrin family	2.062	0.002	0.040
Shoot	A2Z0L8	Enolase	Enolase	0.678	0.001	0.027
Shoot	A2ZBX1	FBA	Fructose-bisphosphate aldolase	0.798	0.012	0.145
Shoot	B8B9S4	GATase	Glutamine amidotransferase	0.653	0.000	0.009
Shoot	Q8GU95	GCS-A	Glutamate-cysteine ligase A	0.702	0.007	0.092
Shoot	A6N1F8	ipiAt1	Isopentenyl pyrophosphate: dimethyllallyl pyrophosphate isomerase	0.650	0.012	0.139

Shoot	A2ZD01	LHC	Chlorophyll a-b binding protein	0.892	0.004	0.067
Shoot	A2XIZ4	LysRS	Lysine-tRNA ligase	0.775	0.006	0.090
Shoot	A2ZCE6	PDI	Protein disulfide-isomerase	0.832	0.005	0.079
Shoot	B8A748	PE	Pectinesterase	1.183	0.000	0.006
Shoot	A2X9Q0	PLAT	PLAT_plant_stress	1.270	0.006	0.087
Shoot	A2Y043	PRX	Peroxidase	0.628	0.000	0.009
Shoot	B8ARU5	PRX	Peroxidase	0.651	0.006	0.087
Shoot	B8ANZ3	Psb27	photosystem II protein Psb27	0.615	0.006	0.087
Shoot	B8AAX3	PsbP	PsbP family	1.300	0.001	0.019
Shoot	P83646	psbQ	Oxygen-evolving enhancer protein 3	0.236	0.002	0.050
Shoot	A2Y7L1	rpL12	ribosomal protein L12	0.624	0.002	0.041
Shoot	A2X4T7	rpS27	40S ribosomal protein S27	1.340	0.010	0.124
Shoot	B8AEE7	rpS30	40S ribosomal protein S30	1.994	0.000	0.002
Shoot	A2WPV4	rpS4	40S ribosomal protein S4	1.420	0.001	0.019
Shoot	A2YNT9	rpS6	40S ribosomal protein S6	1.539	0.002	0.041
Shoot	A2XN63	rpuS5	Universal ribosomal protein uS5 family	2.427	0.000	0.009
Shoot	A2WMG6	RS1	Salt stress root protein RS1	0.495	0.007	0.092
Shoot	A2WPH1	Sar1	Sar1	0.708	0.012	0.145
Shoot	B8B7R8	SCS-α	Succinate-CoA ligase [ADP-forming] subunit alpha	1.260	0.002	0.050
Shoot	B8B7P6	Spiso	Sugar Phosphate Isomerase family	0.362	0.000	0.000
Shoot	A2WYE1	SUMO	Small ubiquitin-related modifier (SUMO)	0.620	0.000	0.009
Shoot	B8A8H2	TAL	Transaldolase	0.564	0.000	0.006
Shoot	B8A797	UL	Ubiquitin ligase	0.578	0.003	0.057
Shoot	B8B5T6	XI	Xylose isomerase	0.616	0.000	0.015
Shoot	A2WTQ2	UnkS-1	Unknown shoot protein 1	1.347	0.007	0.092
Shoot	A2X1M1	UnkS-2	Unknown shoot protein 2	0.416	0.000	0.006
Shoot	A2XL95	UnkS-4	Unknown shoot protein 4	0.426	0.001	0.019

Shoot	B8AFM9	UnkS-5	Unknown shoot protein 5	0.868	0.000	0.009
Shoot	B8ATY0	UnkS-6	Unknown shoot protein 6	0.686	0.004	0.075
Shoot	A2XNV4	UnkS-7	Unknown shoot protein 7	1.151	0.001	0.019
Shoot	A2YVG3	UnkS-8	Unknown shoot protein 8	0.430	0.004	0.067
Shoot	A2YXG2	UnkS-9	Unknown shoot protein 9	1.161	0.001	0.026
Shoot	B8AKC9	UnkS-10	Unknown shoot protein 10	1.320	0.003	0.063
Shoot	B8A7L7	UnkS-11	Unknown shoot protein 11	0.682	0.000	0.002
Shoot	B8A7P4	UnkS-12	Unknown shoot protein 12	0.543	0.000	0.010
Shoot	A2X532	UnkS-13	Unknown shoot protein 13	1.515	0.006	0.087
Shoot	B8B2N3	UnkS-14	Unknown shoot protein 14	1.473	0.005	0.085
Shoot	B8BPU4	UnkS-15	Unknown shoot protein 15	0.627	0.002	0.040
Shoot	B8AW91	UnkS-16	Unknown shoot protein 16	0.730	0.009	0.111
Shoot	B8ACJ6	UnkS-17	Unknown shoot protein 17	0.622	0.000	0.006
Shoot	A2WNX6	UnkS-18	Unknown shoot protein 18	0.512	0.006	0.087
Shoot	B8B1T1	UnkS-19	Unknown shoot protein 19	1.153	0.006	0.087
Shoot	A2WPH9	UnkS-20	Unknown shoot protein 20	0.753	0.002	0.050
Shoot	B8AD81	UnkS-21	Unknown shoot protein 21	0.674	0.001	0.021
Shoot	A2YH08	UnkS-22	Unknown shoot protein 22	0.842	0.000	0.006
Shoot	A2ZDZ0	UnkS-23	Unknown shoot protein 23	0.743	0.012	0.145
Shoot	A2Z468	UnkS-24	Unknown shoot protein 24	1.168	0.010	0.124
Shoot	B8BM57	UnkS-25	Unknown shoot protein 25	1.465	0.005	0.085
Shoot	A2YI21	UnkS-26	Unknown shoot protein 26	1.179	0.002	0.050
Shoot	B8AMV2	UnkS-27	Unknown shoot protein 27	1.465	0.000	0.006
Shoot	A2XZP5	UnkS-28	Unknown shoot protein 28	1.122	0.002	0.050
Shoot	A2XNY1	UnkS-29	Unknown shoot protein 29	1.211	0.000	0.006
Shoot	A2XC67	UnkS-30	Unknown shoot protein 30	0.712	0.001	0.026
Shoot	B8AJF3	UnkS-31	Unknown shoot protein 31	0.868	0.003	0.062
Shoot	B8B879	UnkS-32	Unknown shoot protein 32	1.241	0.000	0.004

Shoot	B8AXV2	UnkS-33	Unknown shoot protein 33	1.356	0.003	0.057
Shoot	B8BGQ8	UnkS-34	Unknown shoot protein 34	0.748	0.000	0.002
Shoot	A2XHW8	UnkS-35	Unknown shoot protein 35	0.398	0.003	0.056
Shoot	A2X6G8	UnkS-36	Unknown shoot protein 36	0.426	0.000	0.018
Shoot	B8ADR9	UnkS-37	Unknown shoot protein 37	1.460	0.007	0.094
Shoot	A2XAI5	UnkS-38	Unknown shoot protein 38	1.157	0.005	0.086
Shoot	A2Y041	UnkS-39	Unknown shoot protein 39	0.802	0.006	0.087
Shoot	A2XA10	UnkS-40	Unknown shoot protein 40	0.555	0.000	0.002
Shoot	A2X6L9	UnkS-41	Unknown shoot protein 41	1.203	0.007	0.093
Shoot	B8AGY1	UnkS-42	Unknown shoot protein 42	0.826	0.002	0.040
Shoot	B8BC94	UnkS-43	Unknown shoot protein 43	1.394	0.001	0.034
Root	A2YAT3	Annexin	Annexin	0.837	0.001	0.054
Root	A2Y628	CPIn	Cysteine proteinase inhibitor	1.288	0.003	0.071
Root	A2ZC38	DirP	Dirigent protein	1.274	0.000	0.035
Root	B8BKI7	EPase1	Probable bifunctional methylthioribulose-1-phosphate dehydratase/enolase-phosphatase E1	1.211	0.001	0.038
Root	A2XMM3	FNR	Ferredoxin-NADP reductase	0.872	0.008	0.142
Root	A2YKG1	G6PD	Glucose-6-phosphate 1-dehydrogenase	0.823	0.000	0.031
Root	Q6WSC2	GST-3	Glutathione S-transferase	1.512	0.001	0.059
Root	A6N0E3	GSTF2	Glutathione s-transferase gstf2	0.870	0.004	0.097
Root	A2XCU2	H2A	Histone H2A	1.282	0.003	0.071
Root	A2WWR4	H4	Histone H4	1.684	0.007	0.131
Root	A2XLT7	LOX	Lipoxygenase	0.721	0.000	0.033
Root	A2XL11	LOX	Lipoxygenase	0.774	0.009	0.149
Root	A2XCT8	MTPene	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase 2	1.331	0.005	0.106
Root	A2XFU4	NAS1	Nicotianamine synthase 1	1.529	0.000	0.036
Root	A2XVK1	PAL	Phenylalanine ammonia-lyase	1.241	0.002	0.059

Root	A2XH53	PDX	Peroxidase	0.688	0.006	0.126
Root	A2YGK4	PDX	Peroxidase	0.735	0.003	0.071
Root	A2WR43	PDX	Peroxidase	0.791	0.001	0.056
Root	A2XIK7	PDX	Peroxidase	0.798	0.002	0.060
Root	A2XH55	PDX	Peroxidase	0.811	0.000	0.031
Root	A2XTH3	PDX	Peroxidase	0.821	0.000	0.036
Root	A2YG06	PGK	Phosphoglycerate kinase	1.146	0.002	0.059
Root	Q9LKM2	PLD	Phospholipase D	0.826	0.003	0.071
Root	A0A0E4G0P6	Pmpoly	Developmentally regulated plasma membrane polypeptide	0.859	0.007	0.134
Root	A2XIT5	rpL13	60S ribosomal protein L13	1.375	0.002	0.059
Root	A2XJ89	rpL15	Ribosomal protein L15	1.348	0.002	0.059
Root	A6MZE1	rpL36	60S ribosomal protein L36	1.216	0.003	0.071
Root	A2Z2J3	rpl9	60S ribosomal protein l9	1.179	0.002	0.059
Root	A2YJM2	rpS12	40S ribosomal protein S12	1.226	0.001	0.056
Root	A2XD89	rpSA	40S ribosomal protein SA	1.142	0.001	0.059
Root	A2YSQ3	XTH	Xyloglucan endotransglucosylase/hydrolase	0.808	0.007	0.134
Root	A2WKD0	UnkR-1	Unknown root protein 1	0.884	0.002	0.059
Root	A2X6I6	UnkR-2	Unknown root protein 2	0.816	0.001	0.059
Root	A2X6L9	UnkR-3	Unknown root protein 3	0.905	0.000	0.033
Root	A2X8M1	UnkR-4	Unknown root protein 4	1.289	0.002	0.059
Root	A2XFZ7	UnkR-5	Unknown root protein 5	0.867	0.000	0.035
Root	A2XI59	UnkR-6	Unknown root protein 6	1.138	0.000	0.035
Root	A2XM46	UnkR-7	Unknown root protein 7	1.223	0.001	0.059
Root	A2XZ41	UnkR-8	Unknown root protein 8	0.890	0.001	0.040
Root	A2Y6T4	UnkR-9	Unknown root protein 9	0.678	0.001	0.059
Root	A2Y8H0	UnkR-10	Unknown root protein 10	0.712	0.000	0.036
Root	A2YIM5	UnkR-11	Unknown root protein 11	1.284	0.004	0.098

Root	A2YIS2	UnkR-12	Unknown root protein 12	1.229	0.002	0.061
Root	A2YKM4	UnkR-13	Unknown root protein 13	0.842	0.002	0.059
Root	A2YP40	UnkR-14	Unknown root protein 14	1.133	0.009	0.149
Root	A2YQ14	UnkR-15	Unknown root protein 15	0.864	0.006	0.127
Root	A2YSN9	UnkR-16	Unknown root protein 16	1.142	0.003	0.071
Root	A2YXU4	UnkR-17	Unknown root protein 17	1.419	0.006	0.124
Root	A2Z480	UnkR-18	Unknown root protein 18	1.173	0.002	0.059
Root	A2Z839	UnkR-19	Unknown root protein 19	1.319	0.000	0.033
Root	A2ZA45	UnkR-20	Unknown root protein 20	0.821	0.004	0.092
Root	A2ZC94	UnkR-21	Unknown root protein 21	1.132	0.008	0.149
Root	A2ZEK3	UnkR-22	Unknown root protein 22	1.125	0.001	0.059
Root	A2ZJA6	UnkR-23	Unknown root protein 23	1.176	0.004	0.092
Root	A2ZLU1	UnkR-24	Unknown root protein 24	0.536	0.000	0.033
Root	B8A7W8	UnkR-25	Unknown root protein 25	0.807	0.003	0.071
Root	B8AK21	UnkR-26	Unknown root protein 26	1.172	0.008	0.143
Root	B8APC5	UnkR-27	Unknown root protein 27	1.194	0.007	0.131
Root	B8AQW5	UnkR-28	Unknown root protein 28	0.853	0.002	0.059
Root	B8AT03	UnkR-29	Unknown root protein 29	1.357	0.004	0.092
Root	B8AXS2	UnkR-30	Unknown root protein 30	0.856	0.000	0.035
Root	B8AYD2	UnkR-31	Unknown root protein 31	0.773	0.000	0.035
Root	B8AZE6	UnkR-32	Unknown root protein 32	0.848	0.002	0.059
Root	B8B8R8	UnkR-33	Unknown root protein 33	1.204	0.006	0.117

CHAPTER 2 - Phytohormone profiling method for rice: effects of *GA20ox* mutation on the gibberellin content of Mediterranean *japonica* rice varieties

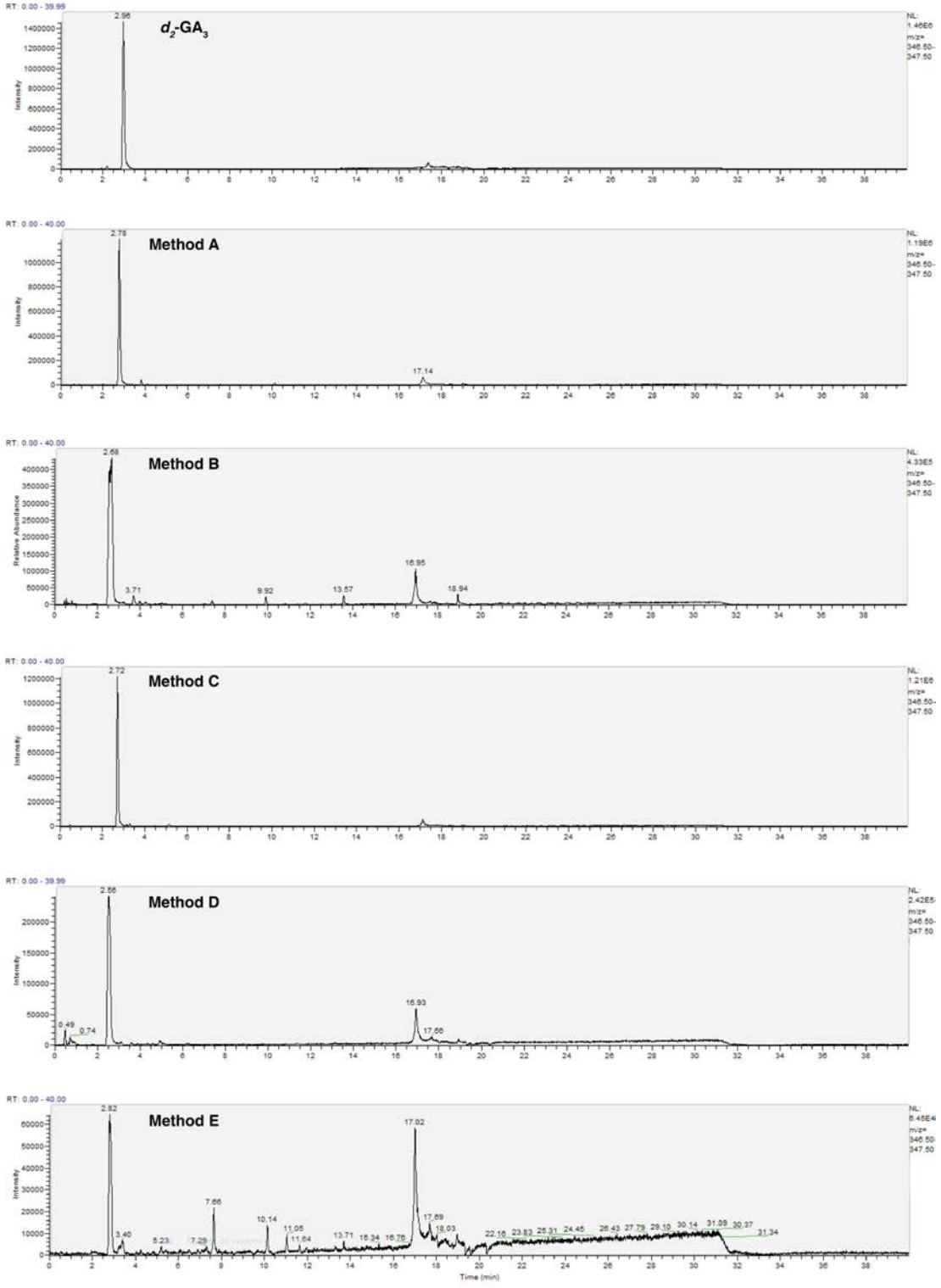


Figure SM.2.1. Trace chromatograms of d_2-GA$_3$ standard, method A, method B, method C, method D and method E for a standard of d_2-GA$_3$ (0.5 mg/L) using method HPLC-1 (LTQ-Orbitrap).

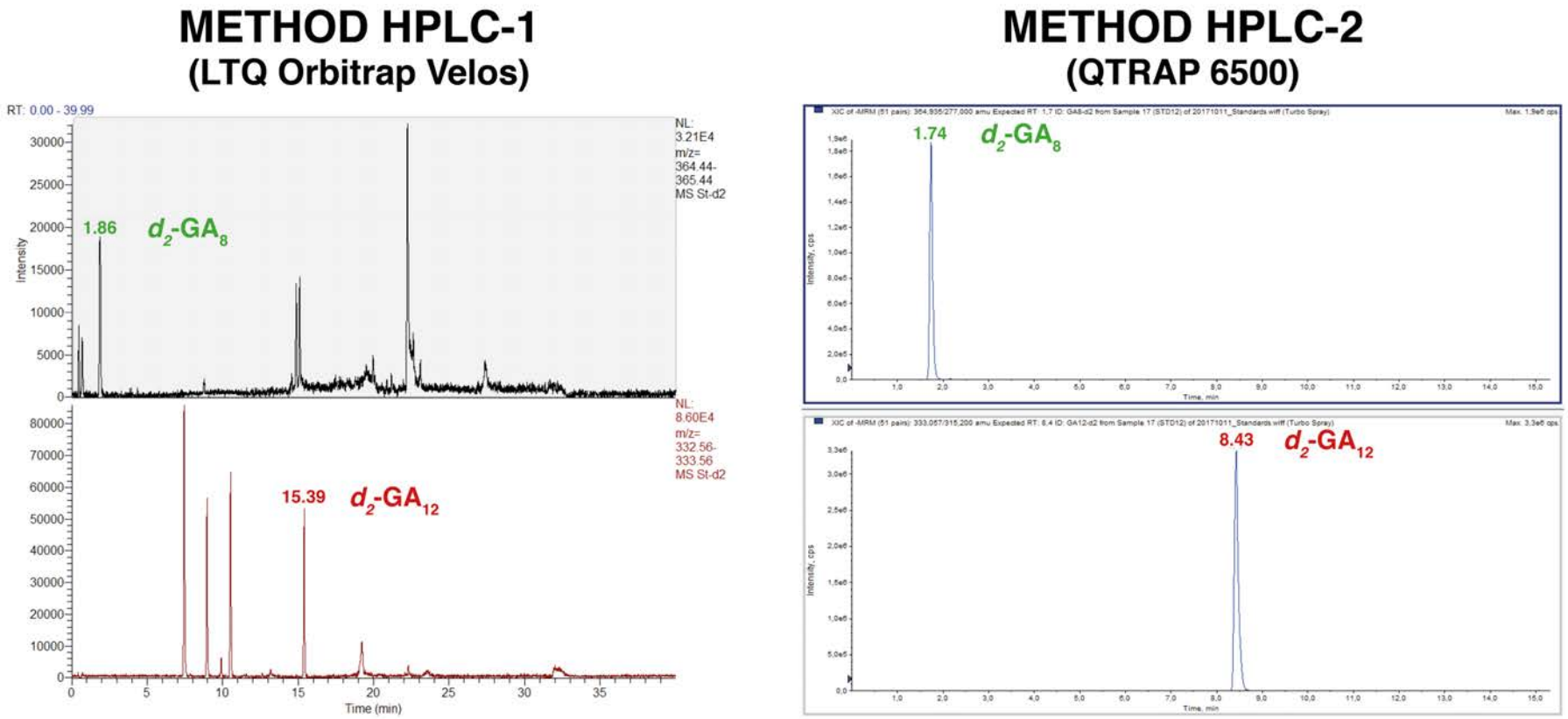


Figure SM.2.2. Trace chromatograms of method HPLC-1 (LTQ-Orbitrap) and HPLC-2 (6500QTRAP) for d_2-GA_8 and d_2-GA_{12}, retention times are shown for each gibberellin deutered form.

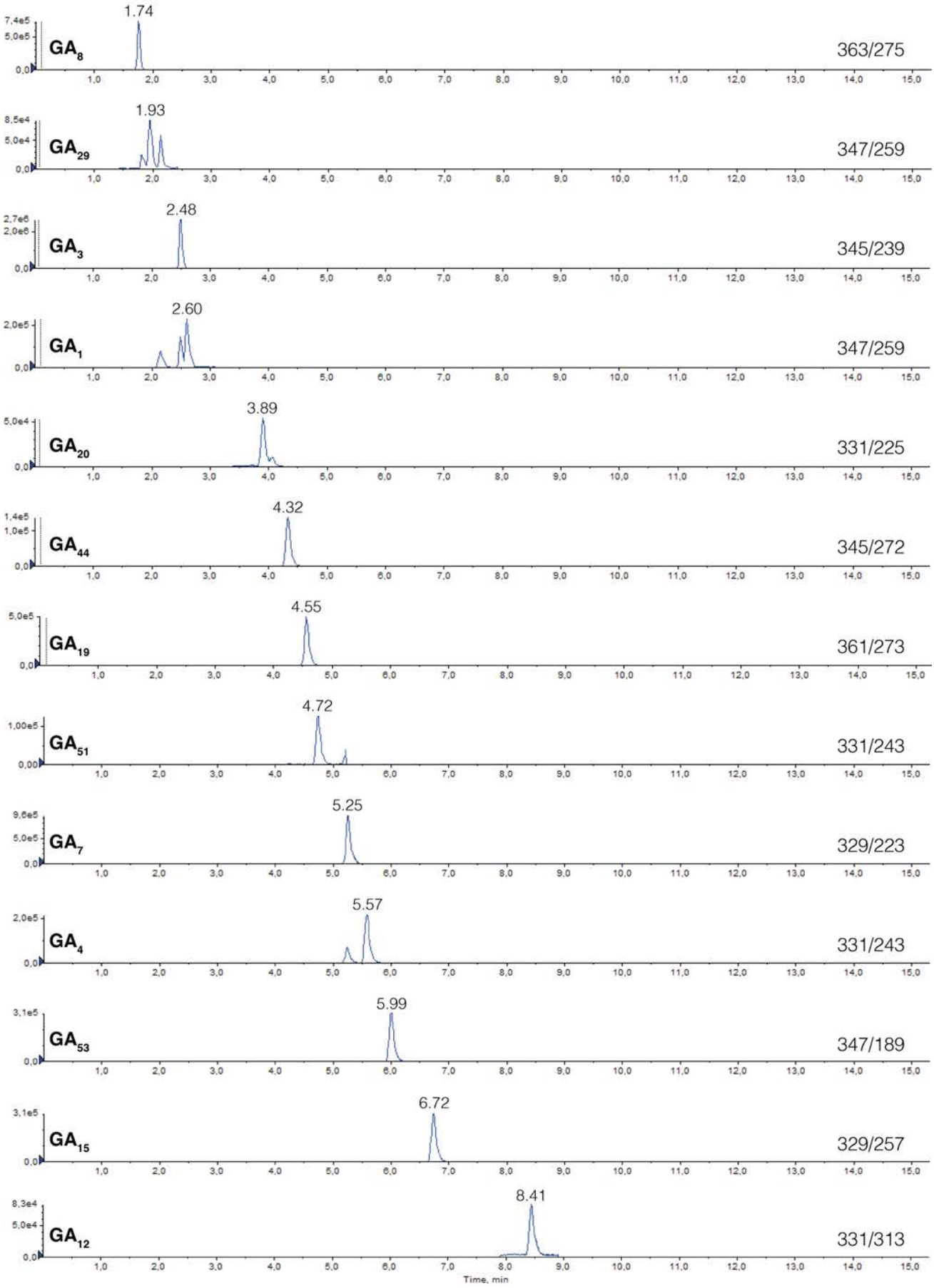


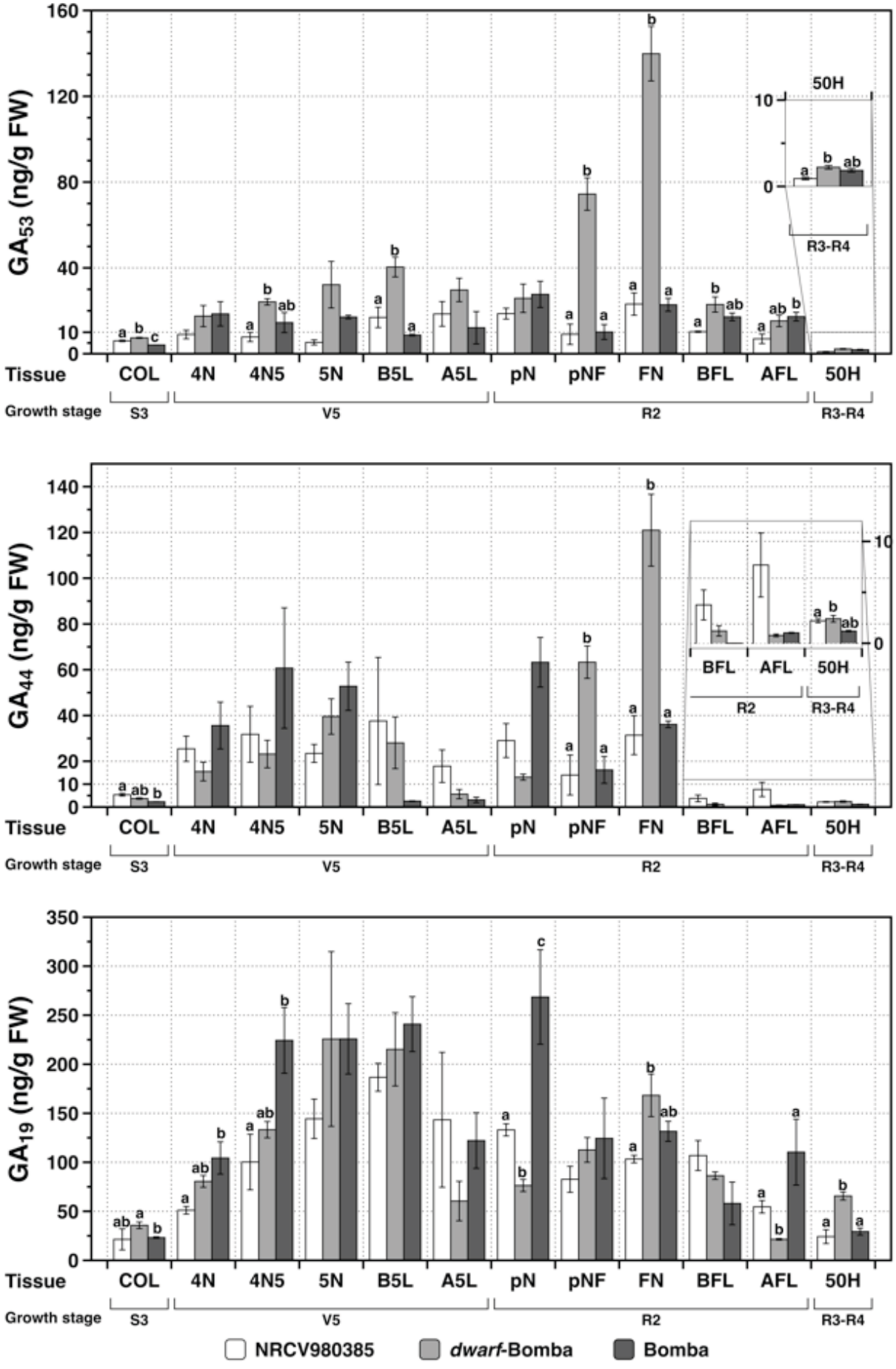
Figure SM.2.3. Trace chromatograms of the 13 detected gibberellins in the flag leaf node (FN) of *dwarf*-Bomba.

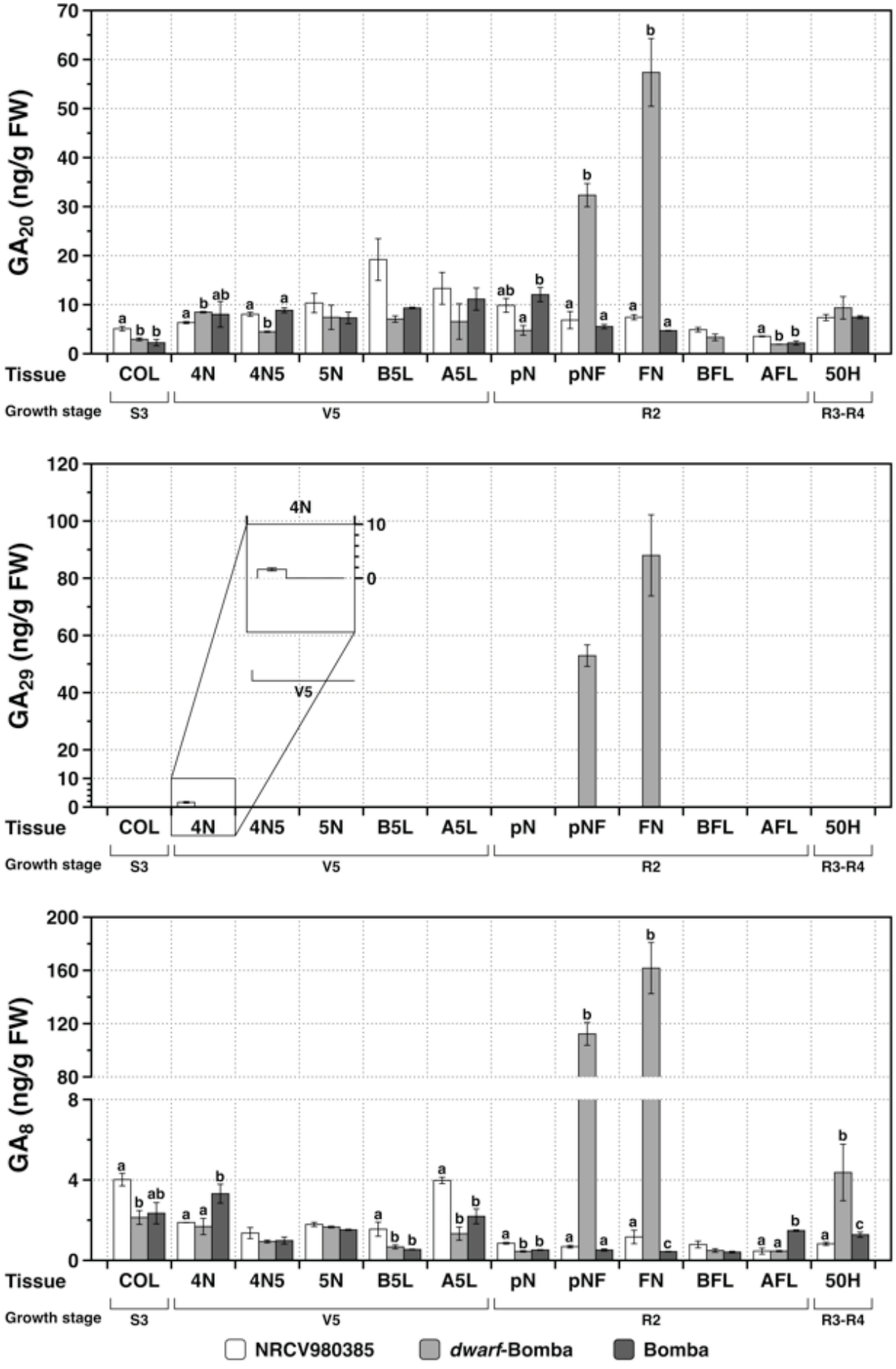
Figure SM.2.4 (page 360). Content of the gibberellins GA_{53}, GA_{44} and GA_{19} in the different tissues for the three analyzed varieties: NRVC980385, *dwarf*-bomba and Bomba. COL: coleoptile; 4N: 4th node; 4N5: internode between 4th and 5th node; 5N: 5th node; B5L: basal part of the 5th leaf; A5L: apical part of the 5th leaf; pN: node previous to the flag leaf node; pNF: internode between flag leaf and previous leaf; FN: flag leaf node; BFL: basal part of the flag leaf; AFL: apical part of the flag leaf. Columns correspond to mean ± SE of 3 replicates, and letters above bars indicate significant differences between varieties for each tissue analyzed.

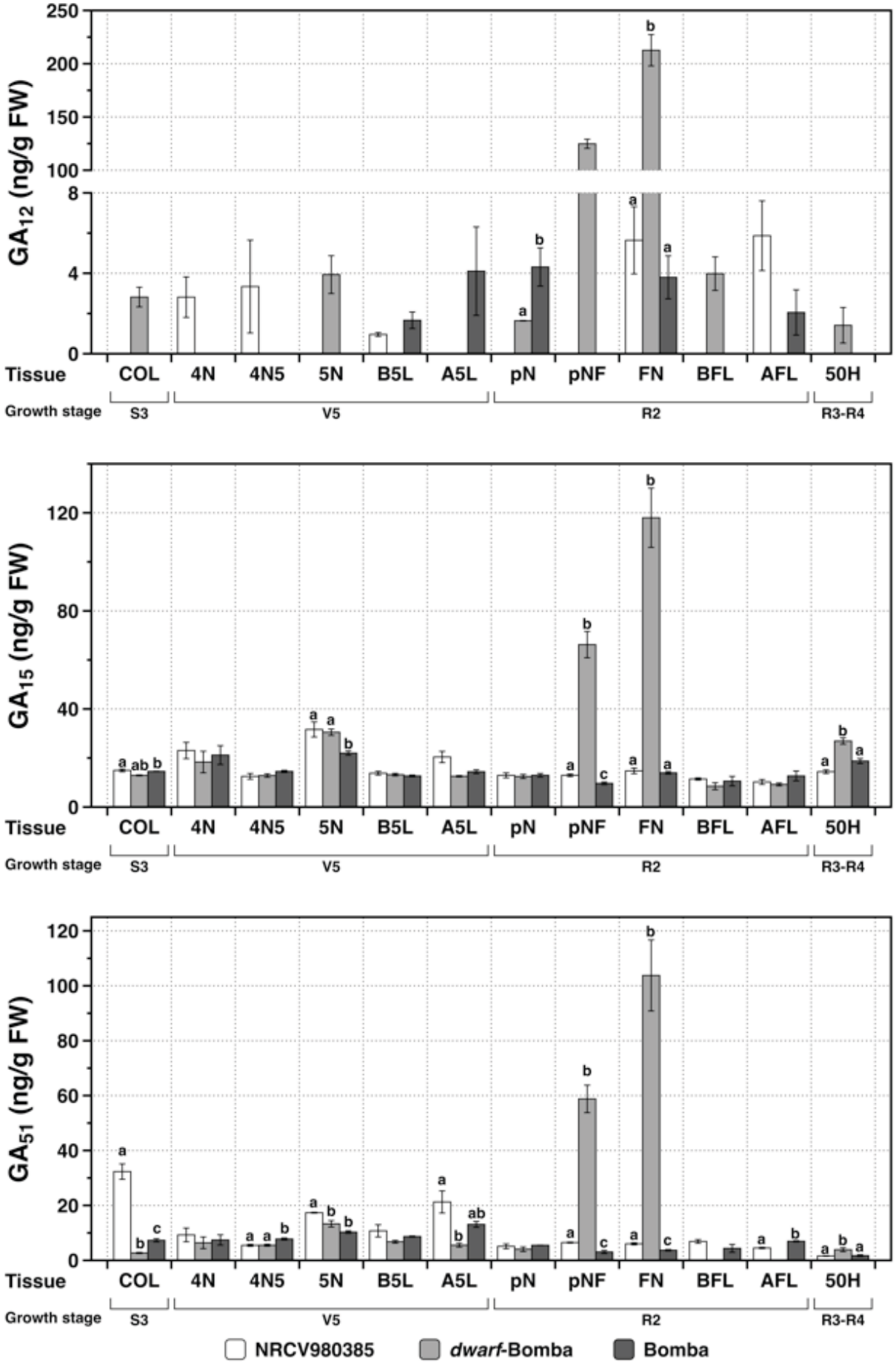
Figure SM.2.5 (page 361). Content of the gibberellins GA_{20}, GA_{29} and GA_{8} in the different tissues for the three analyzed varieties: NRVC980385, *dwarf*-bomba and Bomba. COL: coleoptile; 4N: 4th node; 4N5: internode between 4th and 5th node; 5N: 5th node; B5L: basal part of the 5th leaf; A5L: apical part of the 5th leaf; pN: node previous to the flag leaf node; pNF: internode between flag leaf and previous leaf; FN: flag leaf node; BFL: basal part of the flag leaf; AFL: apical part of the flag leaf. Columns correspond to mean ± SE of 3 replicates, and letters above bars indicate significant differences between varieties for each tissue analyzed.

Figure SM.2.6 (page 362). Content of the gibberellins GA_{12}, GA_{15} and GA_{51} in the different tissues for the three analyzed varieties: NRVC980385, *dwarf*-bomba and Bomba. COL: coleoptile; 4N: 4th node; 4N5: internode between 4th and 5th node; 5N: 5th node; B5L: basal part of the 5th leaf; A5L: apical part of the 5th leaf; pN: node previous to the flag leaf node; pNF: internode between flag leaf and previous leaf; FN: flag leaf node; BFL: basal part of the flag leaf; AFL: apical part of the flag leaf. Columns correspond to mean ± SE of 3 replicates, and letters above bars indicate significant differences between varieties for each tissue analyzed.

Figure SM.2.7 (page 363). Content of the phytohormones ABA, JA and IAA in the different tissues for the three analyzed varieties: NRVC980385, *dwarf*-bomba and Bomba. COL: coleoptile; 4N: 4th node; 4N5: internode between 4th and 5th node; 5N: 5th node; B5L: basal part of the 5th leaf; A5L: apical part of the 5th leaf; pN: node previous to the flag leaf node; pNF: internode between flag leaf and previous leaf; FN: flag leaf node; BFL: basal part of the flag leaf; AFL: apical part of the flag leaf. Columns correspond to mean ± SE of 3 replicates, and letters above bars indicate significant differences between varieties for each tissue analyzed.


GA53 (ng/g FW)
GA44 (ng/g FW)
GA19 (ng/g FW)
Tissue
COL
4N
4N5
5N
B5L
A5L
pN
pNF
FN
BFL
AFL
50H
Growth stage
S3
V5
R2
R3-R4
NRCV980385
dwarf-Bomba
Bomba


GA20 (ng/g FW)
GA29 (ng/g FW)
GA8 (ng/g FW)
Tissue
COL
4N
4N5
5N
B5L
A5L
pN
pNF
FN
BFL
AFL
50H
Growth stage
S3
V5
R2
R3-R4
NRCV980385
dwarf-Bomba
Bomba


GA12 (ng/g FW)
GA15 (ng/g FW)
GA51 (ng/g FW)
Tissue
COL
4N
4N5
5N
B5L
A5L
pN
pNF
FN
BFL
AFL
50H
Growth stage
S3
V5
R2
R3-R4
NRCV980385
dwarf-Bomba
Bomba

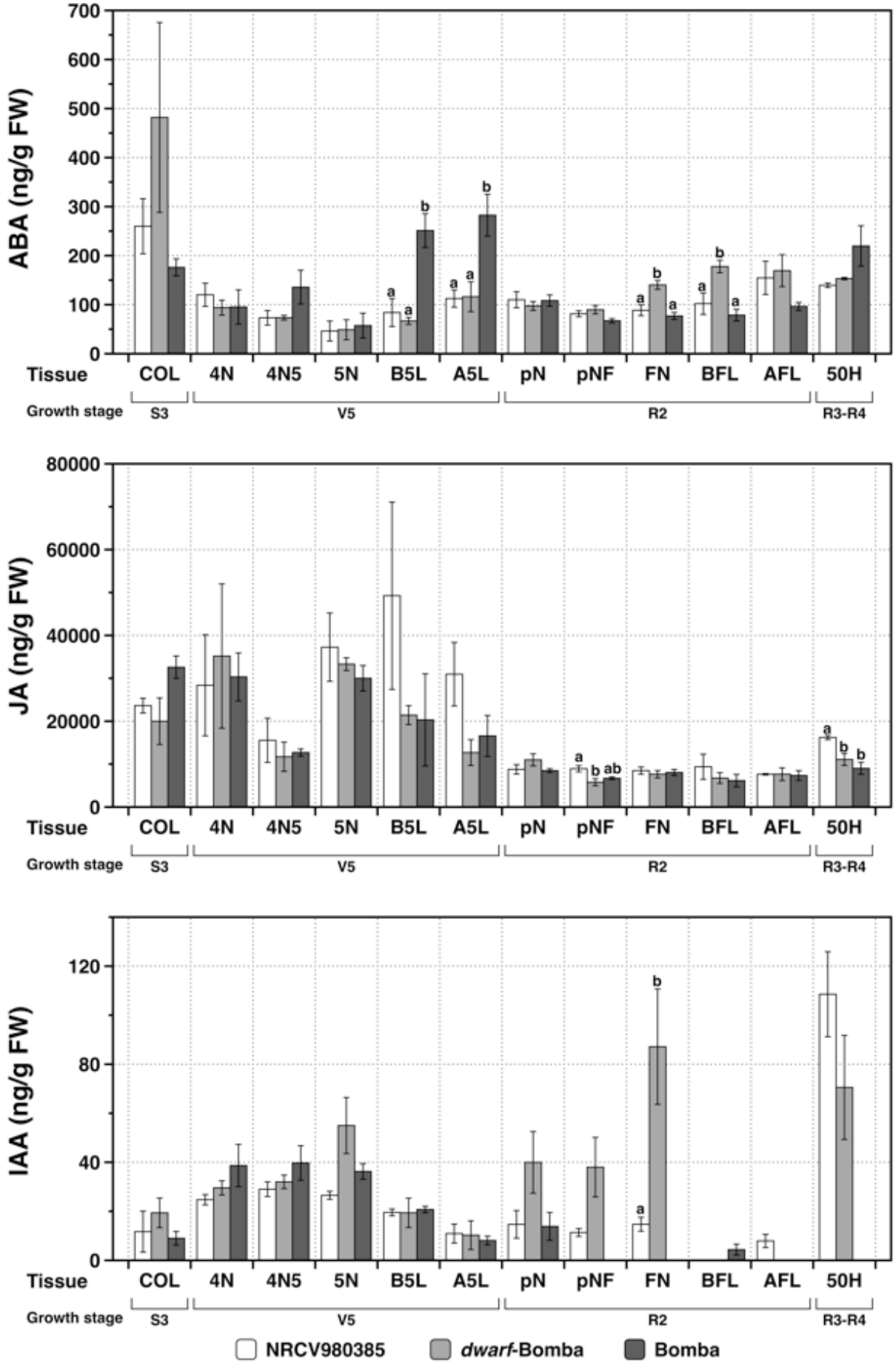

ABA (ng/g FW)
700
600
500
400
300
200
100
0
JA (ng/g FW)
80000
60000
40000
20000
0
IAA (ng/g FW)
120
80
40
0
Tissue
COL
4N
4N5
5N
B5L
A5L
pN
pNF
FN
BFL
AFL
50H
Growth stage
S3
V5
R2
R3-R4
NRCV980385
dwarf-Bomba
Bomba

Table SM.2.1. Statistical values (*p*-values and F) for the test used for analyzing each phytohormone in each tissue comparing the three varieties. GA_1, GA_3, GA_4, GA_7, GA_8 and GA_{12} in page 365; GA_{15}, GA_{19}, GA_{20}, GA_{29}, GA_{44}, GA_{51} and GA_{53} in page 366; ABA, JA and IAA in page 367.

Growth stage	Tissue	GA_1		GA_3		GA_4		GA_7		GA_8		GA_{12}	
		F	*p*-value	F	*p*-value	F	*p*-value	F	*p*-value	F	*p*-value	F	*p*-value
S3	**COL**	-	-	4.02	0.07820[a]	2.81	**0.21930[c]**	1.12	0.38690[a]	6.47	**0.03180[a]**	-	-
	4N	7.56	0.05140[a]	4.11	0.07520[a]	0.61	0.60130[c]	0.85	0.47190[a]	6.18	**0.03490[a]**	-	-
	4N5	59.50	**0.00010[a]**	11.67	**0.00860[a]**	3.38	0.10390[a]	0.57	0.59520[a]	1.44	0.30750[a]	-	-
V5	**5N**	2.10	0.22110[a]	5.60	**0.06080[b]**	5.42	0.06650[b]	7.95	**0.02060[a]**	3.99	0.07900[a]	-	-
	B5L	-	-	0.55	0.62140[c]	3.06	0.12160[a]	1.31	0.33620[a]	5.60	**0.06080[b]**	2.81	0.16890[a]
	A5L	-	-	128.83	**0.00080[c]**	7.20	**0.02730[b]**	3.75	0.14910[c]	20.30	**0.00210[a]**	-	-
	pN	2.65	0.20940[c]	14.07	**0.03620[c]**	3.44	0.10130[a]	1.86	0.23500[a]	40.49	**0.00030[a]**	3.52	**0.10620[c]**
	pNF	7.20	**0.02730[b]**	61.72	**0.00450[c]**	56.36	**0.00260[c]**	52.96	**0.00240[c]**	73.31	**0.00140[c]**	-	-
R2	**FN**	58.63	**0.00590[c]**	24.63	**0.01880[c]**	43.00	**0.00820[c]**	41.38	**0.00470[c]**	30.12	**0.01470[c]**	83.65	**0.00130[c]**
	BFL	-	-	1.14	0.38160[a]	5.96	**0.05090[b]**	0.36	0.83710[b]	4.36	0.11330[b]	-	-
	AFL	3.86	**0.04950[b]**	2.11	0.25050[c]	3.58	0.09470[a]	0.75	0.51320[a]	5.42	**0.06650[b]**	3.41	0.13870[a]
R3-R4	**50H**	202.46	**0.00000[a]**	7.20	**0.02730[b]**	12.15	**0.03890[c]**	27.68	**0.01570[c]**	7.20	**0.02730[b]**	-	-

[a]ANOVA test, [b]Kruskal-Wallis, [c]Welch's ANOVA.

Growth stage	Tissue	GA_{15}		GA_{19}		GA_{20}		GA_{29}		GA_{44}		GA_{51}		GA_{53}	
		F	*p*-value	F	*p*-value	F	*p*-value	F	*p*-value	F	*p*-value	F	*p*-value	F	*p*-value
S3	**COL**	22.49	**0.01370[c]**	7.94	**0.02810[a]**	9.52	**0.01380[a]**	-	-	16.21	0.00380[a]	66.71	0.00350[c]	43.41	0.00030[a]
	4N	0.37	0.70460[a]	10.04	**0.03630[c]**	27.16	**0.00750[c]**	-	-	2.00	0.21660[a]	0.45	0.66020[a]	1.38	0.32050[a]
	4N5	1.74	0.25380[a]	6.22	**0.03440[a]**	38.02	**0.00040[a]**	-	-	1.32	0.33380[a]	13.37	0.00610[a]	7.16	0.02570[a]
V5	**5N**	7.09	**0.02630[a]**	0.69	0.53770[a]	0.78	0.50140[a]	-	-	3.47	0.09960[a]	23.69	0.00140[a]	4.54	0.06290[a]
	B5L	0.94	0.44280[a]	0.93	0.44520[a]	6.88	0.08260[c]	-	-	2.68	0.23020[c]	5.76	0.09260[c]	18.57	0.00270[a]
	A5L	6.51	0.08080[c]	0.94	0.44270[a]	1.10	0.43000[c]	-	-	3.82	0.14790[b]	10.37	0.01130[a]	1.98	0.21800[a]
	pN	0.10	0.91020[a]	7.20	**0.02730[b]**	8.33	**0.01860[a]**	-	-	10.19	0.05240[c]	1.27	0.40870[c]	0.77	0.50240[a]
	pNF	51.84	**0.00230[c]**	0.69	0.53780[b]	5.60	**0.06080[b]**	-	-	14.49	0.00500[a]	58.85	0.00440[c]	5.42	0.06650[a]
R2	**FN**	30.63	**0.00880[c]**	5.40	**0.04550[a]**	34.15	**0.01200[c]**	-	-	5.60	0.06080[b]	35.37	0.00510[c]	5.42	0.06650[a]
	BFL	1.14	0.38020[a]	2.50	0.16200[a]	3.82	0.12240[a]	-	-	2.69	0.17600[a]	2.51	0.18850[a]	7.70	0.02200[a]
	AFL	1.80	0.24390[a]	6.49	**0.03900[a]**	12.88	**0.00670[a]**	-	-	3.19	0.17940[c]	52.51	0.00190_{a}	5.67	0.04140[a]
R3-R4	**50H**	37.40	**0.00040[a]**	19.18	**0.00250[a]**	0.66	0.55120[a]	-	-	8.51	0.01770[a]	4.14	0.14100[c]	13.39	0.01860[c]

a: ANOVA test; b: Kruskal-Wallis test; c: Welch's ANOVA.

Growth stage	Tissue	ABA		JA		IAA	
		F	*p*-value	F	*p*-value	F	*p*-value
S3	**COL**	3.20	0.20190[b]	3.20	0.11350[a]	0.76	0.50720[a]
	4N	0.33	0.73350[a]	0.08	0.92270[a]	2.76	0.25210[b]
	4N5	1.31	0.39070[c]	0.16	0.86060[c]	1.38	0.32070[a]
V5	**5N**	0.07	0.93370[a]	1.87	0.39320[b]	4.38	0.06720[a]
	B5L	15.16	**0.00450[a]**	1.42	0.49110[b]	0.17	0.84700[c]
	A5L	9.28	**0.01460[a]**	3.20	0.11310[a]	0.12	0.88480[a]
	pN	0.29	0.75610[a]	1.68	0.26340[a]	2.99	0.12570[a]
	pNF	3.07	0.12100[a]	6.15	**0.03530[a]**	4.74	0.09510[a]
R2	**FN**	13.53	**0.00600[a]**	0.27	0.77500[a]	9.34	**0.03780[a]**
	BFL	10.35	**0.01140[a]**	0.80	0.67030[a]	-	-
	AFL	1.96	0.22070[a]	0.02	0.97620[a]	-	-
R3-R4	**50H**	4.20	0.12710[c]	10.09	**0.01200[a]**	1.92	0.23770[a]

a: ANOVA test; b: Kruskal-Wallis test; c: Welch's ANOVA.