Polyamine signaling pathway during environmental stress: Metabolomic approaches to elucidate spermine down-stream targets

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POLYAMINE SIGNALING PATHWAY DURING ENVIRONMENTAL STRESS:

METABOLOMIC APPROACHES TO ELUCIDATE SPERMINE DOWN-STREAM TARGETS

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METABOLOMIC APPROACHES TO ELUCIDATE SPERMINE DOWN-STREAM TARGETS

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A mamá y papá

“If god did not exist, it would be necessary to invent him”

Voltaire

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INDEX

FIGURE INDEX

TABLE INDEX

ANNEX INDEX

ABBREVIATIONS

Polyamines, PAs
Putrescine, PUT
Spermidine, SPD
Spermine, SPM
Thermospermine, T-SPM
Abscisic acid, ABA
Ethylene, ET
Salicylic acid, SA
Jasmonate, JA
Proline, Pro
γ-aminobutyric acid, GABA
Paraquat, PQ
Ornithine decarboxylase, ODC
Arginine decarboxylase, ADC
Ornithine, Orn
Arginine, Arg
Agmatine, Agm
Agmatine iminohydrolase, AIH
N-carbamoylputrescine amidohydrolase, CPA
Aminopropyltransferases, APT
Decarboxylated *S*-adenosylmethionine, dSAM
S-adenosylmethionine, SAM
SAM decarboxylase, SAMDC
Spermidine synthase, SPDS
Spermine synthase, SPMS
Amine oxidases, AO
Copper-containing amine oxidases, CuAO
Polyamine oxidases, PAO
Reactive oxygen species, ROS
Cadaverine, Cad

Alternative oxidase pathway, AOX
Reactive oxygen and nitrogen species, RONS
Reactive nitrogen species, RNS
Proline, Pro
Primary root, PR
Lateral root, LR
Root system architecture, RSA
Ribulose-1,5-bisphosphate carboxylase/oxygenase, RuBisCO
Wild-Type, WT
Left border, LB
Gas Chromatography-Time-of-Flight-Mass Spectrometry, GC-TOF-MS
Raffinose Family Oligosaccharides, RFO
Aspartate, Asp
Asparagine, Asn
Glutamate, Glu
Glutamate-like receptor, GLR
Gluthation, GSH
Galactinol Synthase, GolS
Phosphatidylinositol, PtdIns
Inositol-6-phosphate, Ins6P
Inositol-phosphate kinase 2, AtIPK2
Methionine, Met
Ascorbate, ASC
Dehydroascorbate, DHA
Threonine, Thr
Phenylalanine, Phe
Tryptophan, Trp
Indole-3-Acetic acid. IAA

CHEMICALS AND REAGENTS

Superoxide anion, $O_2^{\bullet -}$

Sodium Nitroprusside, SNP

Perchloric acid, PCA

1,7-Diaminеheptane, HTD

Sodium Carbonate, Na_2CO_3

Putrescine dihydrochloride, $NH_2(CH_2)_4NH_2$

Spermidine trihydroclhoride, $NH_2(CH_2)_3NH(CH_2)_4NH_2$

Spermine tetrahydrochloride, $C_{10}H_{26}N_4 \cdot 4HCl$

Hydrogen peroxide, H2O2

Malondehaldehyde, MDA

Nitric oxide, NO

3,3’-Diaminobenzidine, DAB

Trichloroacetic acid, TCA

Potassium Iodide, KI

Diaminobenzydine, DAB

Thiobarbituric acid, TBA

Sodium Nitrate, $NaNO_2$

Zinc Acetate, $Zn(O_2CCH_3)_2(H_2O)_2$

Polyvinylpyrrolidone, PVP

Ammonium acetate, $CH_3CO_2NH_4$

Sodium dodecyl sulfate, SDS

DL-Dithiothreitol, DTT

Dimethyl Sulfoxide, DMSO

CarboxyPTIO, cPTIO

ABSTRACT

Environmental stress is increasingly wearing down crop productivity. Nowadays, one of the main aims of plant research is to elucidate tolerance mechanisms to diverse stresses, in order to provide solutions by generating stress-tolerant plants. In regard with this matter, polyamine signaling pathway is of crucial importance. The major polyamines in plants (putrescine, spermidine and spermine) tend to accumulate in response to stress and are associated with a protective role. The trend of their accumulation is related to the stress the plant is sensing; in fact, polyamine biosynthetic pathway is activated at different levels depending on the stimulus, which gives a selective role to these molecules. Spermine, one of the higher polyamines, is not essential for plant growth; however, it is presently known that this molecule plays diverse protective roles under several stress factors and triggers signaling cascades implicated in plant defense. Therefore, research on spermine down-stream targets has become necessary towards the elucidation of plant tolerance responses. By the use of model plant *Arabidopsis thaliana* this study demonstrated the implication of this polycation on enhancement of anti-oxidative capacity by signaling connections to central hub metabolites for sugar, lipid and amino acid metabolism such as pyruvate or *myo*-Inositol, as well as its involvement on root morphology.

RESUMEN

El estrés medioambiental está afectando de forma paulatina la productividad de los cultivos. En la búsqueda de soluciones, uno de los principales objetivos de la investigación en fisiología de plantas, es dilucidar los mecanismos de tolerancia que se presentan ante diversos estreses, con la finalidad de generar plantas con fenotipos resistentes. En referencia a este asunto, las poliaminas y sus rutas señalizadoras son de importancia crucial. Las más abundantes en plantas (putrescina, espermidina y espermina) tienden a acumularse en respuesta al estrés por lo cual se les asocia a un rol protector, sin embargo, las tendencias de acumulación dependen del tipo de estrés que la planta es capaz de percibir. De hecho, su ruta biosintética se activa a diferentes niveles dependiendo del estímulo, lo cual les confiere un carácter selectivo. La Espermina (una de las poliaminas superiores) no es esencial para el crecimiento de la planta, no obstante, actualmente se sabe que esta molécula ejerce diversos roles protectores en una gran variedad de condiciones y además activa cascadas señalizadoras implicadas en la respuesta defensiva de la planta. En consecuencia, para dilucidar los mecanismos de tolerancia, se ha hecho necesario profundizar en las dianas de señalización por parte de la espermina. Empleando como modelo experimental *Arabidopsis thaliana,* el presente estudio ha demostrado la implicación de este policatión en el aumento de la capacidad anti-oxidativa a través de conexiones con metabolitos centrales en el metabolismo de azucares, lípidos y aminoácidos como es el caso del piruvato y el *mio*-Inositol, así como también, la implicación de esta poliamina en la morfología y ramificación de las raíces, reforzando la noción de implicación esencial por parte de esta poliamina, en la fisiología del estrés en plantas.

INTRODUCTION

Environmental stress is negatively affecting plant development and productivity. One of the biggest challenges of modern science is applied basic research to find solutions for increasing food crop yield. Moreover, the increment of the world population has added more pressure on the demand (Pathak *et al.*, 2014). Global climate change is expected to intensify the frequency and severity of drought and flooding events in many regions world-wide, severely affecting crop production (Pottosin and Shabala, 2014). As sessile organisms, plants are exposed to a large array of environmental stresses and have therefore evolved diverse strategies to combat various life-threatening situations (Berberich *et al.*, 2015). In consequence, plant stress physiology has been pointed out towards dissection of genetic elements involved in stress tolerance. In regard with this topic, polyamines (PAs) are essential molecules. Compelling evidence indicates participation of PAs in abiotic and biotic stress responses in plants (Alcázar and Tiburcio, 2014). The biological functions of PAs were initially associated with their ability to bind anionic macromolecules, and they were thus considered to be polycations with unique structural roles. Later studies showed that PAs also act as regulatory molecules in fundamental cellular processes, including cell division, differentiation and gene expression (Tiburcio *et al.*, 2014).

1. Polyamines: current knowledge

PAs are organic polycations having variable hydro-carbon chains and two or more primary amino groups (Takahashi and Kakehi, 2010). They have an aliphatic structure and are found in all cells across all kingdoms (Moschou *et al.*, 2008*c*). The structure and chemistry of the most abundant PAs in plants diamine putrescine (PUT), triamine spermidine (SPD) and tetraamine spermine (SPM) were elucidated in the late 1920s. It was revealed that they are nitrogen-containing compounds of low molecular weight (Alcázar *et al.*, 2010*a*). Later on, it was shown that thermospermine (T-SPM), an isomer of spermine, was also present in higher plants (Moschou *et al.*, 2008*c*). To the present, it is accepted that all of them are major sinks of assimilated nitrogen due to their intracellular high concentration (Moschou *et al.*, 2012*a*). Over the last two decades, several

results have supported the notion that PAs are essential for life. In plants, depletion of PUT or SPD biosynthesis has resulted in lethal phenotypes in plants, while lack of SPM production results in viable individuals although all present several degrees of dysfunction, which led to the conclusion that SPM, even if is not essential, must play very important roles in growth and development (Alcázar *et al.*, 2010*a*). Therefore, full elucidation of their homeostasis mechanisms became an important aim to be investigated.

1.1 Possible mechanisms of action of individual PAs during stress events

1.1.1 Putrescine

The beneficial, general stimulatory effect of PUT has long been known. However, this effect is not direct (Pál *et al.*, 2015). It is clear that PUT is important as a precursor for the biosynthesis of higher PAs. According to a study using transgenic plants with altered PA levels, it was observed that PUT levels must exceed a certain threshold to enhance the synthesis of SPD and SPM under stress (Capell *et al.*, 2004), such synthesis being necessary for recovery from stress conditions. High levels of PUT are closely related to osmotic stress tolerance (Soyka and Heyer, 1999; Urano *et al.*, 2004), drought (Alcázar *et al.*, 2010*b*), wounding (Perez-Amador *et al.*, 2002) and freezing tolerance by modulation of abscisic acid (ABA) levels (Cuevas *et al.*, 2008).

Recently it was found that PUT acts as a buffer and osmolite, inducing increment in proline (Pro) content, which leads to maintenance of leaf water status under stress conditions (Kotakis *et al.*, 2014). Microarray analysis of one overexpressor of PUT revealed both the up- and down-regulation of various stress-responsive, hormone and signaling-related genes, involved in the biosynthesis of auxin, ethylene (ET), ABA, gibberellin and salicylic acid (SA). Furthermore, genes for auxin transport, genes coding for auxin-responsive proteins, ET and ABA-responsive transcriptional factors, and also jasmonate

(JA)-induced proteins (Marco *et al.*, 2011) were also identified, which confirm the dual role of PUT and PAs in general: direct protection and participation in acclimation signaling pathways (Pál *et al.*, 2015). PUT oxidation in plants produces 4-aminobutanal, which spontaneously cyclizes to Δ^1-pyrroline and can be further converted to γ-aminobutyric acid (GABA) (Petřivalský *et al.*, 2007), an important metabolite, of which the levels tend to be altered during stress response, although its function is currently unknown (Shelp *et al.*, 2012)

1.1.2 Spermidine

SPD is a higher PA that is essential during embryogenesis in *Arabidopsis* (Imai *et al.*, 2004*b*). Overexpression of a spermidine synthase gene up-regulated the expression of various putative stress-related genes in chilling-stressed transgenic *Arabidopsis* compared with the corresponding wild type. These genes putatively encode transcription factors, calmodulin-related protein and stress-protective proteins, such as rd29A (Kasukabe *et al.*, 2004).

Exogenous application of this PA has resulted in salt stress alleviation by improvement of anti-oxidative defense system in *ginseng* seedlings (Parvin *et al.*, 2014) and alteration of tomato transcriptome after high temperature stress. More than 30 genes were up-regulated in SPD-treated tomato fruits as compared with non-treated fruits. These genes are putatively involved in primary metabolism, signal transduction, hormone responses, transcription factors and stress responses, while 55 genes that are putatively involved in energy metabolism, cell wall metabolism and photosynthesis were down-regulated (Cheng *et al.*, 2012).

SPD conjugates are implicated in protection against pathogens, detoxifying phenolic compounds, and/or serving as a reserve of PAs that are available to actively proliferating tissues, although not always essential for survival (Takahashi and Kakehi, 2010).

1.1.3 Spermine

In plants, no requirement for SPM under normal growth conditions has been demonstrated in an *Arabidopsis* mutant which cannot produce this higher PA (Imai *et al.*, 2004*a*). However, this mutant showed sensitivity to drought and salt stress (Yamaguchi *et al.*, 2007). On the other hand, one of the most important roles of SPM in the nucleus, is the protection of DNA from free radical attack and subsequent mutation (Takahashi and Kakehi, 2010).

The existence of a 'spermine signaling pathway' has been proposed, which involves accumulation of SPM in the apoplast and upregulation of a subset of defense-related genes, mostly involved in what is known as 'hypersensitive response' during plant pathogenic infection (Takahashi *et al.*, 2003). However, this activation also required H_2O_2, derived from SPM degradation. This suggested that the generation of H_2O_2 via the oxidative degradation of PAs plays a role in PA-related signaling processes, which has been corroborated in some cases (Cona *et al.*, 2006; Moschou *et al.*, 2008*b*, 2012*b*; Mitsuya *et al.*, 2009).

Arabidopsis plants with increased SPM levels showed altered expression of genes involved in the biosynthesis of JA, ABA and SA, receptor-like kinases, mitogen-activated protein kinases and genes with a role in calcium regulation (Marco *et al.*, 2011). In tobacco, SPM accumulation caused up-regulation of transcripts for anti-oxidative enzymes, especially those induced by abiotic stresses, such as salt, cold or acidic stress (Wi *et al.*, 2006), which lead to the notion that this PA, although not essential, plays versatile roles in stress response (Takahashi and Kakehi, 2010)

1.1.4 Thermospermine

Recent notions have stablished that T-SPM is not a minor PA in plants (Takano *et al.*, 2012). An *Arabidopsis* mutant deficient in T-SPM synthesis displays

growth-arrested phenotype (Kim *et al.*, 2014). In agreement to this, it was demonstrated that T-SPM modifies the expression of auxin-related genes (Tong *et al.*, 2014). Although the potential role of T-SPM in biotic stress protection has already been noted on *Arabidopsis* (Sagor *et al.*, 2012; Marina *et al.*, 2013), its role in abiotic stress has not been established yet.

1.2 Polyamines: homeostasis mechanisms

1.2.1 Polyamine biosynthethic pathway

Metabolic studies indicate that the intracellular levels of PAs in plants are mostly regulated by anabolic and catabolic processes, as well as by their conjugation to hydroxycinnamic acids (Alcázar *et al.*, 2010*a*). PA biosynthesis begins with PUT formation. This PA is derived either directly from ornithine (Orn) by ornithine decarboxylase (ODC) or from arginine (Arg) through several steps catalysed by arginine decarboxylase (ADC), which produces agmatine (Agm), agmatine iminohydrolase, (AIH) and N-carbamoylputrescine amidohydrolase (CPA). In contrast to animals and fungi, in which ODC is the first and rate-limiting enzyme in the synthesis of PAs, plants typically use ADC (Takahashi and Kakehi, 2010). In *Arabidopsis*, PUT content is modulated by the expression of two gene isoforms encoding ADC (*ADC1* and *ADC2*), with contrasting expression patterns depending on the nature of the stress (Alcázar *et al.*, 2010*b*). SPD, SPM and T-SPM are synthesized by aminopropyltransferases (APT), which transfer aminopropyl residues to amine acceptors PUT or SPD, producing SPD, SPM, or its isomers.

The donor of the aminopropyl groups is decarboxylated *S*-adenosylmethionine (dSAM), which is formed by decarboxylation of *S*-adenosylmethionine (SAM), a universal methyl donor, due to enzymatic reaction catalyzed by SAM decarboxylase (SAMDC). The APTs donating aminopropyl residues to PUT or SPD for production of SPD or SPM are spermidine synthase (SPDS) and spermine synthase (SPMS) respectively. SDPS and SPMS from *Arabidopsis*

thaliana are encoded by two gene paralogs (*SPDS1* and *SPDS2*) for SPDS and one for SPMS respectively (Fig. 1).

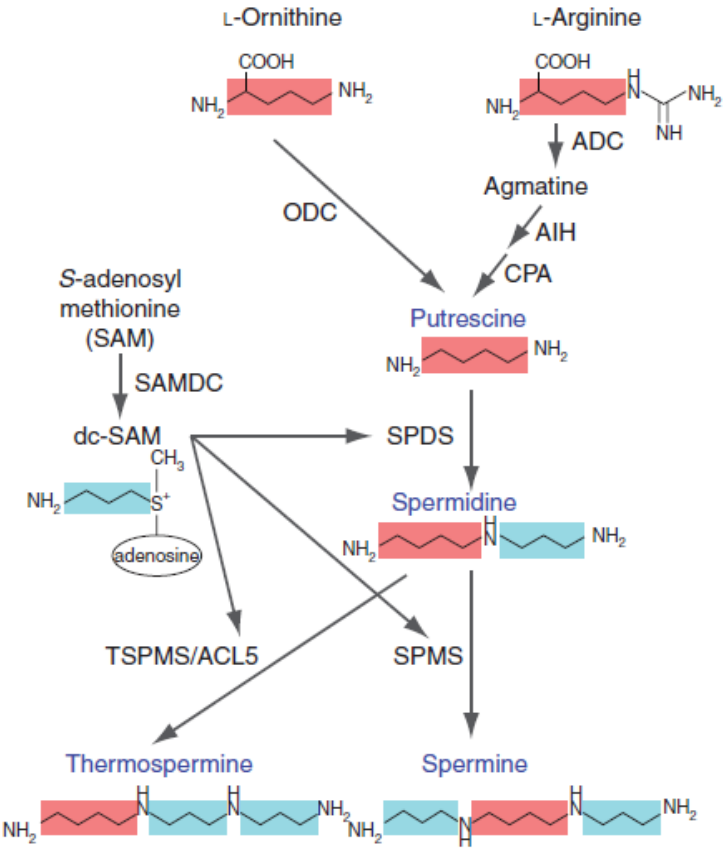

Fig. 1 Polyamine biosynthetic pathway in plants (Takahashi and Kakehi, 2010)

1.2.2 Polyamine conjugation

In plants, PAs occur in free form but also as conjugated to hydroxycinnamic acids to form hydroxycinnamic acid amides, in a process catalyzed by N-acyltransferases. During the reaction an N-acylation of PA with thioesters occurs which is activated by coenzyme A (Fuell *et al.*, 2010). Recently, genes encoding several SPD *N*-acyltransferases have been characterized in *Arabidopsis* supporting the role of these conjugates in pollen tube development (Fuell *et al.*, 2010). Previous ideas considered that PA conjugates were inactive

forms. However, recent evidence indicates that they are essential for certain developmental processes (Tiburcio *et al.*, 2014). PA conjugates are not rare since hydroxycinnamoyl PUT, SPD, and SPM conjugates are found in many plant species. An emerging area of study is the characterization of PA conjugate biosynthetic pathways and the enzymes involved (Tiburcio *et al.*, 2014).

1.2.3 Polyamine transport

In general, PA uptake is affected by the PA cell requirement. Higher growth rates are usually accompanied by higher rates of exogenous PA uptake and intracellular de novo synthesis; therefore, PA transport also plays a vital role in the regulation of intracellular PA levels (Fujita and Shinozaki, 2014). To date, few PA transporters have been identified in plants, with special attention to rice (Mulangi *et al.*, 2012) and *Arabidopsis* (Fujita *et al.*, 2012). An SPD-preferential uptake system (OsPUT1) has been characterized in rice using heterologous expression and yeast complementation assays. SPM competes with SPD in this uptake system, while PUT, as well as the PA precursors Orn and Agm, did not compete (Mulangi *et al.*, 2012). On the other side, a recent screening for natural variation in paraquat (PQ) resistance identified an *Arabidopsis* L-type amino acid transporter (LAT), called *Resistant to Methylviologen 1* (*RMV1*), responsible for the uptake of PQ and PAs (Fujita *et al.*, 2012). Genetic variation at *RMV1* provides differential responses to exogenous PQ and PA applications in root growth. RMV1-overexpressing plants were significantly more sensitive to PAs such as SPM, SPD and PUT, and displayed higher PA uptake activity than that of the control lines. It was established that exogenous application of PAs counteracts PQ toxicity (Fujita *et al.*, 2012). Overall, the results suggest that differences in substrate specificity, subcellular localization and tissue specificity of PA transporters resulted in variations in the response to PQ, but not to PAs (Fig. 2) (Fujita *et al.*, 2012). This was promising since it enabled the identification of PA transporters by genetic approaches using PQ tolerance, which provided a deeper insight into the molecular function of PAs and PA

homeostasis and provided better understanding into the PA anti-oxidative properties (Fujita and Shinozaki, 2014).

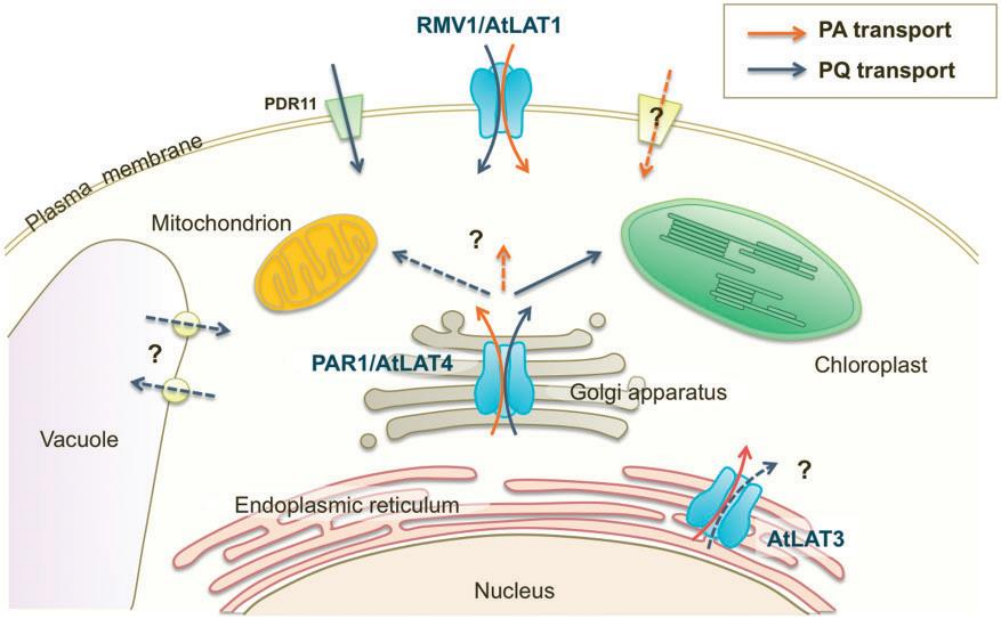

Fig. 2 Subcellular localization and substrate preference of characterized PA/PQ transporters in *Arabidopsis.* (Fujita and Shinozaki, 2014)

1.2.4 Polyamine Oxidation

Endogenous PA levels mostly depend on the dynamic balance between *de novo* biosynthesis and catabolism. PA oxidation is catalyzed by two types of amine oxidases (AO), copper-containing amine oxidases (CuAO) and FAD-dependent polyamine oxidases (PAO) (Tiburcio *et al.*, 2014) releasing in all cases reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2). CuAO are homodimeric enzymes with high affinity for oxidizing the primary amino groups of PUT and cadaverine (Cad), another PA, and lower affinity for SPD and SPM (Moschou *et al.*, 2012*a*). This group of enzymes participate in PA final catabolism in then apoplast and peroxisomes (Planas-Portell *et al.*, 2013). *Arabidopsis* carries ten putative CuAO-encoding genes, four of which (*ATAO1* and *AtCuAO1–3*) have been characterized. *CuAO* genes are differentially

modulated during development, wounding, and treatment with hormones or elicitors. CuAO proteins also differ in their localization, with AtCuAO1 and ATAO1 being apoplastic, whereas AtCuAO2 and AtCuAO3 are peroxisomal enzymes (Møller and McPherson, 1998; Reumann *et al.*, 2009; Planas-Portell *et al.*, 2013), all of them involved in terminal catabolism of PUT and SPD.

PAO catalyzes the oxidation of SPD, SPM, and/or acetylated derivatives at their secondary amino groups (Tavladoraki *et al.*, 2012). They are classified into one of two families depending on whether they terminally oxidize PAs or catalyze PA back-conversion. PAOs of the first family oxidize the carbon at the endo side of the N^4 of SPD and SPM, producing 4-aminobutanal and N-(3-aminopropyl)-4-aminobutanal, respectively (Moschou *et al.*, 2012*b*). PAOs catalyzing PA back-conversion oxidize the carbon at the exo side of the N^4 of SPD and SPM (and/or their acetylated derivatives) producing PUT and SPD, respectively. Essentially the PA pool is dynamic, changing over time, and PAs also undergo rapid interconversion in what was called the "polyamine cycle" in which higher PAs, such as SPD and SPM are synthesized from PUT. Furthermore, degradation of SPM or SPD may also lead to lower PA-releasing H_2O_2 (Fig. 3) (Pál *et al.*, 2015).

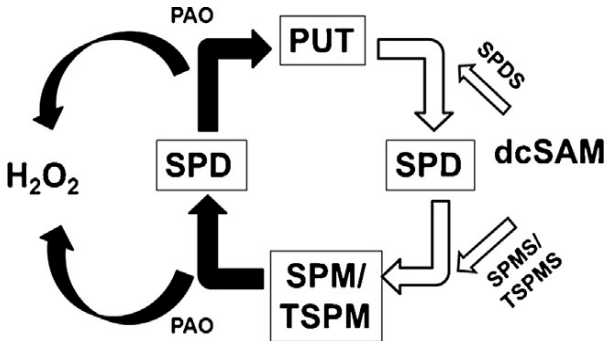

Fig.3 Polyamine cycle (Pál *et al.*, 2015)

The best-characterized plant PAOs involved in PA back-conversion are from *Arabidopsis*. This plant carries five PAO-encoding genes (*AtPAO1–5*) (Takahashi *et al.*, 2010; Fincato *et al.*, 2011). Tissue- and organ-specific expression studies of various *AtPAO* genes have shown some overlapping

patterns but also important differences. This, together with their contrasted substrate specificity, suggests a functional diversity of *AtPAO* genes (Takahashi *et al.*, 2010).

The different subcellular localizations of AtPAO proteins also support the view that AtPAO2–4 are localized in peroxisomes, whereas AtPAO1 and AtPAO5 are predicted to be cytosolic. Thus, PA catabolism in the *Arabidopsis* apoplast is mediated predominantly by CuAO. Recently, it has been demonstrated that PA back-conversion mediated by PAO and terminal catabolism mediated by CuAO are co-localized in peroxisomes of *Arabidopsis* (Planas-Portell *et al.*, 2013). PUT level at certain points is able to inhibit peroxisomal PAO enzymes, which goes in favor of SPD or PUT terminal degradation. A model was proposed where PA homeostasis is maintained by a tight coordination between both catabolic enzyme machineries (Planas-Portell *et al.*, 2013). Thus, SPM homeostasis relies in part on the activity of PAO enzymes (Fig. 4).

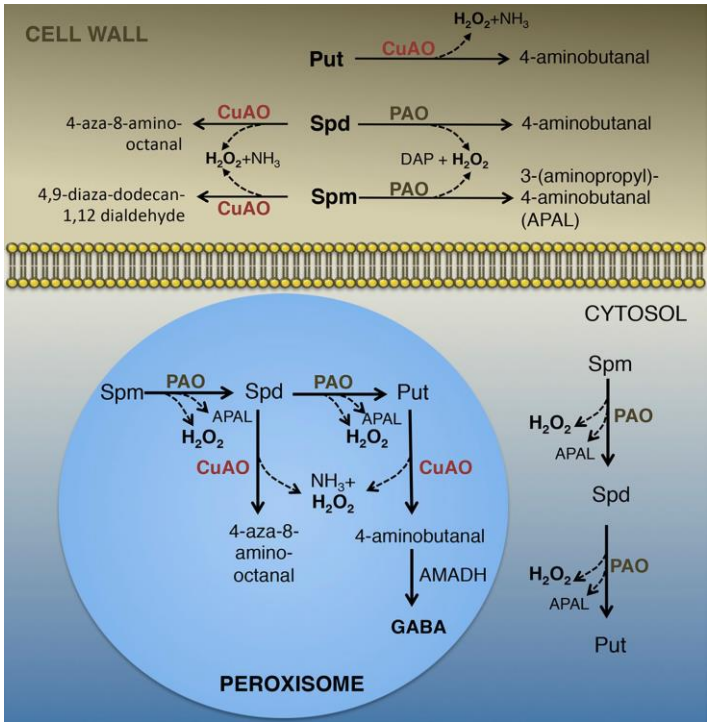

Fig. 4 Polyamine oxidation pathways present in the cytosol, apoplast and peroxisomes (Tiburcio *et al.*, 2014).

1.3 Polyamine oxidase 4. The aim of this study

Peroxisomal PAO subfamily (AtPAO2, AtPAO3 and AtPAO4) are proven to fall into one phylogenetic branch (Takahashi *et al.*, 2010). AtPAO4 is similar with AtPAO2 and AtPAO3 in 58% and 50% of the sequence, respectively (Fincato *et al.*, 2011), which have suggested distinct physiological roles. In fact, a recent study demonstrated that SPD oxidation by PAO3 is required for a balanced respiration, through mitochondrial cytochrome *c*-dependent process or an alternative oxidase pathway (AOX), proposing that this peroxisomal PAO is a key element for balancing superoxide/hydrogen peroxide ($O_2^{\bullet-}$/H_2O_2) production, which is highly implicated in cell respiration (Andronis *et al.*, 2014).
PAO4, the focus of this study, is characterized as the major isoform in *Arabidopsis* roots (Kamada-Nobusada *et al.*, 2008). It is reported to oxidize both SPD and SPM although *Kcat* value for the latter is 10-fold higher than that for SPD (Fincato *et al.*, 2011) and is proved to participate in PA back-conversion pathway (Kamada-Nobusada *et al.*, 2008; Fincato *et al.*, 2011). Expression was observed on the whole plant body irrespective of developmental stages (Takahashi *et al.*, 2010). T-DNA insertional *AtPAO4* mutants accumulate SPM on a high extent and showed decreased SPD levels compared with WT in *Arabidopsis* roots (Kamada-Nobusada *et al.*, 2008). Microarray analysis of loss-of-function PAO4 mutants detected several alterations in gene expression including the up-regulation of genes encoding drought stress response proteins, gluthation homeostasis as well as genes involved in flavonoid and/or lignin biosynthesis, such as phenylalanine ammonia lyase 1 (Kamada-Nobusada *et al.*, 2008). Thus implication of PA homeostasis by PAO4 in tolerance response after environmental stress might be expected.

2. Evidence of cross-talk between polyamines and reactive oxygen and nitrogen species (RONS) during stress response

The peroxisome (the organelle where PAO4 is localized) participates in different cellular processes involved in development, morphogenesis and cell responses

to stress (Linka and Theodoulou, 2013). In recent years biochemical, transcriptomic and proteomic approaches have demonstrated that these highly dynamic and metabolically active organelles are much more complex and perform functions hitherto unknown (Sandalio and Romero-Puertas, 2015). One of the main features of these organelles is that they are an extremely important source of RONS (del Rio, 2015). ROS are mainly produced through different metabolic pathways, including fatty acid β-oxidation, photorespiration, nucleic acid and PA catabolism, most of them occurring at the peroxisome. On the other hand, it has been demonstrated that *Arabidopsis* peroxisomes are essential for reactive nitrogen species (RNS) accumulation in the cytosol, as well as for the synthesis of nitric oxide (NO), which is required for peroxynitrite-mediated nitrosative response (Corpas *et al.*, 2009). Hence, the existence of an active nitrogen metabolism and production of NO-derived RNS inside this organelle, is proven to be a very important process for post-translational modification (e.g nitrosylation or nitration) of several peroxisomal proteins, implied in oxidative regulation (Linka and Theodoulou, 2013). Therefore, RONS are known to play important roles in cell metabolic dynamics (Molassiotis and Fotopoulos, 2011; Choudhury *et al.*, 2013; Wang *et al.*, 2013). In parallel, several studies have tried to elucidate the molecular and biochemical mechanisms underlying PA action; Thus, recent reports have demonstrated PAs to have direct relationship with RONS in signaling mechanisms (Alcázar *et al.*, 2010*a*; Wimalasekera *et al.*, 2011*a*; Minocha *et al.*, 2014; Pottosin *et al.*, 2014). In any case, ROS and RNS signals inducing oxidative/nitrosative stress are essential elements in stress signaling.

2.1 Polyamine involvement with ROS signals

Previous observations relate PAs to ROS through H_2O_2 via their catabolism pathway (Fig. 4); nonetheless, their relationship appears to be far more complex. H_2O_2 derived from PA degradation not always leads to the same effect. By using transgenic tobacco plants over-expressing ZmPAO, low apoplastic SPD and SPM levels (because of their degradation), as well as lower

levels of ROS were observed under normal growth conditions (Moschou *et al.*, 2008*a*). Accordingly, an enhancement of anti-oxidative machinery was also observed. Surprisingly, these transgenic plants were sensitive to oxidative stress compared with WT, which led to the suggestion that PA levels were a key element of the oxidative response (Moschou *et al.*, 2008*a*). Another aspect to consider is that ROS derived from PA oxidation is necessary in order to trigger stress response signaling. However, the size and rate of its accumulation determines cell fate, which means that ROS should not exceed specific thresholds; if so, it no longer signals the expression of stress genes but, instead, triggers programmed cell death (Moschou *et al.*, 2008*b*).

Typically, when cellular PA contents are up ,their catabolism also increases, the levels of H_2O_2 increase, and various ROS as well as the anti-oxidative system (enzymatic and non-enzymatic) is also induced, hence their roles in preventing damage from stress are beneficial as well as deleterious (Minocha *et al.*, 2014). This is consistent with the notion that an increase in cellular PA titers contributes to both sides of the ROS-anti-oxidative equation under conditions of stress (Minocha *et al.*, 2014). As mentioned previously, SPD homeostasis by PAO3 was demonstrated to be involved in ROS production other than H_2O_2. The ratio of $O_2^{\cdot-}$/ H_2O_2 showed to be an important signal in transcription (Andronis *et al.*, 2014) and might be the mediator of PA contribution to plant adaptation under unfavorable conditions (Pál *et al.*, 2015)

2.2 Polyamine involvement with RNS signals

At present, PA researchers recognize that NO biosynthesis and PA metabolism are tightly related. SPD and SPM are able to promote NO biosynthesis inn *Arabidopsis* seedlings, root tip and primary leaves (Tun *et al.*, 2006). A recent report on citrus seedlings provided evidence that there is a tissue-specific modulation of PAO expression, when oxidative/nitrosative stress is imposed as an acclimation treatment, after which pre-treated plants show salinity tolerance, suggesting that PAs may represent a molecular link between oxidative and

nitrosative signaling (Tanou *et al.*, 2012). In fact, these authors further demonstrated that PAs are able to reprogram oxidative and nitrosative status as well as the proteome of salt-stressed plants (Tanou *et al.*, 2014). In addition, other authors have demonstrated that NO is able to modulate PA and proline (Pro) metabolism in *Medicago* plants (Filippou *et al.*, 2013). A significant reduction in NO release was observed in *Arabidopsis* CuAO1 loss-of-function mutants (Wimalasekera *et al.*, 2011*b*), suggesting that this AO might be involved in NO biosynthesis induced by PAs. SPM and SPD also modulated the arginine-linked NO synthase and nitrate reductase pathways (Shi and Chan, 2014). It seems that NO can fill the gap between several physiological effects of PA and the mitigation of stress (Molassiotis and Fotopoulos, 2011; Wimalasekera *et al.*, 2011*a*; Pál *et al.*, 2015), which is essential on stress physiology. Thus, RNS are important signaling molecules that influence many aspects of cell function, including differentiation and cell survival in response to environmental cues (Sandalio and Romero-Puertas, 2015), especially the ones derived from peroxisome (Corpas *et al.*, 2009; Begara-Morales *et al.*, 2013; Corpas and Barroso, 2014).

In conclusion, multiple lines of evidence discussed above suggest that there is a biologically active interplay between ROS, RNS and PA signals in plants that modulates cellular responses to environmental stimuli (Molassiotis and Fotopoulos, 2011) (Fig. 5)

3. New targets on polyamine research

In plants, PAs are implicated in physiological processes, including organogenesis, embryogenesis, floral initiation and development, leaf senescence, pollen tube growth, fruit development and ripening and response to both abiotic and biotic stresses (Tiburcio *et al.*, 2014). Several endogenous and exogenous stimuli, as well as PAs themselves, induce changes in the expression levels of genes involved in PA metabolism, through various

regulatory mechanisms (Ahou *et al.*, 2014), which affect cell metabolome in a wide variety of physiological process.

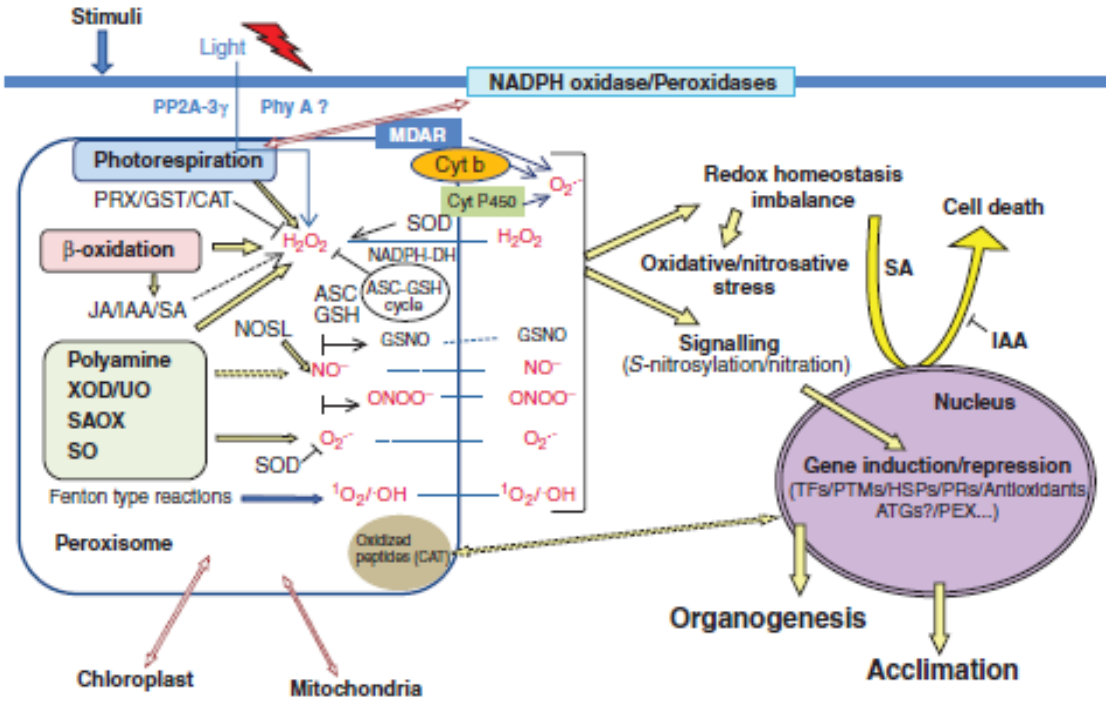

Fig. 5 Schematic overview of the signaling network involving ROS, RNS and PA in peroxisomes (Pál *et al.*, 2015)

On the other hand, although the functional diversity of plant PAOs is established (Takahashi *et al.*, 2010; Fincato *et al.*, 2011), ongoing research is still trying to elucidate the function of these enzymes, which we now know that are not only related with PA regulation, but also with organelle localization and its associated metabolic pathways.

H_2O_2 and NO, the possible links between PAs and stress responses, may act as two independent signaling molecules, but they are also interrelated in the PA-induced plant immune response, which could result in convergence (Pál *et al.*, 2015). Further studies are needed to elucidate the PA signaling pathway, especially their down-stream targets and the connections between PAs and other stress responsive molecules (Shi and Chan, 2014).

3.1 Metabolite profiling as a tool to investigate polyamine signaling pathway

Plants are constantly exposed to changing environments to which they respond by readjusting their cellular metabolome to efficiently utilize available resources and to ensure viability. These transitions often affect almost all levels of cellular organization, starting from gene expression to protein abundances and metabolite levels. Therefore, a systems biology-based analysis is particularly suited for understanding the responses of plants to changes in the environment (Töpfer *et al.*, 2014).

System biology approaches can offer the possibility to integrate data collected across different cellular levels to identify dependence between processes and also to aid in testing hypotheses concerning the behavior of individual component or pathways (Töpfer *et al.*, 2014).

Metabolomics is an approach focused on facilitating an improved understanding of metabolic networks and the subsequent biochemical composition of cells (Dixon *et al.*, 2006). Metabolites are often simply viewed as one of the end products of gene-protein complex dynamics. Nevertheless, it is widely accepted that metabolites themselves modulate macromolecular processes as signaling molecules; therefore, metabolomic studies are intended to provide an integrated view of the functional status of an organism (Dixon *et al.*, 2006).

Metabolite profiling can elucidate links and relationships that occur primarily through regulation at the metabolic level (Fiehn *et al.*, 2000). The essence of this approach is to discover novel marker metabolites and to determine relative changes of metabolite pool sizes in comparison to reference samples (Fernie *et al.*, 2004). In *Arabidopsis,* it has been applied successfully since the early 2000s (Fiehn *et al.*, 2000), and with the passing of time the refining of techniques associated to this approach, became an essential aim in plant system biology.

In the context of META-PHOR project (METAbolomics for Plants Health and OutReach: http://www.meta-phor.eu/index.php), the high reproducibility of Gas Chromatography-Electron Impact-Time of Flight-Mass Spectrometry (GC-EI-TOF-MS), a combination of techniques and equipment, on which several plant metabolomic studies have been based, was proven (Allwood *et al.*, 2009).
At the present time, metabolomics is recognized as a strong tool of plant system biology, to characterize molecular phenotypes (Weckwerth, 2011). It has become increasingly common in plant physiology and biochemistry, since it has been applied to a staggering number of conditions including water, salt, ionic, temperature, light and oxidative stress (Obata and Fernie, 2012), as well as developmental process such as senescence (Gibon *et al.*, 2006; Watanabe *et al.*, 2013). Lately, its application has been extended to nutritional research (O'Gorman and Brennan, 2015).

PAs have been deemed important in preparing the plant for stress tolerance and to directly ameliorate the causes of stress (Minocha *et al.*, 2014); however, it is widely thought that their role on stress signaling remains to be elusive. To understand the interconnections of PA biosynthesis, its catabolism, and conjugation along with PA signaling, requires detailed metabolomic studies among allelic variants, which will provide a framework for unraveling genetic determinants (Rangan *et al.*, 2014). Therefore, metabolite profiling can be an appropriate approach towards elucidation of PA signaling pathway.

3.2 Plant developmental processes related to this study

3.2.1 Physiological aspects of root branching establishment

In *Arabidopsis,* the root system consists of an embryo-derived primary root (PR) from which secondary lateral roots (LR) are continuously produced, shaping what is called root system architecture (RSA) (Vilches-Barro and Maizel, 2015), a structure of tremendous importance for a range of processes, since it is the

main interface between a plant and its soil environment, being responsible for anchoring, storage and nutrient and water uptake (Tian *et al.*, 2014).

RSA is the combined result of PR and LR growth and is influenced by both intrinsic genetic programs and external signals. Formation of LR entails the specification of founder cells. The process initiates when these founders divide and create a dome-shaped formation named primordium, which has to cross three overlying tissues to emerge at the surface of the parent root (Vilches-Barro and Maizel, 2015). The mechanisms that control the development and growth of PR and LR have mainly been explored separately. It is known that the initiation on both is different with respect to origin and tissue. However, there are similarities at the mechanistic level and with respect to key players involved in PR and LR initiation and development (Tian *et al.*, 2014).

In *Arabidopsis* and many crop species, most of the overall RSA is generated by *de novo* formation of LR in a process mediated by the plant hormone auxin (Manzano *et al.*, 2014), which also influence PR formation (Rigas *et al.*, 2013; Sato *et al.*, 2014). It has been established that auxin-mediated NO signaling is an important process on root branching (Pagnussat *et al.*, 2002; Kolbert *et al.*, 2008; Fernández-Marcos *et al.*, 2012; Correa-Aragunde *et al.*, 2013), and ROS signals are of extreme importance during this process (Tsukagoshi *et al.*, 2010; Manzano *et al.*, 2014; Correa-Aragunde *et al.*, 2015). On the other hand, ABA reversibly inhibits LR development (De Smet *et al.*, 2003), and stimulates stem cell dynamics in root meristems in a coordinated process with LR emergence (Zhang *et al.*, 2010).

However, both PR and LR possess distinct growth dynamics in response to environmental cues, which strongly implies that there might be LR-specific regulators mediating these processes (Tian *et al.*, 2014). In fact, recent reports showed that PR and LRs have differential growth dynamics in response to salt stress in a process mediated by ABA signaling (Ding and De Smet, 2013). In

that context, the assumption that PR and LR have additional different hormone signaling components remains a fascinating subject for further study.

Interestingly, perturbation of PA catabolism in *Zea mays* has showed important changes on root phenotype (Tisi *et al.*, 2011). Interactions between PAs and ROS on root signaling have been demonstrated (Pottosin *et al.*, 2012, 2014); however, few studies relate PAs with RONS on this aspect of plant development. Furthermore, few studies relate PAs as molecules with recognized role on stress protection with possible changes on RSA upon stress events. A leap forward might be expected of improved phenotyping approaches enabling the study of root systems as a whole, in combination with focused transcriptomic, proteomic, and metabolomic approaches (Tian *et al.*, 2014).

3.2.2 Polyamine signaling during leaf senescence

Senescence is an integral part of the normal developmental cycle of plants and can be viewed on a cell, tissue, organ or organization level (Pandey *et al.*, 2000). Leaf senescence is basically governed by the developmental age, but is influenced by both internal and environmental signals. The internal factors would include reproductive structures and hormones, whilst environmental factors include biotic and abiotic stresses, which generally accelerate the process (Khanna-Chopra, 2012). A process generally occurs in senescent leaves resulting in the coordinated degradation of macromolecules and the subsequent mobilization of components to other parts of the plants. Chloroplasts are involved in sustaining the energy requirements for the progression of senescence and develop into gerontoplasts (Del Duca *et al.*, 2014). Yellowing is well known to be due to the preferential degradation of chlorophyll over carotenoids (Del Duca *et al.*, 2014). Concomitantly with chlorophyll release and degradation, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is also degraded (Park *et al.*, 2007). Exogenous addition of SPD or SPM inhibited protein degradation, chlorophyll loss and stabilized thylakoid proteins such as RuBisCO (Del Duca *et al.*, 2014)

On the other hand, a recent report demonstrated that NO not only has an important role in dark-induced senescence signaling process (Niu and Guo, 2012) but also, when deficient, is able to cause a major acceleration of chlorophyll breakdown and loss of stability of thylakoid membranes (Liu and Guo, 2013). Other reports have established a clear relationship between endogenous enhancement of anti-oxidative capacity and tolerance response, when oxidative stress was imposed by dark-induced senescence treatment on detached leafs of tobacco and wheat (Hui *et al.*, 2012; Fotopoulos and Kanellis, 2013). Stabilization of plant cell membranes and their protection by PAs from damage produced during dark-induced senescence have been observed in a number of studies (He *et al.*, 2002; Liu *et al.*, 2007; Del Duca *et al.*, 2014). The anti-senescent properties of PAs have been associated with various mechanisms including their ability to act as free radical scavengers and inhibitors of lipid peroxidation (Del Duca *et al.*, 2014). PAs and the senescence-related hormone ET share a common precursor: SAM. Hence, PAs and ET could act in an antagonistic manner competing for the common substrate. Whereas ET would contribute to senescence and fruit ripening, PAs would favor growth and inhibition of aging-associated process. However, the antagonistic effect between PAs and ET is not always observable (Bitrián *et al.*, 2012). Thus, elucidation of PA signaling pathway during senescence might shed light over this process.

Examples have been reported where the PA levels do not correlate with stress tolerance, which also confirms that “the more the better” phrase cannot be true in all cases (Pál *et al.*, 2015). How do PAs act as signal molecules? They may have many modes of action and this is the starting point of this work. Contributions in this matter seem to be necessary. The use of PAO inhibitors as well as loss-of-function mutants could be promising tools in these experiments. Abiotic and biotic stresses are the primary cause of plant losses worldwide, and thus approaches employing genetic modification aimed at overcoming severe environmental stresses need to be quickly implemented (Pathak *et al.*, 2014).

JUSTIFICATION AND AIMS

Peroxisomal PAO subfamily (AtPAO2, AtPAO3 and AtPAO4) are proven to fall into one phylogenetical branch (Takahashi *et al.*, 2010), and even though they share high identity in their amino acid sequence (Fincato *et al.*, 2011), they have been suggested to have distinct physiological roles. AtPAO4 is proven to participate either in PA catabolism or back-conversion pathway. Previous studies have established *knock-out* and *knock-down* mutations of this PAO, resulting in increases of SPM levels (especially in roots).

Understanding the interconnections of PA along with PA signaling is vital. In that sense, a detailed metabolite profiling among PA gene and allelic variants, will provide a framework for unraveling genetic determinants (structural/regulatory) with genomic assisted analyses (Rangan *et al.*, 2014).

Even though a potential signaling role has been recognized for SPM through transcriptional approaches, global metabolomics profiles in engineered plant genotypes endogenously affected in SPM levels are still missing. Such studies might provide clearer links between genotypes and stress-tolerance phenotypes, as well as a better integration of PAs in the context of global metabolic networks (Bitrián *et al.*, 2012). The identification of PA-regulated down-stream targets and the discovery of connections between PA and other stress-responsive molecules have opened up new possibilities to investigate the function of individual PAs at the transcriptional, translational and molecular levels (Pál *et al.*, 2015).

To date, assessing plant metabolome related with enzymes involved in PA homeostasis and nitro-oxidative response is a pending matter. Therefore, the main aim of this study was:

- **To use plant metabolite profiling as a comprehensive approach, in order to elucidate possible metabolic pathways involved with AtPAO4 which could allow us to understand more about PA signaling pathway relying on the function of this *Arabidopsis* PAO**

Specific objects of this project were:

1. Polyamine oxidase 4 (PAO4) has been characterized as the major isoform in *Arabidopsis* roots (Kamada-Nobusada *et al.*, 2008). Consequently, the first aim was to elucidate the role of PAO4 in root architecture phenotype as well as the implication of SPM in stress response after nitro-oxidative stress imposition.

2. *PAO4* expression was observed in the whole plant body irrespective of developmental stages (Takahashi *et al.*, 2010). In consequence, the second aim was to study the effects of abolished *AtPAO4* expression on detached leaves after dark-induced senescence.

3. SPM accumulation is related to protection against drought stress in several species (Wi *et al.*, 2006; Yamaguchi *et al.*, 2007; Li *et al.*, 2015). Consequently, the third aim was to elucidate if SPM accumulation in *pao4* mutants can lead to drought stress tolerance.

MATERIALS AND METHODS

1. Plant material

All *Arabidopsis thaliana* wild type (WT) plants (Lehle seeds, USA) and T-DNA insertional mutants used during the progress of this study were of ecotype Columbia-0 (Col-0) genetic background, with the mutants belonging to the SALK collection (Alonso *et al.*, 2003) and being distributed by the Arabidopsis Biological Resource Center (ABRC).

- SALK_133599, T-DNA insertion on the second intron of *AtPAO4* gene
- SALK_109229, T-DNA insertion on the ninth exon of *AtPAO4* gene

1.1 Isolation of *pao4* mutants and gene expression analyses

Sequences and genetic resources were obtained from The Arabidopsis Information Resources (TAIR). https://www.arabidopsis.org/index.jsp.

1.1.1 Oligonucleotides

Table 1. Oligonucleotides used for isolation of *pao4* mutants.

Oligo	Sequences
Pao4-1 Fw	5'- TTCCGATAAGCTTCGTCGTTG -3'
Pao4-1 Rv	5'- TGGAGTCATCCCCGCTAGTTC -3'
Pao4-2 Fw	5'- GGTGGTCATGGTCTAATGGTG-3'
Pao4-2 Rv	5'- GAGAGGCACAGTTGCAGTTTC-3'
SALK-LB	5'-TTTGGGTGATGGTTCACGTAGTGGG-3'
Actin2-Fw	5'-TCACCACAACAGCAGAGCGGGA -3'
Actin2-Rv	5'-GATTCAGATGCCCAGAAGTCT -3'

Actin oligonucleotides were used for normalization (Sigma-Aldrich; United States). T-DNA left border (LB) and *pao4* primers were used at *AtPAO4* gene sequence (Fig. 6)

Fig. 6 AtPAO4 genomic DNA sequence. T-DNA insertions and primers anneling sequences are marked

1.1.2 Small scale DNA preparation

In order to confirm the presence of T-DNA insertion in mutant plants, total DNA was extracted from 20 independent 4-week-old plants tousing DNeasy Plant Mini kit (QIAGEN; The Netherlands) following manufacturer's instructions. The position of the T-DNA insertion in SALK_109229 was confirmed by PCR using a combination of *AtPAO4* specific gene primers (pao4-2-Fw and pao4-2 Rv) and T-DNA primer SALK-LB. For SALK_133599, SALK-LB was used in combination with *AtPAO4* specific primers (pao4-1 Fw and pao4-1 Rv).

1.1.3 RNA extraction and cDNA synthesis

Total RNA was isolated from 4-week-old *Arabidopsis* leaves using TRIzol (Invitrogen, Life Technologies; United States). Total RNA was treated with DNase I (RNase-free; Promega USA; United States) and reverse-transcribed using the SUPERSCRIPT III First-Strand Synthesis kit (Invitrogen), following manufacturer's instructions using 2.5 µg RNA as starting material.

1.1.4 Lack of expression by sqRT-PCR

PCR from equal amounts of cDNA was performed using *AtPAO4*-specific primers and TaKaRa Ex Taq^{TM}. Amplification of the *Arabidopsis Actin 2* gene (AT3G18780.2) was used for normalization. The PCR conditions were as follows: 96 ºC 5 min, followed by 35 cycles (5 s at 96 °C, 10 s at 64 °C, and 40 s at 72 °C). PCR products were separated on a 1.0% (w/v) agarose gel. The analysis was repeated three times with identical results.

1.2 *Arabidopsis* grown on soil

20-30 seeds were stratified for 3 days in the dark at 4 ºC and sown in 3'' square pots containing a mixture of soil and vermiculite (1:1 [v/v]), irrigated with water

and Hoagland-based mineral solution and grown at 21 ºC under long-day photoperiod (16 h of white fluorescent light, photon flux of 70–90 mmol m^{-2} s^{-1}). 7-9 days after sowing excess plants were removed, leaving 1 plant per pot.

1.3 *Arabidosis* grown *in vitro*

For *in vitro* culture, seeds were vapor-phase sterilized with chlorine gas (Cl_2) for 3 h in a closed desiccator. Chlorine gas was obtained mixing 100 ml of 30% Sodium Hypochlorite (Panreac; Spain) with 5 ml of 9 N HCl (Panreac). Seeds were stratified for 3 d at 4 °C and grown on agar media (described below) on controlled-environment chamber at 21 ºC under long-day photoperiod (16 h of white fluorescent light, photon flux of 70–90 mmol m^{-2} s^{-1}). After 4 d, seedlings were transferred to Petri dishes containing different treatments.

1.3.1 Media composition

Control media were prepared using 4,2 g/L Murashige and Skoog medium (Duchefa; The Netherlands) containing B5 Gamborg vitamins and 1% sucrose, with the pH adjusted to 5.8 with KOH before autoclaving. Solid media were obtained by addition of 0.8% (w/v) Plant agar (Duchefa), with the exception of root elongation assays where 1% (w/v) Plant agar was added. Media were autoclaved at 120 ºC for 30 min. For preparation of treatments, control media were cooled to 50-60 ºC and subsequently supplemented with varying amounts of filter-sterilized H_2O_2 (Merck) and NO-donor Sodium Nitroprusside (SNP) (Sigma-Aldrich; United States) using 0.22 µm membranes (Merck-Millipore; Germany).

1.3.2 Root elongation assays

4 d-old seedlings were transferred approximately 2 cm from the top edge of a 120 x 120 cm square Petri plate containing control media supplemented either with 0.25, 0.5 or 1 mM H_2O_2 for oxidative response observation or 30, 60 or 90

µM SNP for nitrosative response observation. Nine seedlings were placed per plate (three for each mutant and WT). Plates were wrapped with parafilm® and placed vertically in a growth chamber under conditions previously described for 7 d.

1.3.3 Quantification of RSA

Digital images of plant roots were taken using a Canon EOS 450D digital camera (Canon; Japan) and were subsequently traced by hand using ImageJ software with SmartRoot *plug-in* (Lobet *et al.*, 2011).

2. Stress treatments

All treatments were done with at least 5 biological replicates per group and 3 technical replicates per replicate. Samples were prepared pulverizing plant material with TissueLyzer® (QIAGEN; The Netherlands) and metal beads on liquid nitrogen environment. All samples were stored at -80 ºC until further analysis.

2.1 Treatments on *Arabidopsis* grown on soil

2.1.1 Dark-Induced senescence

Dark-induced senescence was carried out on adult plants. Leaves from 4-week-old plants were used for all analyses. Dark-induced senescence was established essentially as described (Fotopoulos and Kanellis, 2013). Fully expanded leaves were excised and floated on dH_2O in 25 mm-diameter Petri dishes, incubating in the dark at ambient temperature for a period of 4 d. 6 biological replicates per group were used with 3 technical replicates for each. After incubation period, excess water was removed from the leaves and samples were maintained on liquid nitrogen until further analysis.

2.1.2 Drought

Drought treatment was carried out on adult plants essentially as described (Alcázar *et al.*, 2010*b*). After stratification, plants were grown in a growth chamber for 4 weeks with normal watering every 3 d. After the fourth week, plants were divided (6 of each group per tray). A total of 8 trays were prepared, 4 trays for control conditions and 4 trays for drought treatment. Control group was watered normally. At the beginning of drought, trays assigned to this treatment were watered. After 1 h, pots were placed above filter paper to remove excess water, and the treatment was completed by withholding watering for 10 d. After water deprivation, plants were re-watered and returned to their original growth conditions. Tolerance to drought was determined as the capacity of plants to resume growth after 7 d of recovery under control conditions.

2.1.2.1 Physiological measurements

Net photosynthetic rates, intercellular CO_2 concentration, and water-related parameters were measured on rosette leaves using a LI-COR 6400 portable gas analysis system with a light-emitting diode light source (LI-COR Inc.; United States) and *Arabidopsis* adapted chamber. Three periods of measuring were stablished: At beginning of drought, before re-watering and 2 d after recovery. Leaves from 6 random plants of each group of samples were measured. The interval set-up for each measurement between leaves was 5 min.

2.2 Treatments on *Arabidopsis* grown *in vitro*

All treatments were initiated with 12 d-old *Arabidopsis* seedlings grown horizontally, on nylon membranes above solid control media.

2.2.1 Oxidative stress

Seedlings were grown on 120 x 120 Petri plates containing control media and sterilized nylon membranes (Nytran®, 0.45). 6 plants of each group were placed per plate. At least 6 plates were used for every experiment. Membranes containing 12 d-old seedlings were transferred to 120 x 120 Petri plates supplemented with 2 mM H_2O_2. Plates were wrapped with parafilm® and placed horizontally in a growth chamber for 10 d under conditions previously described.

2.2.2 Nitrosative stress

Seedlings were grown on 120 x 120 Petri plates containing control media and sterilized nylon membranes (Nytran®, 0.45). 6 plants of each group were placed per plate. At least 6 plates were used for every experiment. Membranes containing 12 d-old seedlings were transferred to 120 x 120 Petri plates supplemented with 500 µM SNP. Plates were wrapped with parafilm® and placed horizontally in a growth chamber for 2 d under conditions previously described.

3. Physiological parameters on *pao4* mutants

3.1 PA analysis by High Performance Liquid Chromatography (HPLC)

PAs were analyzed by high-performance liquid chromatography (HPLC) separation of dansyl chloride-derivatized PA as described (Marcé *et al.*, 1995)

3.1.1 PA extraction from plant material and dansylation

Samples were kept on ice during initial process of extraction until dansylation. 300 mg of frozen and pulverized plant material were homogenized with 1 ml of 5% PCA [v/v] (Sigma-Aldrich). Homogenate was centrifuged at 21.000 g during

20 min. The supernatant contained free and conjugated PAs while the pellet contained non-soluble PAs.

Dansylation was made with an aliquot of supernatant. 100 µl of supernatant fraction was mixed with two volumes of 5 mg/ml Dansyl Chloride (Sigma-Aldrich), one volume of saturated Na_2CO_3 (Sigma-Aldrich) and 20 µl of 1,7-Diaminoheptane (HTD) (Sigma-Aldrich), a synthetic PA that was used as an internal standard. The mix was incubated for 1 h on agitation at 60 ºC in the dark.

Dansyled PAs were extracted with 250 µl of Toluene through vigorous agitation for 1 min, followed by 2 min centrifugation at 21.000 g. 200 µl of organic phase was transferred to 2 ml Eppendorf® tube to be evaporated in a SpeedVac Evaporator (GeneVac; United States) for 30 min. Dry residue was dissolved in 700 µl of acetonitrile (ProLabo; France) and filtrated using 0.45 µm membranes for separation by HPLC.

3.1.2 Preparation of PA standards

PA standards were obtained as hydrochloride salts (Sigma-Aldrich; United States). Solutions of PAs were prepared in water at 10 µM concentration. PAs were dansylated as previously described. Elution times used for calculations are indicated in the following table:

Table 2. Elution time for PA standards separated by HPLC

Polyamine	Elution time (min)
Putrescine	6.690
HTD	8.500
Spermidine	9.340
Spermine	10.570

3.1.3 Separation of PAs by reversed phase HPLC

20 µl aliquots of dansylamines dissolved on acetonitrile, were applied to a reversed phase column Sphereclone ODS(2) C_{-18} (250 x 4.6 mm) with particle size of 5 microns and pore diameter of 80 Å (Phenomenex; Unites States), preceded by a precolumn with similar characteristics. Separation was achieved with a gradient elution solvent system of water:acetonitrile (70:30) maintained by a biphasic pump Perkin-Elmer 200. The eluent was monitored with a fluorescence detector Applied Biosystems Spectroflow 980 (Applied Biosystems, Life Technologies; United States), using an excitation and emission wavelength of 252 and 500 nm, respectively.

3.1.4 Calculations and quantification

Integration of peaks was made with TotalChrom II® software (Perkin Elmer; United States). Standard curves absorbance/concentration were prepared for each PA examined.

$$\text{PA (nmol/ g FW)} = K \frac{\text{PA area}}{\text{HTD area}}$$

3.2 Quantification of RONS and stress-related parameters

3.2.1 H_2O_2 quantification assay

H_2O_2 quantification assay was made essentially as described (Velikova *et al.*, 2000). 0.01 g of pulverized tissue was homogenized in 1 ml 0.1 % [w/v] TCA on ice. Homogenate was centrifuged at 15.000 g for 15 min at 4º C. From each supernatant, an aliquot of 0.5 ml was added to a mix of 0.5 ml of 10 mM phosphate buffer pH 7 and 1 ml of 1 M KI. After gentle agitation, absorbance

was followed for 1 min at 390 nm. The highest value during that period was used for calculations. H_2O_2 was quantified taking into account a calibration curve using solutions with known concentrations. Results were expressed as μmol H_2O_2 g^{-1} fresh weight.

3.2.2 NO quantification

Nitrite-derived NO content was measured using the Griess reagent in homogenates prepared with Na-acetate buffer (pH 3.6) as described by (Zhou *et al.*, 2005). 0.02 g of pulverized tissue was homogenized in 1 ml of buffer containing 50 mM Acetic acid (cool) and 4% [w/v] $Zn(O_2CCH_3)_2(H_2O)_2$ pH 3.6. Homogenate was centrifuged at 10.000 g for 15 min at 4º C. The supernatant was preserved (S1) and pellet was washed with 0.5 ml of extraction buffer. Homogenate was centrifuged at 10.000 g for 15 min at 4º C. The supernatant was mixed with S1 to make a total supernatant. 5 mg of charcoal were added to total supernatant to remove the color in the reaction. The mix was centrifuged at 15.000 g for 15 min at 4º C. Clear total supernatant was added to one volume of Griess reagent (Merck; Germany). Mix was incubated at room temperature for 30 min. The absorbance was detected at 540 nm. NO content was calculated by comparison to a standard curve of $NaNO_2$.

3.2.3 Lipid peroxidation quantification (MDA)

Lipid peroxidation was determined measuring MDA content resulting from the TBA reaction using an extinction coefficient of 155 $mM^{-1}cm^{-1}$, as described by Hodges (Hodges *et al.*, 1999). 0.01 g of pulverized tissue was homogenized in 1 ml of 0.1 % [w/v] TCA on ice. Homogenate was centrifuged at 15.000 g for 15 min at 4º C. From each supernatant an aliquot of 0.5 ml was added to 1.5 ml of 0.5% TBA [w/v] diluted in 20% TCA. The mixture was incubated at 95 ºC for 25 min. Reaction was terminated on ice. The reaction absorbance was determined at 532 and 600 nm.

The concentration of the MDA content was estimated by the following equation.

$$\text{MDA (nmol/ml)} = \left(\frac{\text{A532-A600}}{155000}\right) 10^{6}$$

3.3 Analysis of protein content

3.3.1 Protein extraction

Total protein was extracted with phenol, as previously described (Wang *et al.*, 2006). 0.2 g of pulverized tissue was mixed with 0.05 mg of PVP under liquid nitrogen until a fine powder was obtained, then adding 1.75 ml of 10% TCA. The mix was vortexed and centrifuged at 16.000 g for 3 min at 4 ºC. The supernatant was discarded and pellet was resuspended in 1.7 ml of 0.1 M $CH_3CO_2NH_4$ diluted in methanol. The mix was vortexed and centrifuged at 16.000 g for 3 min at 4 ºC. The supernatant was discarded. Pellet was resuspended in 1.75 ml of 80% cold acetone. The mix was vortexed and centrifuged at 16.000 g for 3 min at 4 ºC. Supernatant was discarded and pellet was dried in Speed-Vac evaporator for 10 min at 35 ºC.

Dried material was resuspended in 0.8 ml of buffer containing 30% sucrose, 2% SDS and 80 mM DTT pH 8. The mix was vortexed for 1 min, incubated 5 min on ice and centrifuged at 16.000 g for 3 min at 4 ºC. An aliquot of 400 µl of upper phase was transferred to a new tube containing 1.6 ml of 0.1 M $CH_3CO_2NH_4$ diluted in methanol, and was incubated at -20 ºC for 30 min. The mix was centrifuged at 16.000 g for 3 min at 4 ºC and the supernatant was discarded. The pellet was resuspended with 2 ml of 100% ice cold methanol and centrifuged at 16.000 g for 3 min at 4 ºC. The supernatant was discarded and the pellet was resuspended with 2 ml of 80% ice cold acetone and centrifuged at 16.000 g for 3 min at 4 ºC. Supernatant was discarded and pellet was dried

in a Speed-Vac evaporator for 10 min at 35 ºC. Pellet was stored at -80 ºC until protein quantification.

3.3.2 Protein quantification

Protein concentration was determined using Bradford reagent (BioRad; United States), diluted to a final concentration of 20 µg/µL, and stored at -20°C. 20 µg of total protein extracts were separated by SDS-PAGE in 12.5% acrylamide gels. Bands were resolved using Colloidal Comassie Brilliant Blue G-250 stain.

3.4 Chlorophyll quantification

Leaf pigments were extracted from 12 mm leaf discs in dimethyl sulfoxide as described by (Richardson *et al.*, 2002). Carotenoid and chlorophyll concentrations were determined using the equations described by (Sims and Gamon, 2002). 700 µl of pre-heated DMSO was added to a 2 ml eppendorff® tube containing 10 mg of fresh tissue. The pigments were extracted by incubating for 30 minutes at 65 ºC. After incubation, 300 µl of DMSO was added to get the extract to a final volume of 1 ml. Absorbance was determined at 470, 645 and 663 nm, calibrating to zero with DMSO.

$$\text{T Chl (mg/g FW)} = \left(8.02*A_{663} + 20.08*A_{645} \right) \frac{V}{1000*W}$$

$$\text{Chla(mg/g FW)} = \left(12.7*A_{663} - 2.69*A_{645} \right) \frac{V}{1000*W}$$

$$\text{Chlb(mg/g FW)} = \left(22.9*A_{645} - *4.68A_{645} \right) \frac{V}{1000*W}$$

$$\text{Carotenoids (mg/g FW) Xanthophylls} = \left(\frac{\left(1000*A_{470} - \right) 1.90\text{Chla} - 63.14\text{Chlb}}{214} \right) \frac{V}{1000*W}$$

4. Metabolomic Profiling by gas chromatography time-of-flight mass spectrometry (GC-TOF-MS)

Metabolite profiling was performed as previously described (Wagner *et al.*, 2003; Erban *et al.*, 2007) by gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) using an Agilent 6890N24 gas chromatograph (Agilent Technologies, Germany) with split and splitless injection onto a FactorFour VF-5ms capillary column, 30 m length, 0.25 mm inner diameter, and 0.25 µm film thickness (Varian-Agilent Technologies), which was connected to a Pegasus III time-of-flight mass spectrometer (LECO Instrumente GmbH, Germany). Soluble metabolites were extracted from 30 mg frozen material (fresh weight ±10 % tolerance) with a 30µL internal standard addition of 0.02 mg/mL $^{13}C_6$-sorbitol. Two successive steps of incubation were performed, methanol (15 min at 70 °C) and chloroform (5 min at 37°C), amounting to a final 330:230 (v/v) solvent ratio. A polar metabolite fraction enriched for primary metabolites and small secondary compounds was obtained by liquid partitioning into water/methanol from a methanol/water/chloroform solvent system (330:400:230, v/v/v). Aliquots of 80 µL from the polar metabolite fraction were dried by vacuum concentration and stored dry under inert gas at -20°C until further processing. Metabolites were methoxyaminated and trimethylsilylated manually prior to GC-TOF-MS analysis (Fiehn *et al.*, 2000; Roessner *et al.*, 2000; Wagner *et al.*, 2003; Lisec *et al.*, 2006; Erban *et al.*, 2007). Retention indices were calibrated by addition of a C_{10}, C_{12}, C_{15}, C_{18}, C_{19}, C_{22}, C_{28}, C_{32}, and C_{36} n-alkane mixture to each sample (Strehmel *et al.*, 2008).

GC-TOF-MS chromatograms were acquired, visually controlled, baseline corrected and exported in NetCDF file format using ChromaTOF software (Version 4.22; LECO, United States). GC-MS data processing into a standardized numerical data matrix and compound identification were performed using the TagFinder software (Luedemann *et al.*, 2008; Allwood *et al.*, 2009). Compounds were identified by mass spectral and retention time index matching to the reference collection of the Golm metabolome database (GMD, http://gmd.mpimpgolm.mpg.de/; (Kopka *et al.*, 2005; Hummel *et al.*,

2010) and to the mass spectra of the NIST08 database (http://www.nist.gov/srd/mslist.htm). The Guidelines for manually supervised metabolite identification were the presence of at least 3 specific mass fragments per compound with a retention index deviation < 1.0% (Strehmel *et al.*, 2008).
All mass features of an experiment were normalized by sample fresh weight, internal standard and maximum scaled. For quantification purposes all mass features were evaluated for best specific, selective and quantitative representation of observed analytes. Laboratory and reagent contaminations were evaluated by non-sample control experiments. Metabolites were routinely assessed by relative changes expressed as response in comparison to control condition, using logarithmic transformed data.

5. Statistical analyses and data visualization

Statistical analyses were performed using IBM® SPSS® Statistics V.22. Biochemical and physiological damage measurements were subjected to Analysis of Variance (ANOVA) or Student's t-test depending on the number of groups. Significant differences between individual means were determined using Tukey's HSD (Honestly significant difference) pairwise comparison test at the 5% confidence level. Data for analysis were $\log_2$-transformed response ratios.
Data from metabolomics were analyzed and heat maps obtained from MeV: MultiExperiment Viewer v.4.9 (A.I. Saeed, V. Sharov *et al.*, 2003). Proportional Venn diagrams were generated according to (Hulsen *et al.*, 2008). The 4-way Venn diagram was obtained by the Venny application (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

6. Metabolome analysis

Metabolic implications of reported altered metabolites in this work, further classification and simplified metabolic maps were made by the use of public database KEGG (Kanehisa and Goto, 1999; Kanehisa *et al.*, 2014) and AraCyc

developed by Plant Metabolic Network project (PMN) (Mueller *et al.*, 2003; Chae *et al.*, 2012).

RESULTS AND DISCUSSION

CHAPTER I

Loss-of-function of PAO4 modulates root system architecture by cross-talk between PA and ROS/RNS signals

ABSTRACT

PAs are molecules with diverse physiological roles on plant life cycle. These amine compounds are essential components of signaling mechanisms under challenging environments, leading to tolerance or survival. However, their metabolic or signaling targets are not well characterized. One of the essential elements regarding PA homeostasis is AtPAO4, a major isoform in *Arabidopsis* roots. T-DNA insertional mutants of AtPAO4 accumulated 10-fold more SPM and presented increased root length and LR density compared with WT Col-0 plants under normal growth conditions, suggesting a role for this enzyme on root architecture. In this study it was demonstrated that exogenous addition of H_2O_2 or NO donor SNP generates changes on *pao4* root architecture by increasing the number of LR and last LR site of insertion, both important elements to increase root surface of absorption. Cross-talk between PAs and RONS under abiotic stress conditions has been established. *Arabidopsis pao4* seedlings also demonstrated increased tolerance to nitro-oxidative stress. After both stresses, *pao4* mutants showed an increase in SPM content suggesting a link between its accumulation and the plant's overall tolerance capacity. In order to identify down-stream signaling targets, metabolomic profiling by GC-TOF-MS after H_2O_2 and SNP treatment revealed that the PA signaling pathway under nitro-oxidative stress conditions is related to PA biosynthetic precursors, glutathione homeostasis, Raffinose Family Oligosaccharides (RFO) biosynthesis pathway and *myo*-Inositol. Our results indicate that PA down-stream signaling following nitro-oxidative stress imposition relies on anti-oxidative capacity regulation, establishing a novel connection between PAs and RFO homeostasis.

1. RESULTS

1.1 Root system architecture on *pao4* mutants

The results reported in this work have been obtained from two PAO4 (At1g65840) T-DNA insertional mutants from the SALK collection. The first was a *knock-down* mutant which had the T-DNA insertion on the second intron of *AtPAO4* gene (SALK_133599) and was selected from 20 independent plants by sqRT-PCR using gene-specific *PAO4* primers. It has been previously reported as *pao4-1* with *knock-out* expression (Liu *et al.*, 2014*a*), although it was found to display residual PAO4 expression compared with WT Col-0. The second, was a mutant that had the T-DNA insertion on the ninth exon of *AtPAO4* gene and it has been reported in previous publications (Kamada-Nobusada *et al.*, 2008) as a *knock-out* mutant. We confirmed the lack of expression of *AtPAO4* using gene-specific *PAO4* primers and named it *pao4-2* (Fig. 7). Meanwhile, no macroscopical differences were observed under normal growth conditions, between plants carrying the T-DNA insertion and WT Col-0.

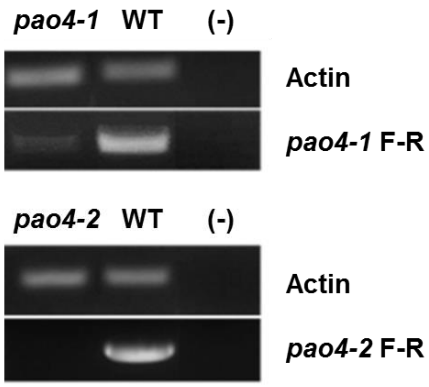

Fig. 7 *AtPAO4* expression. **G**ene expression of *AtPAO4* in *Arabidopsis thaliana* compared with WT Col-0. *AtPAO4* gene-specific primers were designed to confirm the partial or total lack of expression as previously described. The *pao4-1* mutants demonstrated considerably lower expression compared with WT (Liu *et al.*, 2014*a*), while *pao4-2* demonstrated total lack of expression (Kamada-Nobusada *et al.*, 2008).

1.1.1 Roots of *pao4* mutant presented alterations in root system architecture during normal growth conditions

It has been reported that perturbations of PA catabolism affect root development and root xylem differentiation in *Zea mays* (Tisi *et al.*, 2011). Although AtPAO4 has been detected in all plant organs independently of developmental stage (Takahashi *et al.*, 2010), this enzyme it is the major isoform in *Arabidopsis* roots (Kamada-Nobusada *et al.*, 2008); therefore, we evaluated if the lack of AtPAO4 has a consequence on RSA using root elongation assays (Fig. 8).

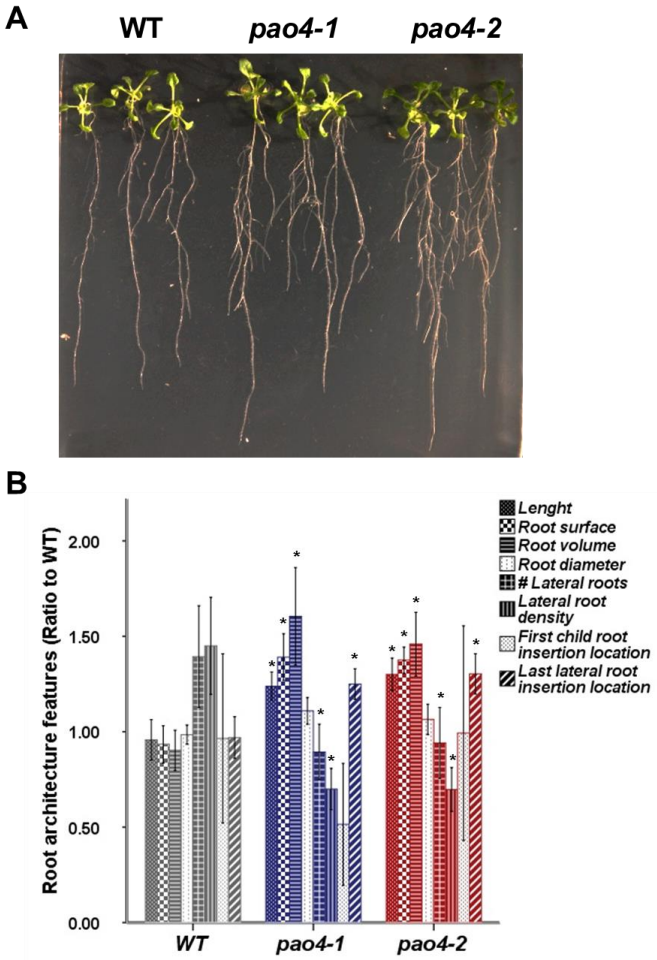

Fig 8. Root phenotypic analysis of *pao4* mutants under control conditions. **A** Seedlings after 7 days growing on vertically oriented plates containing MS medium supplemented with 1% (w/v) sucrose. **B** Root architecture features after 7 days growing on vertically oriented plates under control conditions. Results are presented as ratios relative to WT Col-0. Each point represents the mean value of at least six independent analyses, error bars refer to standard deviation. Asterisks indicate values which are significally different from those of the corresponding WT plants, as determined by both one-way ANOVA and Tukey HSD post-hoc tests (P<0.05).

Results revealed that some PR and LR features are altered in *pao4* mutants. By measuring the length of meristems and elongation zone, it was observed that LR of *pao4* mutants is significantly longer and presents increase on surface and

volume compared with WT (Fig. 8B). Regarding LR emergence, a decrease in the number of LR can be observed, a subsequent reduction in the density (i.e. measure of number of LR per unit length) and significant increase in last LR insertion location, which means that last LR is closer to root meristem (Fig. 8B). Previous studies on *Arabidopsis* roots had established that AtPAO4 has 10-times more affinity for SPM than SPD or T-SPM (Kamada-Nobusada *et al.*, 2008; Takahashi *et al.*, 2010; Fincato *et al.*, 2011).PAO4 has in fact been proposed as a key modulator of SPM homeostasis (Takahashi *et al.*, 2010); results presented herein demonstrated that loss-of-function mutants at seedling stage accumulate more than 2-fold SPM and have 0.5-fold SPD decreased compared with WT, when the expression is either totally null or partially interrupted in agreement with a number of previously reports (Kamada-Nobusada *et al.*, 2008; Takahashi *et al.*, 2010; Liu *et al.*, 2014*a*).

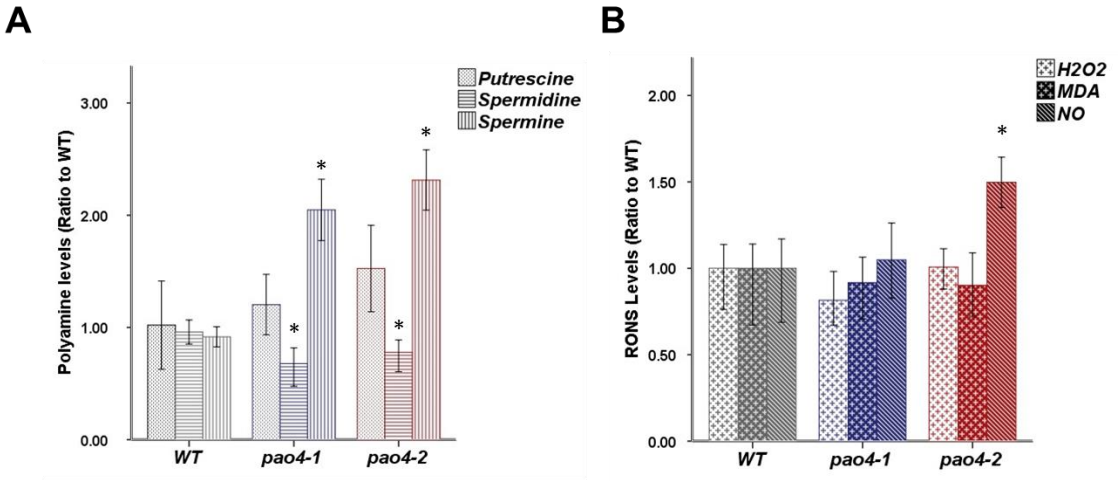

Fig. 9. Physiological measurements in *pao4* seedlings under control conditions. **A** PAs levels in *pao4* seedlings after 7 days growing on vertically oriented plates containing MS medium supplemented with 1% sucrose. **B** RONS levels in *pao4* seedlings after 7 days growing on vertically oriented plates containing MS medium supplemented with 1% sucrose . Results are presented as ratios relative to WT Col-0. Each point represents the mean value of at least three independent analyses, error bars refer to standard deviation. Asterisks indicate values which are significally different from those of the corresponding WT plants, as determined by both one-way ANOVA and Tukey HSD post-hoc tests (P<0.05).

Total lack of expression results in PUT increase with no significant difference compared with WT (Fig. 9A). Cross-talk between PAs and RONS molecules has been reported (Tanou *et al.*, 2012, 2014; Filippou *et al.*, 2013).In addition, RONS are known to be involved in root development and LR emergence (Flores *et al.*, 2008; Tsukagoshi *et al.*, 2010; Fernández-Marcos *et al.*, 2012; Manzano *et al.*, 2014; Tian *et al.*, 2014); subsequent measurements revealed no significant difference in ROS levels between *pao4* mutants and WT, while a significant increase in NO content was observed in *pao4-2* compared with WT (Fig, 9B).

Recent work established that PAs are able to reprogram oxidative and nitrosative status of citrus seedlings, leading to prime-like state that conferred salt stress tolerance (Tanou *et al.*, 2014). Therefore, we considered to evaluate if the mutants were tolerant to oxidative and nitrosative stress, and if the nature of a possible tolerance response relies on changes on RSA.

1.1.2 ROS and RNS donors enhanced LR features on *pao4* mutants

Recently it has been established that ROS signaling is specifically required during LR emergence (Manzano *et al.*, 2014). In consequence, 4 d-old *pao4* mutants were exposed to increasing concentrations of H_2O_2 ranging from 250 uM to 2 mM during 7 d. Tolerance to oxidative stress was observed starting from 500 μM H_2O_2 imposition (Fig. 10A). Significant increase in *pao4* mutants compared with WT was found not only in LR length but also in the number of LR and last LR insertion location (Fig. 10B).

NO is a molecule tightly related to RSA (Forde, 2014). More than a decade ago, this RNS was found to be necessary for root initiation (Pagnussat *et al.*, 2002). At present, it is known that NO plays an important role on cell elongation during LR growth (Fernández-Marcos *et al.*, 2012) and LR initiation (Lira-Ruan *et al.*, 2013) in *Arabidopsis*.

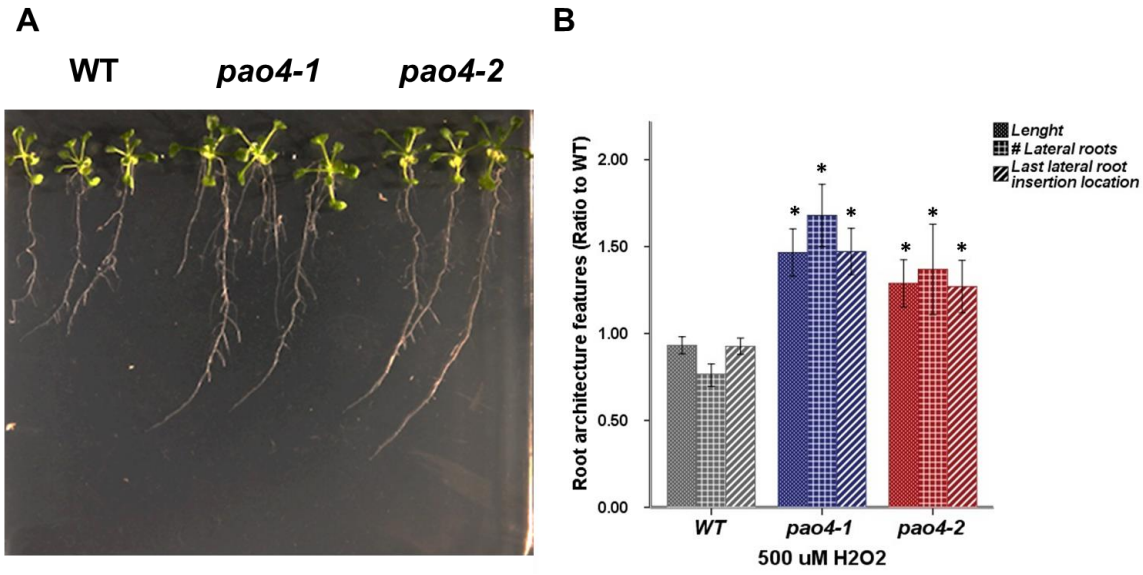

Fig. 10. Root phenotypic analysis of *pao4* mutants during oxidative stress imposition. **A**. Seedlings after 7 d growing on vertically oriented plates containing MS medium supplemented with 1% sucrose and 500 μM H_2O_2. **B** Root architecture features after 7 d growing on vertically oriented plates under oxidative stress. Results are presented as ratios relative to WT Col-0. Each point represents the mean value of at least six independent analyses, error bars refer to standard deviation. Asterisks indicate values which are significally different from those of the corresponding WT plants, as determined by both one-way ANOVA and Tukey HSD post-hoc tests (P<0.05)

Recently, the interconnection between PAs and nitrogen metabolism on root signaling has been reviewed (Moschou *et al.*, 2012*a*), although the contribution of PA oxidation by back-conversion pathway remains to be elusive. 4 d-old *pao4* mutants were exposed to increasing concentrations of NO-donor SNP, ranging between 30 μM to 1 mM. Nitrosative stress tolerance was observed starting from 60 μM (Fig. 11A). Significant increase was found on several features of root branching such as root length, surface, number of LR, last LR insertion location and also LR density (Fig. 11B).

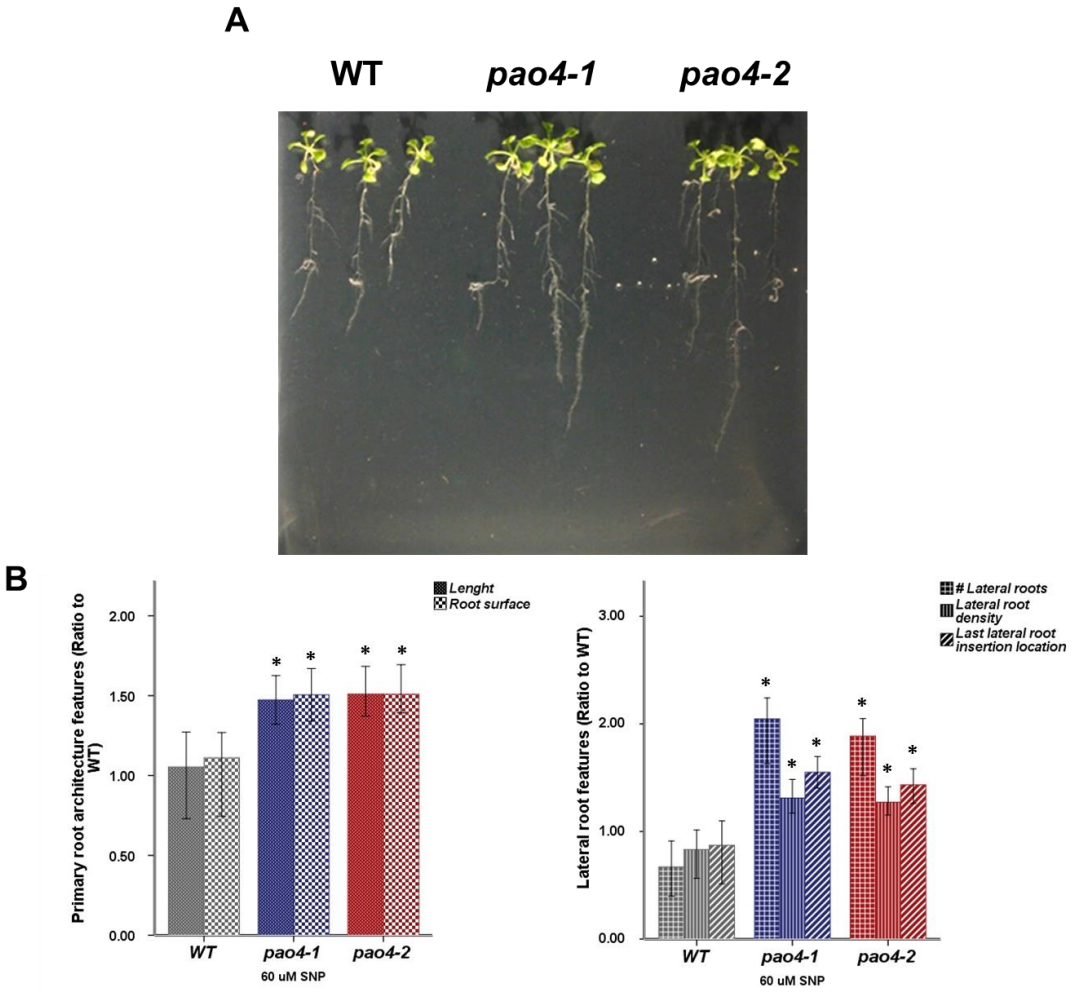

Fig. 11. Root phenotypic analysis of *pao4* mutants during nitrosative stress imposition. **A**. Seedlings after 7 days growing on vertically oriented plates containing MS medium supplemented with 1% sucrose and 60 μM SNP. **B** Root architecture features after 7 days growing on vertically oriented plates under nitrosative stress. Results are presented as ratios relative to WT Col-0. Each point represents the mean value of at least six independent analyses, error bars refer to standard deviation. Asterisks indicate values which are significally different from those of the corresponding WT plants, as determined by both one-way ANOVA and Tukey HSD post-hoc tests (P<0.05)

Given the fact that H_2O_2 and SNP cause similar increases in some features of *pao4* RSA, we attempted to elucidate *pao4* response after combining both ROS/RNS donors at the minimum concentration where an effect was observed, in order to establish if combination of both agents might result in an additive effect on *pao4* root phenotype (Fig. 12).

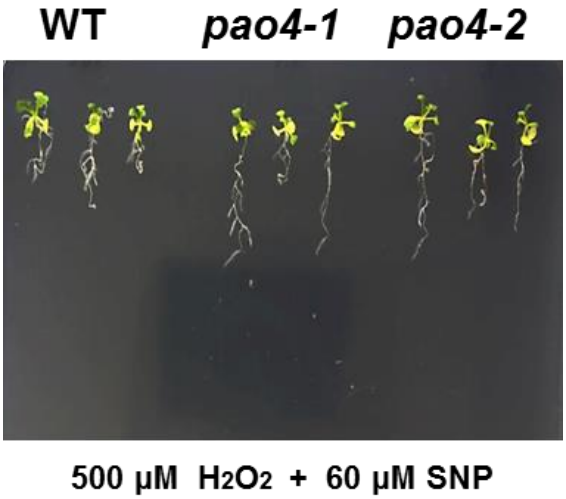

Fig. 12. Root phenotypic analysis of *pao4* mutants during nitro-oxidative stress imposition. Seedlings after 7 days growing on vertically oriented plates containing MS medium supplemented with 1% sucrose 500 µM H_2O_2 and 60 uM SNP.

Surprisingly, even though both H_2O_2 and SNP commonly result in a promoted increase on root features such as root length, number of LR and last root insertion location, *pao4* mutants were not able to cope with nitro-oxidative stress after exposure to both donors, suggesting a certain incompatibility on both responses (Fig. 12).

1.2 SPM homeostasis regulated by AtPAO4 is implied on a long-term oxidative response and a short-term nitrosative response

In order to determine the extent of oxidative and nitrosative stress tolerance on *pao4* mutants, we decided to expose 12 d-old seedlings to higher concentration of RONS donors on separate-experiments basis. Interestingly, both mutants were able to maintain stress tolerance phenotypes, although not in the same period. Tolerance to oxidative stress was evident after 10 d of 2 mM H_2O_2 treatment (Fig. 13AB), while tolerance to nitrosative stress was observed after 2 d of 500 µM SNP imposition (Fig. 14AB), suggesting a long-term *pao4* oxidative response and a short-term nitrosative response. In order to confirm the occurrence of oxidative stress, H_2O_2 and MDA levels were determined. Significant increase in H_2O_2 content between stressed and control samples could be observed after stress imposition; interestingly, MDA content only increased in *pao4* mutants suggesting the existence of a moderate hypersensitivity to this oxidative agent at the concentration applied (Fig. 13C).

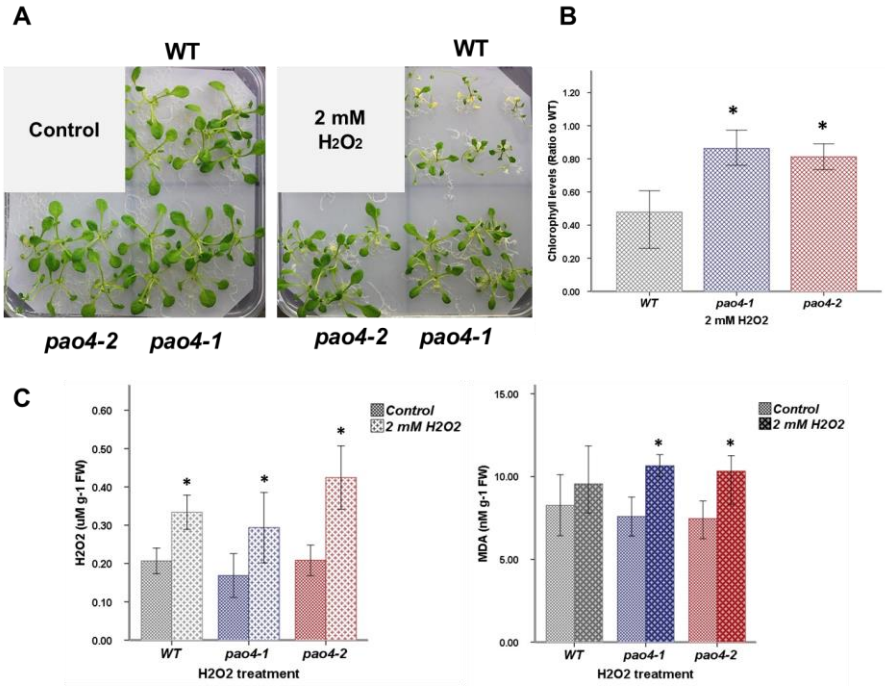

Fig. 13. Phenotypic analysis of *pao4* mutants after oxidative stress imposition. **A** 22 d-old *pao4* seedlings after 10 d of continuous exposure to 2mM H_2O_2 (See Materials and methods). **B** Total chlorophyll levels **C** ROS Levels, H_2O_2 (Left chart) MDA (Right chart). Each point represents the mean value of at least 3 independent analyses, error bars refer to standard deviation. Asterisks indicate values which are significally different from those of the corresponding WT plants or control samples (in case of ROS), as determined by both one-way ANOVA and Tukey HSD post-hoc tests (P<0.05)

Similarly, NO and MDA levels were determined to confirm nitrosative stress by SNP exposition.NO levels showed a significant increase compared with control samples, while MDA content did not show any difference compared with control samples suggesting that 500 µM SNP was not a toxic concentration (Fig. 14C). In both cases, SPM showed 5-fold increase while SPD maintained 0.5-fold levels compared with WT. Interestingly, PUT content was significantly different in *pao4-2* mutants compared with WT showing a 0.5-fold increase after oxidative stress and a 0.5-fold decrease after nitrosative stress, suggesting that PA homeostasis regulated by PAO4 is a key element of stress response (Fig. 15).

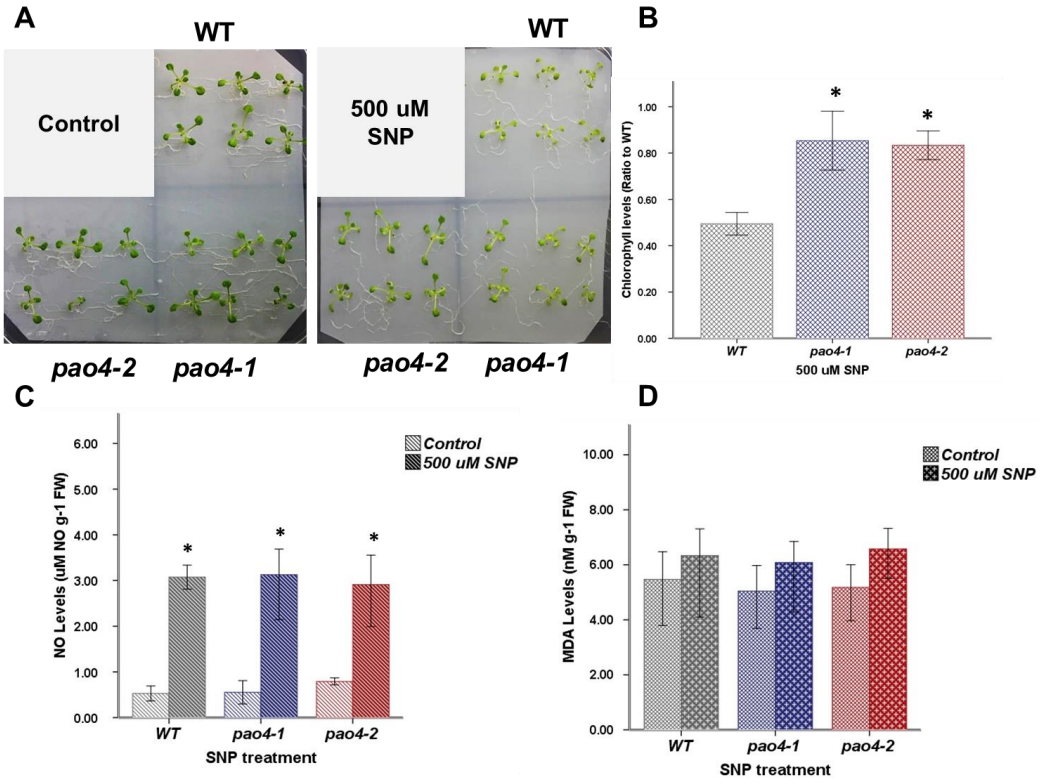

Fig. 14. Phenotypic analysis of *pao4* mutants after nitrosative stress imposition. **A** 14 d-old *pao4* seedlings after 2 days of continuous exposure to 500 μM SNP (See Materials and methods). **B** total chlorophyll content **C** NOcontent, **D** MDA content. Each point represents the mean value of at least 3 independent analyses, error bars refer to standard deviation. Asterisks indicate values which are significally different from those of the corresponding WT plants or control samples (In case of RONS), as determined by both one-way ANOVA and Tukey HSD post-hoc tests (P<0.05)

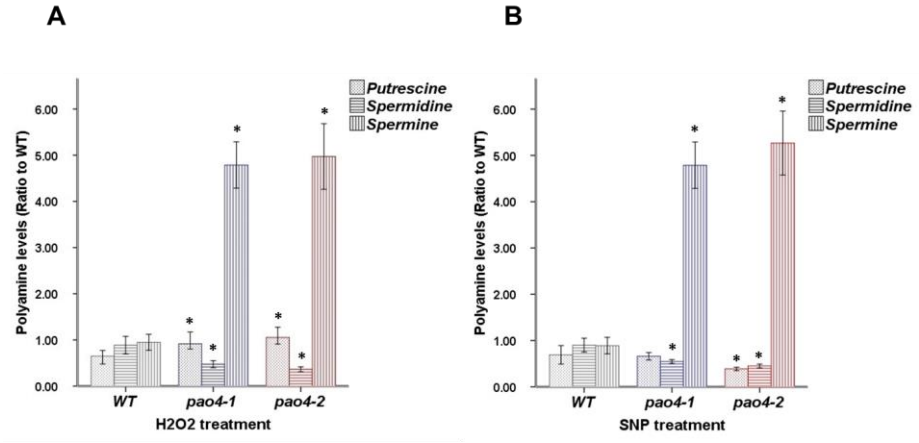

Fig. 15. Polyamine analysis of *pao4* mutants after oxidative and nitrosative stress imposition. **A** 22 d-old *pao4* seedlings after 10 days of continuous exposure to 2 mM H_2O_2 (See Materials and methods). **B** 14 d-old *pao4* seedlings after 2 days of continuous exposure to 500 μM SNP (See Materials and methods). Results are presented as ratios relative to WT Col-0. Each point represents the mean value of at least 3 independent analyses, error bars refer to standard deviation. Asterisks indicate values which are significally different from those of the corresponding WT plants, as determined by both one-way ANOVA and Tukey HSD post-hoc tests (P<0.05)

1.3 Metabolomic profiling after oxidative and nitrosative stress on *pao4* mutants revealed modulation of key elements on PA biosynthesis, glutathione homeostasis and RFOs pathway.

Systems biology analysis has identified AtPAO4 as a PA oxidation-related enzyme, positively correlated with genes involved in either biotic or abiotic stress responses and developmental process (Tiburcio *et al.*, 2014). Transcriptomic analysis in roots of AtPAO4-deficient mutants revealed that the majority of altered genes were grouped into two categories, the first one related with abiotic stress response with special mention to glutathione metabolism and the second with flavonoid and/or lignin metabolism (Kamada-Nobusada *et al.*, 2008). Nonetheless, down-stream targets of PAs in plant stress response remain unclear (Shi and Chan, 2014). In that sense, metabolomic studies provide a comprehensive view of how metabolic networks are regulated and identify metabolic flows in adverse environments (Obata and Fernie, 2012); therefore, we carried out metabolomic profiling of *pao4* mutant seedlings after long-term oxidative and short-term nitrosative stress imposition, in order to elucidate possible SPM targets on PA signaling pathway and also find common metabolites on nitro-oxidative response to identify possible metabolic fluxes. After oxidative stress 106 metabolites were detected. 45 altered metabolites were identified (Table 3), 14 of which presented lack of annotation data because the structure is currently unknown, although reported (Kopka *et al.*, 2005). Metabolites presenting lack of significant difference compared with WT were also described (*See Annex 1*).

Altered metabolites after oxidative stress are related with amino acid metabolism, pathways involved with oxidative response and fatty acid mobilization. Interestingly, most amino acids showed a significant strong correlation with pyroglutamate, PUT and glycerophosphoglycerol ($P \leq 0.05$; *See Annex 3*).

Table 3. Altered metabolites after oxidative stress. Metabolite levels were determined using seedling tissue after oxidative stress treatment (*see materials and methods*). Compound abundance is presented as log_2-transformed fold-changes realtive to the mean of WT-Col 0 samples. Altered metabolites were detected with MeV tool V.4.9 by rank product statistical test (P<0.05).

	ALTERED METABOLITES AFTER OXIDATIVE STRESS IMPOSITION					
	WT		***pao4-1***		***pao4-2***	
	Mean	**Std. Deviation**	**Mean**	**Std. Deviation**	**Mean**	**Std. Deviation**
Oxalate	-.197	.086	.330	.050	.776	.065
Arginine	-.111	.061	.826	.036	1.210	.082
Glutamate	.162	.062	.669	.049	.592	.051
Isoleucine	-.495	.050	.510	.049	.278	.047
Leucine	-.339	.110	.544	.061	.370	.021
Ornithine	-.034	.034	1.283	.063	1.289	.085
Pyroglutamate	-.305	.116	.422	.051	.305	.035
Tyrosine	-.214	.087	1.142	.053	.730	.087
Valine	-.254	.092	.509	.058	.427	.054
Carbodiimide	.037	.037	1.155	.061	1.124	.079
Putrescine	-.220	.104	.358	.031	.392	.035
Uridine	-.051	.044	.847	.043	.707	.040
Glycerophosphoglycerol	-.086	.055	.606	.035	.427	.039
Threonate	-.057	.044	.279	.036	.283	.050
Threonate-1,4-lactone	-.054	.042	.275	.022	.247	.058
Galactose	-.095	.126	.782	.032	.979	.045
Raffinose	.493	.081	.730	.075	.681	.072
A194007	-.092	.057	.586	.033	.309	.027
A217004	-.075	.051	.684	.038	.487	.040
A290003	-.064	.047	.591	.091	.254	.024
A296004	-.039	.081	.282	.022	.305	.074
A302001	-.118	.065	.620	.074	.462	.039
A308003	-.110	.008	.211	.031	.302	.022
Fumarate	-.121	.068	-.668	.048	-.963	.079
Proline	-.330	.205	-1.301	.091	-1.977	.088
Succinate	-.094	.056	-.905	.052	-.801	.082
Asparagine	-.306	.105	-.905	.231	-1.685	.193
Glutamine	-.027	.086	-.750	.208	-1.279	.128
Phenylalanine	-.277	.152	-.634	.069	-1.350	.133
Palmitate	-.287	.240	-1.132	.074	-1.289	.096
Estearate	-.563	.256	-1.122	.064	-1.150	.064
Sinapate	-.257	.096	-1.253	.097	-1.053	.092
Glycerate	-.112	.062	-.746	.074	-.854	.075
Glycerol	-.235	.145	-.724	.030	-.956	.035
***myo*-Inositol**	-.071	.120	-.619	.095	-1.626	.084
Galactinol	-.359	.113	-1.107	.048	-1.507	.038
Ribose	.013	.145	-1.385	.121	-3.463	.215
A143002	-.744	.285	-1.147	.077	-1.145	.086
A145016	-.299	.252	-1.569	.153	-1.722	.128
A203003	-.345	.116	-1.420	.139	-1.495	.174
A207003	-.060	.047	-.943	.175	-1.358	.104
A214003	-.154	.080	-.411	.065	-1.051	.044
A260482	-.088	.388	-1.756	.073	-1.714	.045
A278931	-.532	.253	-.951	.056	-1.306	.087
A311002	-.186	.164	-1.775	.055	-2.437	.040

Among the increased metabolites we could observe branched chain and aromatic amino acids, both reported to be involved on oxidative stress response

as alternative substrates for mitochondrial respiratory chain (Araújo *et al.*, 2010, 2011; Obata *et al.*, 2011) as well as precursors of flavonoids biosynthesis in the case of aromatic amino acids (Vlot *et al.*, 2009; Luis A, 2013), the anti-oxidative defense role of which has been established (Xie *et al.*, 2012; Nakabayashi *et al.*, 2014). Raffinose was also found to increase, which is involved in galactose and stachiose metabolism according to cited databases by means of raffinose family oligosaccharides pathway (RFO), lately associated with plant protection by anti-oxidative capacity acquisition in several species including *Arabidopsis* (Van den Ende, 2013; Elsayed *et al.*, 2014). Among decreased metabolites, intermediates of non-decarboxylating part of TCA cycle such as fumarate and succinate were observed in agreement with previous reports (Lehmann *et al.*, 2009; Obata *et al.*, 2011), as well as metabolites related with peroxisomal fatty acid β-oxidation and secondary metabolites biosynthesis, such as palmitate, stearate or glycerate.

After nitrosative stress, 104 metabolites were detected. 35 altered metabolites were identified (Table 4), 10 out of which presented lack of annotation data and have also been previously reported (Kopka *et al.*, 2005). Metabolites presenting lack of significant difference compared with WT were also described (*See Annex 2*). Altered metabolites after SNP treatment were identified as amino acids or compounds highly involved with C:N mobilization pathways (e.g. glycolysis, gluconeogenesis or glyoxylate pathway). With regard to increased metabolites, intermediates of TCA cycle such as citrate, aspartate (Asp), glycerate-3-phosphate and sucrose were identified. GABA was identified as one of the decreased metabolites, with its role being related with C:N balancing during stress response (Buchanan-Wollaston *et al.*, 2005). Interestingly, significant strong correlation was found between the levels of this molecule and nitrogen related metabolites (e.g. Orn, pyroglutamate) or carbon fixation metabolites (e.g. gluconate, erythronate, galactose, glycolate and ribose) ($P \leq 0.05$; *See Annex 4*), all of which decreased after nitrosative stress.

Table 4. Altered metabolites after nitrosative stress. Metabolite levels were determined using seedling tissue after nitrosative stress treatment (*see materials and methods*). Compound abundance is presented as log_2-transformed fold-changes realtive to the mean of WT-Col 0 samples. Altered metabolites were detected with MeV tool V.4.9 by rank product statistical test (P<0.05).

	ALTERED METABOLITES AFTER NITROSATIVE STRESS IMPOSITION					
	WT		***pao4-1***		***pao4-2***	
	Mean	**Std. Deviation**	**Mean**	**Std. Deviation**	**Mean**	**Std. Deviation**
Citrate	.183	.043	.706	.062	1.049	.108
Proline	.131	.094	.692	.154	.507	.049
Aspartate	-.145	.074	1.089	.100	.809	.074
Ethanolamine	-.184	.081	.348	.056	.269	.020
Glycerate-3-phosphate	-.158	.080	.486	.056	.466	.065
Glycerophosphoglycerol	-.013	.022	.219	.029	.477	.054
Phosphoric acid	-.024	.029	.137	.069	.156	.014
***myo*-Inositol**	-.098	.058	.593	.114	.599	.070
Galactinol	-.172	.082	.413	.049	.633	.065
Sucrose	-.019	.026	.153	.056	.190	.009
A112003	-.373	.197	.432	.226	.359	.142
A116201	-.215	.091	.276	.241	.652	.045
A196004	-.027	.030	.253	.033	.342	.036
A278931	-.166	.163	.707	.143	1.144	.091
A290003	-.012	.021	.422	.011	.452	.049
A296004	-.080	.056	.597	.086	.691	.053
A302001	-.122	.062	.525	.061	.569	.064
A313001	-.177	.079	.739	.062	.790	.093
Glycolate	.012	.080	-.578	.062	-.882	.156
Arginine	-.304	.108	-2.024	.237	-3.866	.219
Asparagine	.101	.142	-1.871	.033	-2.765	.258
4-Aminobutanoate (GABA)	-.037	.102	-.793	.056	-2.286	.131
Glutamate	-.018	.113	-.966	.227	-1.132	.231
Ornithine	-.700	.208	-2.106	.053	-2.589	.124
Putrescine	-.049	.073	-.397	.063	-.398	.118
Pyroglutamate	-.016	.101	-1.469	.058	-1.167	.095
Serine	.070	.086	-1.324	.179	-1.505	.166
2,3-Dihydroxy-Pyridine	-.125	.102	-.944	.102	-1.156	.040
3-Hydroxy-Pyridine	.346	.077	-1.614	.178	-2.680	.128
Erythronate-1,4-lactone	-.055	.103	-1.167	.104	-1.552	.090
Gluconate	-.139	.072	-.613	.020	-1.052	.031
Galactose	-.134	.069	-.586	.026	-1.228	.054
Ribose	.097	.064	-.641	.033	-1.977	.081
A124002	-.051	.044	-.753	.024	-1.262	.066
A207003	-.007	.137	-1.597	.235	-1.573	.207

Common metabolites on nitro-oxidative response were Arg, Orn, Asparagine (Asn), Pro, Glutamate (Glu), Pyroglutamate, Ribose, Galactose, Galactinol, *myo*-Inositol, Glycerophosphoglycerol, and five with structure currently unknown (Kopka *et al.*, 2005) (Fig. 16).

Glu, pyroglutamate, PUT, glycerophosphoglycerol and galactinol were the metabolites which demonstrated more significant strong correlations with all altered metabolites after oxidative stress (*P*≤0.05; *See Annex 3*), while Arg, Asn, Orn, pyroglutamate and galactose exhibited this trend with altered metabolites after nitrosative stress (*P*≤0.05; *See Annex 4*).

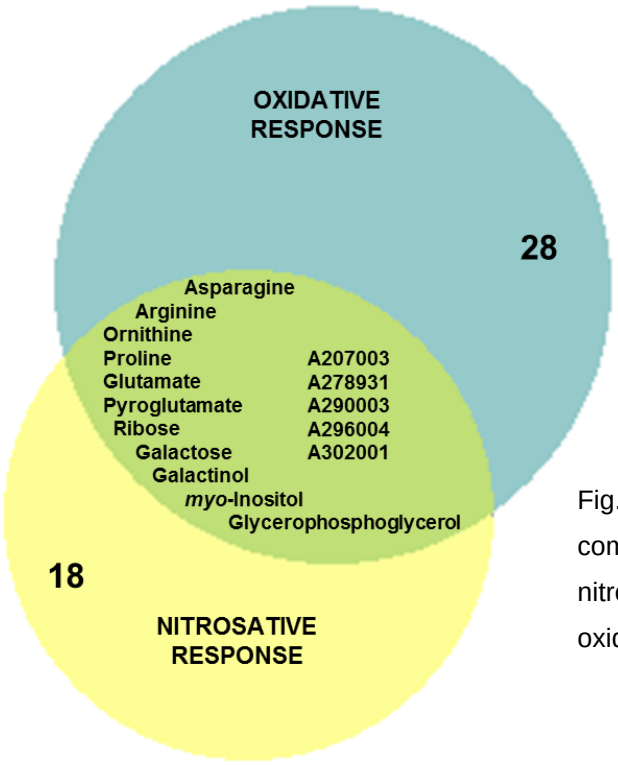

Fig. 16 Proportional Venn diagram comparing metabolites implicatedin nitro-oxidative response after oxidative/nitrosative stress imposition

In-depth analysis in search of specific patterns on both responses, revealed Arg, Orn and Glu as part of polyamine biosynthetic pathway (Alcázar *et al.*, 2010*a*), as well as pyroglutamate followed an opposite trend in content, which means oxidative stress promoted a significant increase compared with WT, whereas nitrosative stress provoked the opposite response (Fig. 17A). The same pattern was observed in metabolites related with RFO pathway such as Galactose, Galactinol and *myo*-Inositol (Fig. 17B), suggesting that SPM homeostasis regulated by AtPAO4 has a direct influence on the levels of these metabolites which seem to play an essential role on nitro-oxidative response altering RSA.

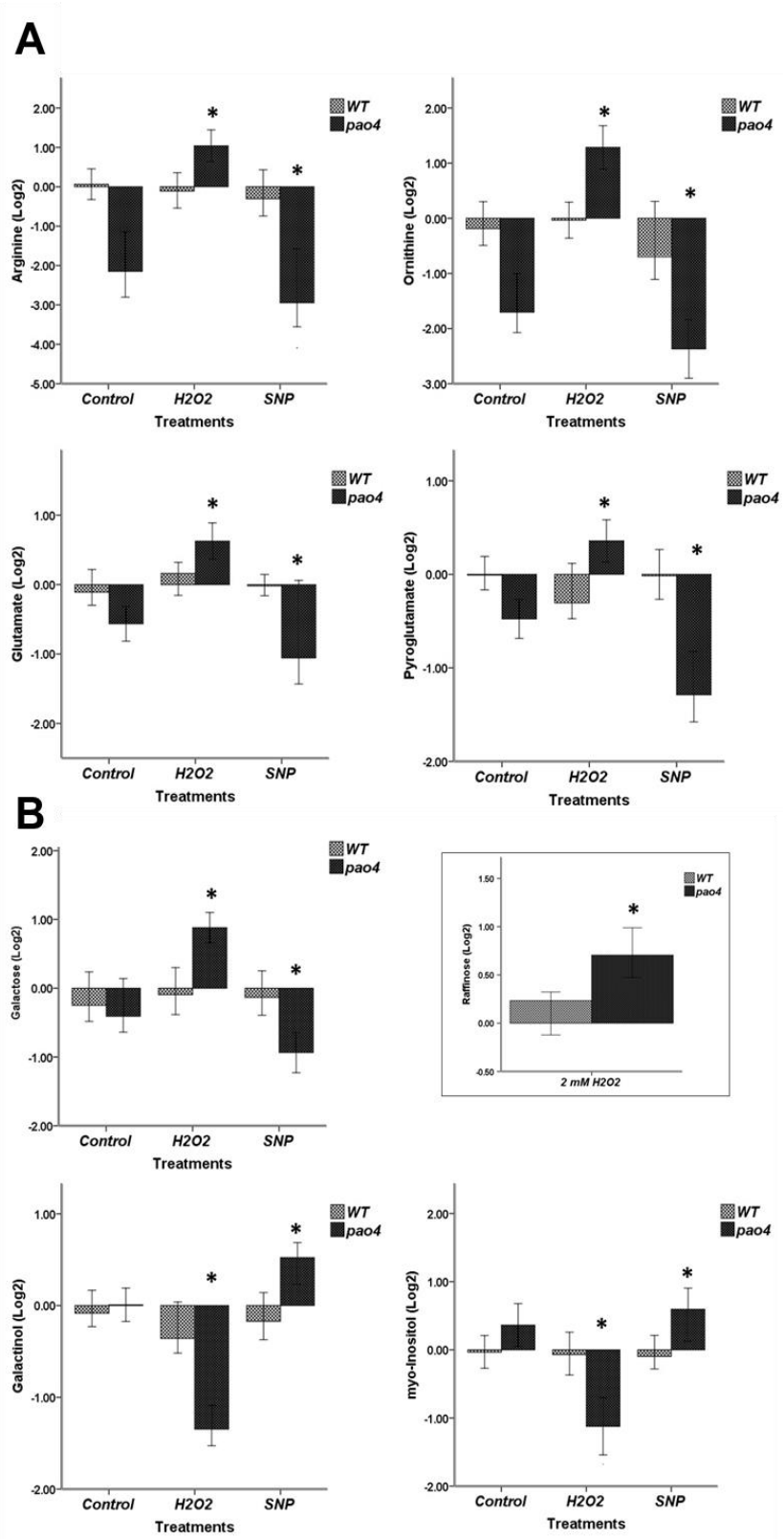

Fig. 17. Levels of commonly altered metabolites in *pao4* mutants after oxidative and nitrosative stress imposition.

A Metabolites related with PA biosynthetic pathway.

B Metabolites related to RFO pathway. Results are presented as logarithmic transformed ratios relative to WT Col-0. Each point represents the mean value of at least 3 independent analyses, error bars refer to standard deviation. Asterisks indicate values which are significally different from those of the corresponding WT plants, as determined by both one-way ANOVA and Tukey HSD post-hoc tests (P<0.05)

2. DISCUSSION

2.1AtPAO4 as major isoform in *Arabidopsis* roots is involved in root architecture system by SPM modulation

Over the last decades, PA role on plant growth and development as well as their role on signaling under diverse environmental stresses has been a matter of attention in plant physiology. The ability to identify unique or common regulatory nodes of metabolic pathways, and the cross- talk among the different pathways that are affected by genetic manipulation of PA metabolism, will provide us effective targets to genetically engineer plants that are tolerant to different abiotic stresses (Minocha *et al.*, 2014). PAs play important roles in plant multiple stress tolerance; however, how they interact with other physiological and biochemical pathways is the key question (Shi and Chan, 2014). Results from previous work showed that SPM oxidation by AtPAO4, as the major isoform identified in *Arabidopsis* roots (Kamada-Nobusada *et al.*, 2008), is involved in RSA modeling given that LRs as wells as LR features are significantly affected (Fig. 8). Intercellular communication and coordination of responses between cells and tissues are of paramount important for LR emergence. It is known that auxin plays a pivotal role for the structural and functional patterning of LR primordium, nonetheless despite several lines of evidence the concrete mechanism by which this hormone gradient governs cell identity and behavior is poorly understood (Vilches-Barro and Maizel, 2015). A recent report showed that tetraamines containing core structure 3.3 (e.g thermospermine, norspermine and norspermidine) were actively inhibiting LR emergence by down-regulation of auxin signaling elements (Tong *et al.*, 2014). The number of LR in *pao4* mutants during normal growth conditions, suggests that PA homeostasis regulated by AtPAO4 (Fig. 9A) might be an important element on LR development, which is intriguing since it has been established that SPM is the best substrate for this enzyme, although it is also proved that it can oxidize T-spermine and norspermine (an uncommon PA) (Takahashi *et al.*, 2010; Fincato *et al.*, 2011). Hence, further investigation must be made in order

to clarify its role in this process. Nevertheless, the idea of SPM as upstream modulator on auxin signaling could not be ruled out.

2.2 AtPAO4 mutants are tolerant to oxidative and nitrosative stress by changes in root architecture system related to this species

Either ROS or RNS induced changes in *pao4* root features (Fig. 10; Fig. 11), which suggest that these signal molecules could contribute to abiotic stress adaptation of *pao4* mutants (e.g. drought). To date there is consensus on the fact that ROS participate in developmental and regulatory physiological process by acting as signal molecules. Together, ROS and anti-oxidative components contribute to the redox homeostasis in the cell, playing a fundamental function in the physiological process in whole plants including root development (Correa-Aragunde *et al.*, 2013). Previous authors have reported that *Arabidopsis* root growth and elongation are highly related with ROS homeostasis (Lehmann *et al.*, 2009; Tsukagoshi *et al.*, 2010), while they also identified ROS-mediated Ca^{2+} influx on elongation zone and mature tissue of *Arabidopsis* roots, where LR emergence occurs (Demidchik *et al.*, 2007). Moreover, there is increasing evidence of plasma membrane remodeling or cation channel activation as product of cross-talk between PAs and ROS-induced membrane conductance (Pottosin *et al.*, 2012, 2014). On the other hand, over the last decade robust evidence has emerged regarding NO implication on auxin-mediated root organogenesis (Zhang *et al.*, 2007*a*; Schlicht *et al.*, 2013; Forde, 2014). It was shown that NO was required for adventitious root development in cucumber seedlings induced by auxin by using specific NO-scavenger (cPTIO) (Pagnussat *et al.*, 2002). Supressed LR initiation has been found on a nitrate-poor area as well as differences in LR length which was correlated with the age of the plants (Linkohr *et al.*, 2002). In addition, by using *Arabidopsis nos1* and *nia1 nia2* mutants with impaired NO production, a significant reduction in root hair elongation was found, establishing that endogenous NO is a critical parameter for root hair formation in a process mediated by auxin-signaling cascades (Lombardo *et al.*, 2006). In any event, LR is more sensitive to

variations in N supply than PR (Tian *et al.*, 2014) and it is known that root branching process is highly regulated by auxin, N supply and Glu concentrations (Forde, 2014). A major research aim nowadays is to elucidate the redundancy and even antagonism between these components and signaling pathways related to it (Forde, 2014). The fact that ROS or RNS imposition provoked a significant change on *pao4* RSA, suggests that PA homeostasis might be an important element on root signaling as upstream element of RONS and hormone mediated root modeling.

2.3 Oxidative and nitrosative response on *pao4* mutants is related with PA biosynthetic pathway, gluthatione and RFOs pathway modulation

Both oxidative and nitrosative stress induced similar changes in *pao4* RSA by enhancing characteristic root features of branching process and last root insertion location when compared with WT (Fig. 10; Fig. 11). There is evidence of a relationship between ROS and RNS metabolism under physiological and adverse environmental conditions. In that sense, several stresses are reported to generate oxidative and nitrosative stress as well (Tanou *et al.*, 2009; Molassiotis and Fotopoulos, 2011; Corpas and Barroso, 2013); even so, few studies carried out in plant biology have adopted this position (Corpas and Barroso, 2013). After combination of both ROS and RNS donors, tolerance could not be observed in *pao4* mutants (Fig. 12). On one hand, oxidative and nitrosative stress showed different time of responses. While oxidative stress tolerance was evidenced at 10 d of H_2O_2 imposition (Fig. 13), nitrosative stress tolerance was pronounced at 2 d after SNP exposition (Fig. 14). In both cases SPM levels were increased 5-fold compared with WT (Fig. 15), suggesting an implication of SPM homeostasis in stress response signaling, which has been previously observed during biotic stress in *Arabidopsis* (Mitsuya *et al.*, 2009) and salt stress in citrus roots (Tanou *et al.*, 2014), although this implication seems to occur on different periods of nitro-oxidative response. On the other hand, metabolomic profiling by GC-TOF-MS displayed alteration of 11 metabolites common to nitro-oxidative response (Fig. 16). Interestingly, 8 of

them demonstrated a stress-dependent opposite trend in content (Fig. 17), and also presented strong correlations (P≤ 0.05; *See Annex 5*) with altered metabolites after oxidative (Table 3) or nitrosative (Table 4) response. The above lead to the idea that these metabolites might be crucial for SPM pathway linked with ROS or RNS signaling.

Arg, Orn, Glu and even pyroglutamate are precursors of PA biosynthetic pathway (Bitrián *et al.*, 2012). All of them significantly increased after oxidative stress and decreased after nitrosative stress (Fig. 17A). Arg metabolism has been related to PA-induced NO production in root tissue of apple (Gao *et al.*, 2009), and *Arabidopsis* (Tun *et al.*, 2006; Flores *et al.*, 2008; Wimalasekera *et al.*, 2011*b*), leading to an enhancement of NO-mediated root signaling. In consequence, Arg decrease after nitrosative stress is an expected result (Fig. 17A). Glu is a tremendously important signaling molecule in roots. *Arabidopsis* has 20 glutamate-like receptor genes (GLRs), all of them expressed in root tissue (Chiu *et al.*, 2002). Even though the number of receptors might lead to the idea of functional redundancy among the GLR family members, single-gene knock-out mutations of GLR showing impairment on LR features have been isolated (Vincill *et al.*, 2013). Previous reports showed that Glu inhibited LR growth while it stimulated the outgrowth of LR in a spatio-temporal manner, proposing a role for this amino acid as a signaling molecule (Walch-Liu *et al.*, 2006). This study evidenced that SPM homeostasis has a more complex relationship with Glu than merely PA biosynthesis. The levels of these amino acids are decreased during normal growth (Fig. 17A) which might explain, at least in part, the phenotype where the mutants presented less LR features during normal growth and longer LR (Fig. 8). However, after nitro-oxidative stress a change in the metabolic flux of this amino acid could be observed (Fig. 17A) that did not affect the increase of LR features, which suggests that Glu might be a signaling connector between ROS and RNS during stress response, although this requires further investigation. In any case, the hypothesis of such an important role for Glu on root signaling is related with the ability of the plant to sense N-rich patches as an adaptive response during challenging

environments (Forde and Lea, 2007), therefore cross-talk between PAs and this amino acid would not be out of the question. Gluthatione (GSH) is one of the major non-enzymatic anti-oxidatives playing a major role in detoxifying oxidative stress in plants (Foyer and Noctor, 2011). It has been proposed as a biomarker of the redox state and has been implicated in direct ROS scavenging and meristem redox homeostasis during root growth (Correa-Aragunde *et al.*, 2015). it Pyroglutamate has been described as a GSH degradation product in *Arabidopsis*, establishing a link between GSH and glutamate levels during oxidative stress events (Ohkama-Ohtsu *et al.*, 2008). Interestingly, increased expression of enzymes of γ-glutamyl-cycle and strong coordination between them has been observed in *Arabidosis* roots, after heavy metal stress, where a high N assimilation rate is necessary to detoxification, suggesting that this cycle is highly activated during stress events in a N-dependent manner (Paulose *et al.*, 2013). Nitrosative stress lowered Glu and pyroglumate levels (both strongly correlated to GABA), while oxidative stress seems to be enhancing their production (Fig. 17A) probably to alleviate oxidative stress by means of GSH. Either way, SPM signaling pathway and cross-talk with ROS or RNS influence the dynamic of these two important nitrogen components, suggesting another function of PAs as stress protector compounds. The remaining question regarding this topic is how alterations on PA homeostasis are able to influence Glu, pyroglutamate and GSH turnover. Thus, it might possible that PA biosynthetic pathway is stimulated during oxidative stress with consequences in GSH and NO production, while nitrosative stress switches it off as a way to impair NO production.

A strong interest on sugar signaling, sensing and metabolism is on the rise (Van den Ende, 2014). Sugar interaction with auxin-mediated apical dominance depends on growth conditions, tissues and developmental stage making this response variable (Kelly *et al.*, 2012). Recent studies on sugar metabolism are challenging classical models in plant physiology; for instance, it has been recently shown that sugar signals, and not auxin, initiates the signals for apical dominance in pea (Mason *et al.*, 2014), while sugar status modulates auxin-

related transcripts in tomato which is implicated in root and shoot branching (Golan *et al.*, 2013). In any case, understanding the complexity of cellular sugar homeostasis and deciphering connections with hormonal regulatory mechanism is far from complete (Van den Ende, 2014). RFOs have been related to membrane protection during dehydration or freezing, ROS scavenging in leaves during cold stress and phloem-mobile stress signals during biotic or abiotic stress in *Arabidopsis* (Van den Ende, 2013, 2014; Elsayed *et al.*, 2014). Our results suggest that oxidative and nitrosative responses related to PA levels have a role in this process. Galactinol and *myo*-Inositol are strong correlated metabolites of nitro-oxidative response on *pao4*, both being considered as principal metabolites of the classical RFO pathway (Elsayed *et al.*, 2014). The first step in RFO biosynthesis is the production of galactinol from *myo*-inositol and UDP-galactose in one step reaction catalyzed by galactinol synthase (GolS). Galactinol is then used as a donor to deliver galactose to sucrose producing raffinose (Van den Ende, 2013). After oxidative stress, *pao4* mutants showed 2-fold increase in galactose and raffinose content (Fig. 17B), which correlates with recently found anti-oxidative properties for certain RFOs (Dos Santos *et al.*, 2013; Elsayed *et al.*, 2014; Van den Ende, 2014). Results from this study suggest that SPM signaling pathway after oxidative stress is implicated on the synthesis of this sugar metabolite. On the other hand, galactinol and *myo*-inositol (also part of RFO pathway) are increased after nitrosative stress. *M*yo-inositol is an essential molecule on plant signal transduction given that it constitutes the majority of inositol in average eukaryotic cell (Gillaspy, 2011). It plays several roles due to possible reversible addition of both lipids and phosphates, resulting in the production of new intracellular second messenger molecules that impart specific information to the cell (Gillaspy, 2011). Inside this group of second messengers are membrane-associated phosphatidylinositol (PtdIns), which can be phosphorylated or dephosphorylated producing molecules that takes part on diverse signaling mechanism as well (e.g. diacylglycerol). Mutations on PtdIns kinases and phosphatases, can impact root hair development and tip growth process driven by membrane trafficking (Ischebeck *et al.*, 2010). For instance, auxin receptor

TIR1, which degrades transcription repressors of auxin, contains a binding site of inositol-6-phosphate (Ins6P) implicated in the recognition of repressor (Tan *et al.*, 2007). Moreover, over-expression of Ins6P Kinase 1, commonly named inositol-phosphate kinase 2 (AtIPK2), was found to be directly involved in Ins6P degradation. AtIPK2 over-expressors exhibited auxin-insensitive phenotype, with the consequence of impaired LR features, which was strongly correlated with low levels of Ins6P (Zhang *et al.*, 2007*b*). There is evidence of interaction between phospholipids and RNS in a hormone-mediated mechanism (Distéfano *et al.*, 2012). Oxidative stress might therefore be stimulating the RFOs pathway while nitrosative stress might be promoting phospholipid-mediated signaling with a consequence on sugar metabolism. It is known that there is a strong interaction between nitrogen supply and the response of photosynthesis, metabolism and growth when the plant is exposed to adverse environments, having ultimately impact on sugar availability (Stitt and Krapp, 1999). In fact, nitrogen metabolism needs to be regulated by carbon metabolism, at least in part, since nitrate assimilation and amino acid biosynthesis requires reducing equivalents and ATP and therefore competes with photosynthetic carbon fixation and carbohydrate synthesis (Stitt and Krapp, 1999). After nitrosative stress citrate was increased.This TCA intermediary is reported to be altered in response to NO at the mitochondrial level (Blokhina and Fagerstedt, 2010). Citrate is also associated with N detoxification in other species (Paulose *et al.*, 2013) and showed high increased and correlation with SPM treatment in tomato (Mattoo *et al.*, 2010). Asp also showed significant increase, which is in agreement with previous work where a dramatic rise on this amino acid was found in seeds and rosettes under high nitrogen supply (Lemaître *et al.*, 2008). Another increased metabolite was glycerate 3-phosphate, an essential intermediary of sugar signaling; its biosynthetic enzymes are reported to be modulated throughout *S*-nitrosylation in response to exogenous SPM (Tanou *et al.*, 2014). Hence, it is not surprising that most altered metabolites were related to sugar metabolism after nitrosative stress (Table 4), most of which strongly correlated to GABA. Therefore SPM-mediated nitrosative response suggests a role in C:N balancing probably mediated by GABA.

Overall, SPM-related nitro-oxidative response relies on common metabolites essentials for nitrogen, sugar and lipid metabolism (Table 5). However, most important metabolites converged towards different metabolic fluxes in a stress-dependent manner. SPM signaling pathway was found to be part of both processes, reinforcing the idea of a cross-talk with RONS elements. Nonetheless, the nature of the responses is what makes a difference. SPM-mediated oxidative response seems to be a long-term process while nitrosative response could be more immediate. This fact enriches the nature of PA signaling pathway and opens the gates to novel connections. PAs' recognized role as protector molecules might also rely on their involvement on the production of essential elements of stress physiology such as GSH, *myo*-inositol or raffinose as well as sugar signaling modulation.

Table 5. Metabolic implications of common metabolites to nitro-oxidative response with highest numbers of strong correlations with listed altered metabolites

METABOLITE	METABOLIC IMPLICATIONS
Arginine	Arginine and proline metabolism (KEGG; AraCyc) Arginine and ornithine metabolism (KEGG; AraCyc) Biosynthesis of amino acids (KEGG) Alanine, aspartate and glutamate metabolism (KEGG; AraCyc) Nitric oxide biosynthesis (AraCyc) Putrescine biosynthesis (AraCyc)
Ornithine	Arginine and proline metabolism (KEGG; AraCyc) Arginine and ornithine metabolism (KEGG) Biosynthesis of amino acids (KEGG) Glutathione metabolism (KEGG)
Glutamate	Arginine and proline metabolism (KEGG; AraCyc) Biosynthesis of amino acids (KEGG) Alanine, aspartate and glutamate metabolism (KEGG; AraCyc) Putrescine biosynthesis (AraCyc) γ-Glutamyl Cycle (AraCyc)
Pyroglutamate	Glutathione metabolism (KEGG) γ-Glutamyl Cycle (AraCyc)
Galactose	Galactose metabolism (KEGG) RFOs pathway (el saeed, van den ende 2014) Amino sugar and nucleotide sugar metabolism (KEGG) Ascorbate and aldarate metabolism (KEGG) Pentose Phosphate Pathway (KEGG)
***myo*-Inositol**	Inositol phosphate metabolism (KEGG) Phosphatidylinositol signaling system (KEGG) Galactose metabolism (KEGG) Plant ascorbate biosynthesis (Lorence, 2004)

CHAPTER II

Loss-of-function of pao4 delays dark-induced senescence through metabolic interactions with primary metabolism

ABSTRACT

In the present work, we investigate the role of a major regulator of SPM homeostasis, *Arabidopsis Polyamine Oxidase 4* (*PAO4*), on dark-induced senescence. Two *PAO4* (*pao4-1* and *pao4-2*) T-DNA-insertion mutants have been characterized which accumulate 10-fold higher SPM than the wild-type (WT). Interestingly, *pao4* mutants exhibit delayed dark-induced senescence suggesting that loss of PAO4 activity alleviates oxidative damage. Global metabolomic profiling of *pao4* mutant under normal growth conditions indicates that *pao4* has constitutively higher levels of metabolites involved in redox state regulation (e.g. ascorbic acid), central metabolism (e.g. pyruvate) and signaling metabolites (e.g. *myo*-Inositol). During dark-induced senescence, we observed a possible interaction between molecules of oxidative, sugar and nitrogen metabolism, some of them involved in senescence process such as glutamate, asparagine and tryptophan, being higher the levels in WT comparing to *pao* mutants. The results suggest an interaction between the enzymes of PA catabolism, cell oxidative balance and transport/biosynthesis of amino acids as a strategy to cope with oxidative damage produced by abiotic stress.

1. RESULTS

1.1 Polyamine levels of *atpao4* mutants during normal growth on adult plants

Analysis of free PUT, SPD and SPM levels in leaves from 4 week-old *pao4-1* and *pao4-2* mutants indicated a consistent accumulation of SPM up to 10-fold and 5-fold lower SPD than the wild-type in both *pao4* mutant alleles (Fig. 18). Conversely, PUT levels only differed in *pao4-2* and not in *pao4-1*, probably as a result of the residual *PAO4* expression in the latter.

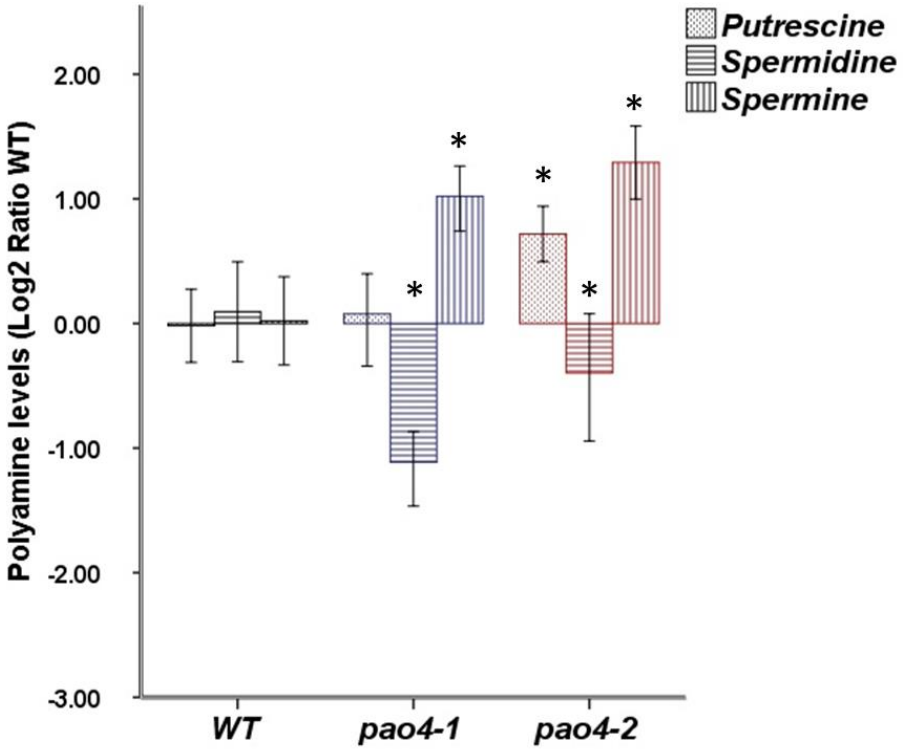

Fig. 18. PA quantification during normal growth on adult plants. **A** Determination of the gene expression of *AtPAO4* in *Arabidopsis thaliana pao*4 mutants compared to WT Col-0. *AtPAO4* gene-specific primers were designed to confirm the partial or the total lack of expression as was previously described (Kamada-Nobusada *et al.*, 2008; Liu *et al.*, 2014*a*). The *pao4-1* mutant has a considerably reduced expression compared to WT, while the *pao4-2* lacks of expression completely. **B** Polyamine levels were quantified by chloroform/methanol extraction in leaf tissue grown under conditions previously described (*see materials and methods*). Results are presented as logarithmic transformed ratios relative to WT Col-0. Each point represents the mean value of at least five independent analyses, error bars are referred to standard deviation. Asterisks indicate values which are significally different from those of the corresponding WT plants, as determined by both one-way ANOVA and Tukey HSD post-hoc tests (P<0.05)

1.2 Metabolomic profiling of *pao4* mutants under normal growth conditions

In order to obtain a broader view of the metabolic consequences driven by the loss of *PAO4* expression under normal growth conditions, GC-TOF/MS metabolomic profiling (Erban *et al.*, 2007; Allwood *et al.*, 2011) was performed in *pao4* mutants and wild-type. Primary metabolite profiling generated from detached leaves under normal growth conditions identified a total of 75 metabolites, 37 of which did not show significant differences respect to the wild-type (*See Annex 6*). From the remaining 38 metabolites, 28 were increased (Fig. 19) and 10 decreased. Most down-regulated metabolites could not be classified in metabolic groups, due to lack of available annotation data since the structure is currently unknown (*See Annex 7*). Increased metabolites in *pao4* mutants could be sorted into four major metabolic categories belonging to oxidative, nitrogen, sugar and lipid metabolism according to cited public databases (Fig. 19A). Some metabolic features associated with these metabolites are summarized in Table 6, as well as metabolic pathways in which they participate (*See Annex 8*).

Pearson's correlation analysis ($P<0.05$; Fig. 19B) confirmed the occurrence of a correlation between high SPM levels and increased levels of other metabolites. Therefore, most metabolic changes are directly or indirectly associated with SPM accumulation induced by *PAO4* loss-of-function mutations. Metabolite classification within categories identified amino acids as the largest group of up-regulated metabolites. These included 1) SAM precursor methionine (Met) and Orn, both precursors of PAs. 2) Branched-chain and aromatic amino acids, both used as substrates of alternative respiratory chain during dark-induced senescence (Araújo *et al.*, 2010, 2011). 3) Polar uncharged amino acids, which are essential for post-translational modifications. In addition most of altered amino acids are either involved in transitions on day/night cycle (Gibon *et al.*, 2006) or adaptation to extended dark (Gibon *et al.*, 2006, 2009) in *Arabidopsis*.

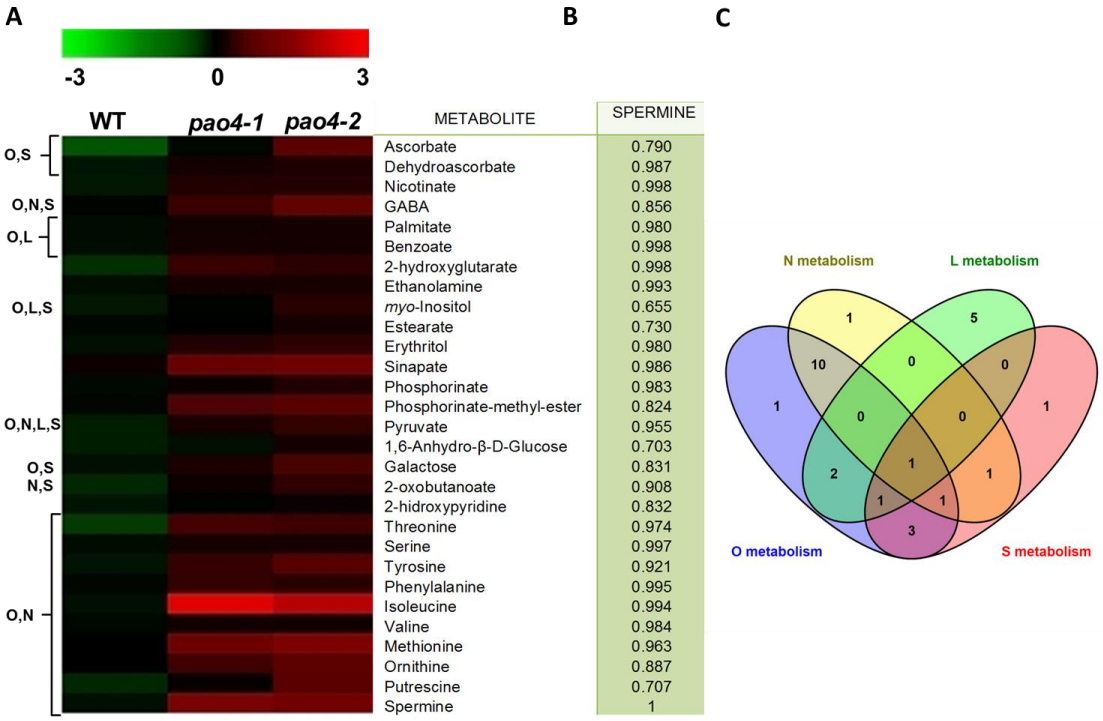

Fig. 19 Heat map of *pao4* mutants up-regulated metabolites under normal growth condition. **A** Metabolite levels were determined using detached leaf tissue from 4 week-old plants grown on the conditions previously described (*see materials and methods*), compounds abundance is presented as $\log_2$-transformed fold-changes relative to the mean of WT-Col 0 samples. Altered metabolites were detected with MeV tool V.4.9 by rank product statistical test (P<0.05). Classification of metabolites (to the left) according to metabolic context, O = Oxidative; N = Nitrogen; L = Lipid; S = Sugar. **B** Pearson correlation values (r) related to Spermine, values were obtained from at least four independent biological experiments (P<0.05). **C** Proportional Venn diagram. Up-regulated metabolites on *pao4* mutants according metabolic implications.

Other important upregulated metabolites included pyruvate, a crucial metabolic hub, GABA which accumulates during abiotic stress (e.g. Bitrián *et al.*, 2012; Shelp *et al.*, 2012), *myo*-Inositol, a key building block of signaling metabolites involved in sugar and phospholipid-mediated signaling, (e.g. Gillaspy, 2011; Williams *et al.*, 2015), and ascorbate-dehydroascorbate (ASC-DHA) essential metabolites for maintenance of cell anti-oxidative capacity and oxidative stress alleviation (Wang *et al.*, 2010; Foyer and Noctor, 2011). In summary, most *pao4* up-regulated metabolites detected belong to nitrogen, oxidative, sugar and lipid metabolism, with some of them shared in different categories due to their relevance on central metabolism (Fig. 19C). The majority of up-regulated metabolites were related to abiotic stress and senescence (Table. 6).

Table 6. Metabolic features related with senescence signaling process, according to previous research, on some up-regulated metabolites of *pao4* mutants under normal growth conditions.

METABOLITE	METABOLIC FEATURES
Nicotinate	Guard cells protection against ROS in ABA-mediated stomatal movement (Hashida *et al.*, 2010)
2-hydroxiglutarate	Associated with dark-induced senescence by electron donation to mitochondrial respiratory chain (Araújo *et al.*, 2010; Engqvist *et al.*, 2011)
Pyruvate	Associated with regulation of gene expression during extended darkness and essential diurnal changes during day/night cycles in *Arabidopsis* (Gibon *et al.*, 2006) Involvement on the enhancement of 2-Oxoglutarate production during carbon deprivation, for alternative electron transfer from protein degradation (Araújo *et al.*, 2011) Involvement in GABA conversion to succinic semialdehyde for TCA cycle during abiotic stress response (Shelp *et al.*, 2012)
Galactose	Associated with Phosphatidylinositol 3-kinase activity upon salt stress (Leprince *et al.*, 2015) Responsible for the 70-80% of the bulk of ascorbate in *Arabidopsis* during normal growth and during stress response (Gallie, 2012)
Serine	Associated with highly efficient carbon recovery from pyruvate production to fuel seedling establishment in *Arabidopsis* (Eastmond *et al.*, 2015) Associated with metabolic mitochondrial regulation as a molecular mechanism to alleviate short-term oxidative stress (Obata *et al.*, 2011) Associated with active site of essential protein kinases on signal transduction during leaf senescence (Guo *et al.*, 2004) Increased levels are associated with late senescing phenotypes (Diaz Céline *et al.*, 2005) Correlates with GSH foliar content in a light-dependent modulation (Noctor *et al.*, 1997)
Tyrosine	Established Senescence marker (Engqvist *et al.*, 2011) Associated with regulation of gene expression during extended darkness and essential diurnal changes during day/night cycles in *Arabidopsis* (Gibon *et al.*, 2006) Associated with metabolic mitochondrial regulation as a molecular mechanism to alleviate short-term oxidative stress (Obata *et al.*, 2011) Increased levels are associated with decreased PhosphatydilInositol 3-Kinase activity (Leprince *et al.*, 2015)
Valine	Associated with regulation of gene expression during extended darkness and essential diurnal changes during day/night cycles in *Arabidopsis* (Gibon *et al.*, 2006) Associated with ETF complex activity for survival of *Arabidopsis* in extended darkness (Ishizaki *et al.*, 2006) Alternative electron donor to mitochondrial respiratory chain via Isovaleryl-CoA degradation during extended darkness (Araújo *et al.*, 2011) Accumulation associated with general abiotic stress response (Obata and Fernie, 2012)
Methionine	Precursor metabolite for SPD, SPM and tSPM biosynthesis (Tiburcio *et al.*, 2014) Important intermediate metabolite on ethylene biosynthetic pathway (Yamagami *et al.*, 2003) Associated with regulation of gene expression during extended darkness and essential diurnal changes during day/night cycles in *Arabidopsis* (Gibon *et al.*, 2006) Associated with coordination of auxin and ethylene levels by its amino group donation to aminotransferase VAS1 in response to light (Zheng *et al.*, 2013) Accumulation associated with general abiotic stress response (Obata and Fernie, 2012)

1.3 Effects of dark-induced senescence on *pao4* mutants

Dark-induced senescence symptoms were investigated in *pao4-1*, *pao4-2* and wild-type plants. Both genotypes evidenced signs of delayed entry on senescence after 4 d of continuous dark (Fig. 20A). Protein degradation plays a role in natural senescence by removing target proteins required to protect from senescence (Araújo *et al.*, 2011). Accordingly, total protein levels were measured to quantify the extent of senescence delay induced by *PAO4* mutation. Protein levels were significantly higher in *pao4-1* and *pao4-2* than the wild type, thus suggesting a lower rate of protein degradation (Fig. 20B).
Quantification of chlorophyll and other pigments in *pao4-1* and *pao4-2* further supported these observations (Fig. 20C). PA levels generally remained constant throughout the induced senescence, except for SPD levels which dropped from 5-fold lower than the wild-type under normal growth conditions to 10-fold lower under senescence induction (Fig. 20D).

Metabolomic profiling after dark-induced senescence identified 103 metabolites (*See Annex 9)*. In dark-induced senescent *pao4* mutants, we detected 28 altered metabolites (13 up- and 15 down-regulated; Fig 22A) whose metabolic features were related to senescence (Table 7). Interestingly, 8 out of 13 *pao4* up-regulated metabolites were already detected under normal growth conditions and their levels remained high after senescence treatment. Such permanently up-regulated metabolites were PUT, SPM, ASC, DHA, *myo*-Inositol, GABA, Threonine (Thr) and Phenylalanine (Phe).

In order to establish the occurrence of correlations between SPM, SPD and PUT with altered metabolites in *pao4* mutants, we performed Pearson's analysis. A strong positive correlation was found between up-regulated metabolites and SPM levels, but negative with SPD levels (P<0.05; Figure 22B). Down-regulated metabolites showed an opposite pattern with strong positive correlation with SPD but negative with SPM, suggesting that SPD and SPM

interplay may be relevant. Additional correlations between altered metabolites and PUT levels were detected, however its significance was more moderate (*P*<0.05; Figure 22B).

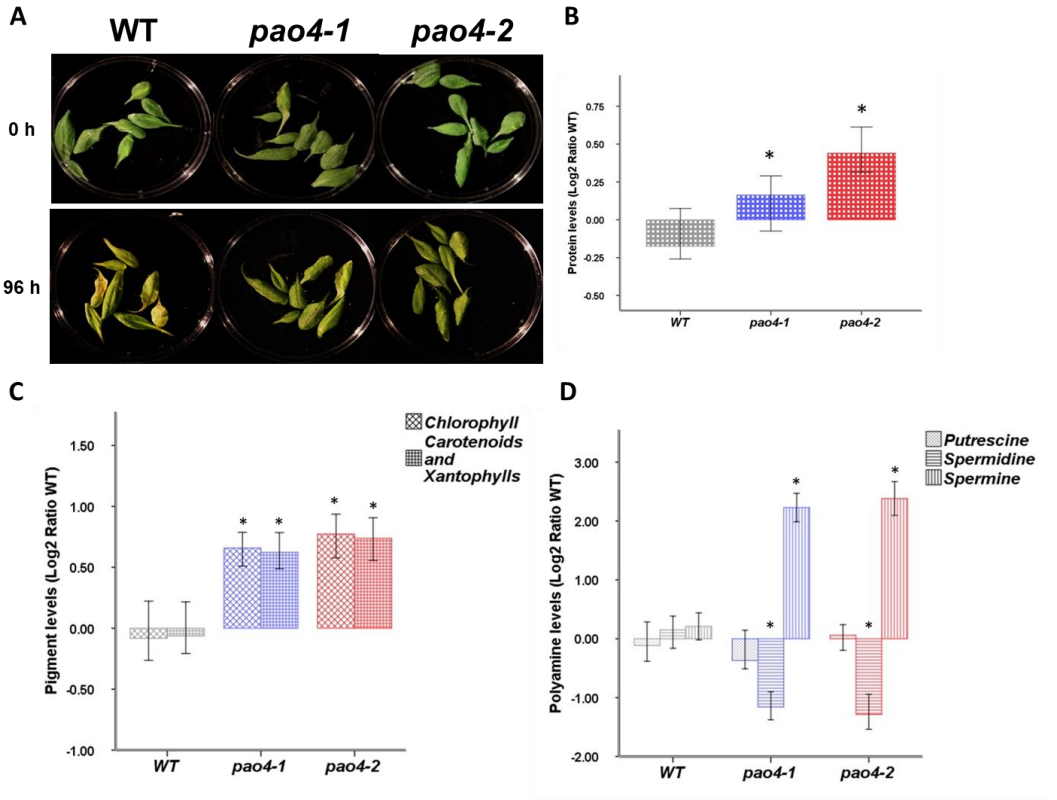

Fig. 20 Effect of dark-induced senescence on WT and *PAso4* mutants. **A** Leaves macroscopic observation following 4 day incubation on dH_2O in the dark. **B** Total proteins were extracted from senescent leaves after 4 d incubation on dH_2O. **C** Chlorophyll, carotenoids and xantophylls loss had been quantified by DMSO extraction in senescent leaves after 4 day incubation on dH_2O. **D**. PAs were determined in leaf tissue after 4 d incubation on dH_2O by chloroform/methanol extraction. Results are presented as logarithmic transformed ratios relative to WT Col-0. Each point represents the mean value of at least five independent analyses, error bars are referred to standard deviation. Asterisks indicate values which are significally different from those of the corresponding WT plants, as determined by both one-way ANOVA and Tukey HSD post-hoc tests (*P*<0.05)

Among the up-regulated metabolites, it can be distinguished three major types: (1) Those involved in oxidative detoxification and/or producers of reducing agents (e.g. ASC, DHA, xylose, etc.) (2) Amino acids, amines and nitrogen metabolism (e.g. 2-Oxoglutarate, GABA, Phe, etc.) and (3) sugar metabolism and signaling (e.g. Lactate, *myo*-Inositol, etc.) (Table 7). Among categories, oxidative metabolism was the one with most up-regulated metabolites, followed

by nitrogen metabolism. GABA, *myo*-inositol and lactate were common for three or more groups (Fig. 22A).

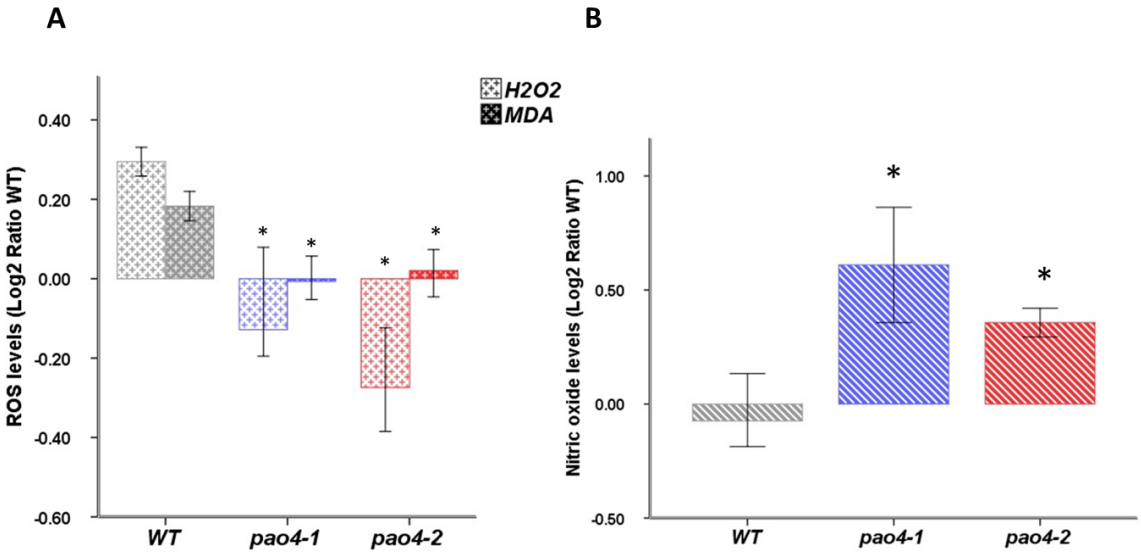

Fig. 21 RONS levels in *pao4* mutants and WT after dark-induced senescence treatment. **A** H_2O_2 and MDA levels **B** NO levels. Free radical levels were determined in detached leaf tissue after 4 days of dark-induced senescence treatment. Results are presented as logarithmic transformed ratios relative to WT Col-0. Each point represents the mean value of at least five independent analyses, error bars are referred to standard deviation. Asterisks indicate values which are significally different from those of the corresponding WT plants, as determined by both one-way ANOVA and Tukey HSD post-hoc tests (P<0.05)

Among down-regulated metabolites, the most abundant category belonged to amino acids and compounds involved in their metabolism which is consistent with high nitrogen mobilization during senescence (Soudry *et al.*, 2005). Interestingly, amino acids within this group have important implications on senescence signaling. Glu influences adaptation to dark periods in *Arabidopsis* (Gibon *et al.*, 2009). Glu is involved in glutathione catabolism along with pyroglutamate (Ohkama-Ohtsu *et al.*, 2007, 2008), oxidative response (Obata *et al.*, 2011) and GABA formation which is reported to be increased during senescence (Soudry *et al.*, 2005; Watanabe *et al.*, 2013). Tryptophan (Trp), main precursor of the plant hormone Indole-3-Acetic acid (IAA) (Zhao, 2014) , is reported to have implications on developmental and dark-induced senescence signaling (Van der Graaff *et al.*, 2006). Asparagine (Asn) and 3-Cyanoalanine,

products of cyanide detoxification pathway (Piotrowski *et al.*, 2001), activated after ET final biosynthetic reaction (Yamagami *et al.*, 2003), are up-regulated along with ET signal transduction components during senescense (Van der Graaff *et al.*, 2006; Watanabe *et al.*, 2013). Other molecules involved in glucose biosynthesis/degradation and enhancement of oxidative burst were also identified such as α,α, Trehalose, with recognized implication on redox signaling (O'Hara *et al.*, 2013) and degradation to glucose production during dark-induced senescence (Buchanan-Wollaston *et al.*, 2005; Gibon *et al.*, 2006).

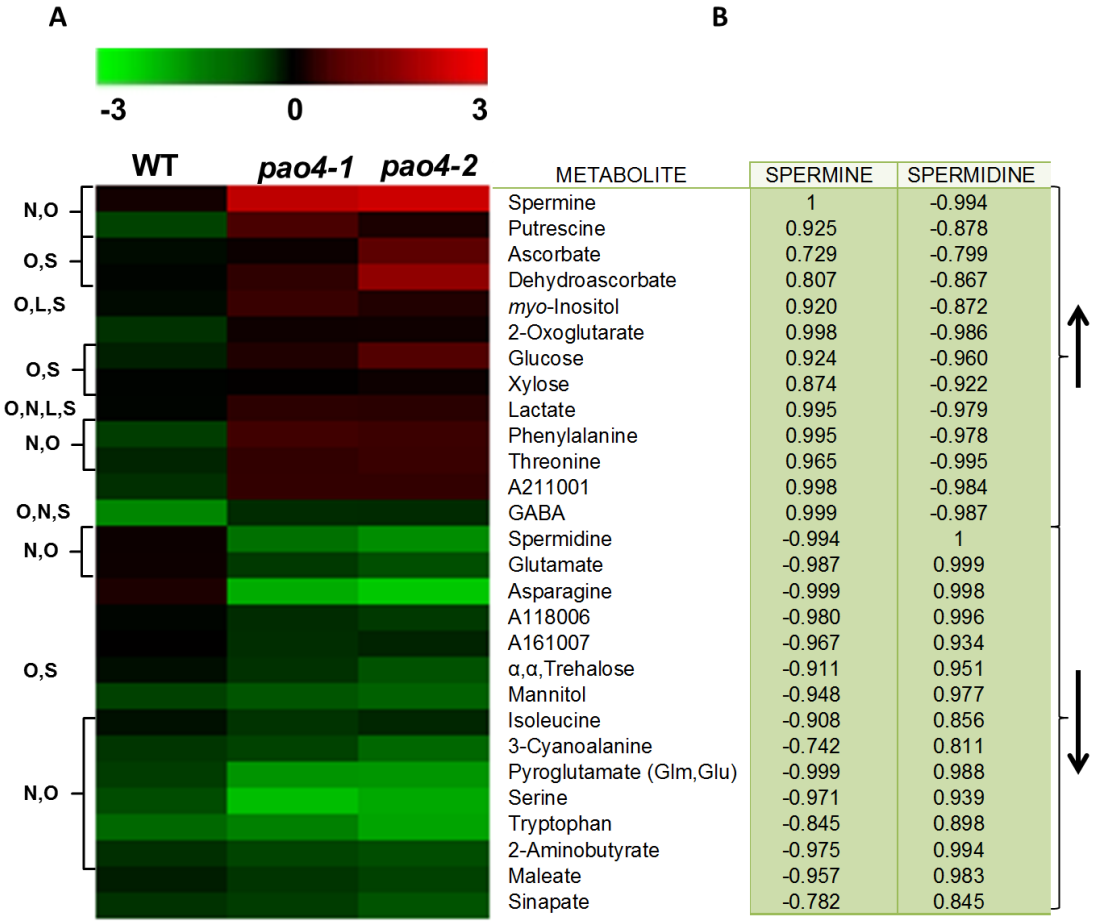

Fig. 22 Heat map of *pao4* mutants altered metabolite pools after dark-induced senescence. **A** Metabolite levels were determined using detached leaf tissue from 4 week-old plants grown on the conditions previously described (*see materials and methods*), compounds abundance is presented as log_2-transformed fold-changes relative to the mean of WT-Col 0 samples. From the top, first group represents the up-regulated metabolites and second group the down-regulated. Altered metabolites were detected with MeV tool V.4.9 by rank product statistical test (P<0.05). **B** Pearson correlation values (r) related to Spermine and Spermidine, values were obtained from at least four independent biological experiments (P<0.05).

In senescent wild-type, nine metabolites were found altered (3p- and 6 down-regulated). Seven of such metabolites also differed in senescent *pao4* mutant plants. Interestingly, all up-regulated metabolites in the wild-type (Asn, Glu and 3-cyanoalanine) were down-regulated in *pao4* (Fig. 23B), which was expected since low Glu and Asn are associated with late senescing phenotypes (Diaz Céline *et al.*, 2005), and is consistent with the delay of senescence observed in *pao4*. Conversely, Phe, which was down-regulated in wild-type, resulted upregulated in *pao4* (Fig. 23B).

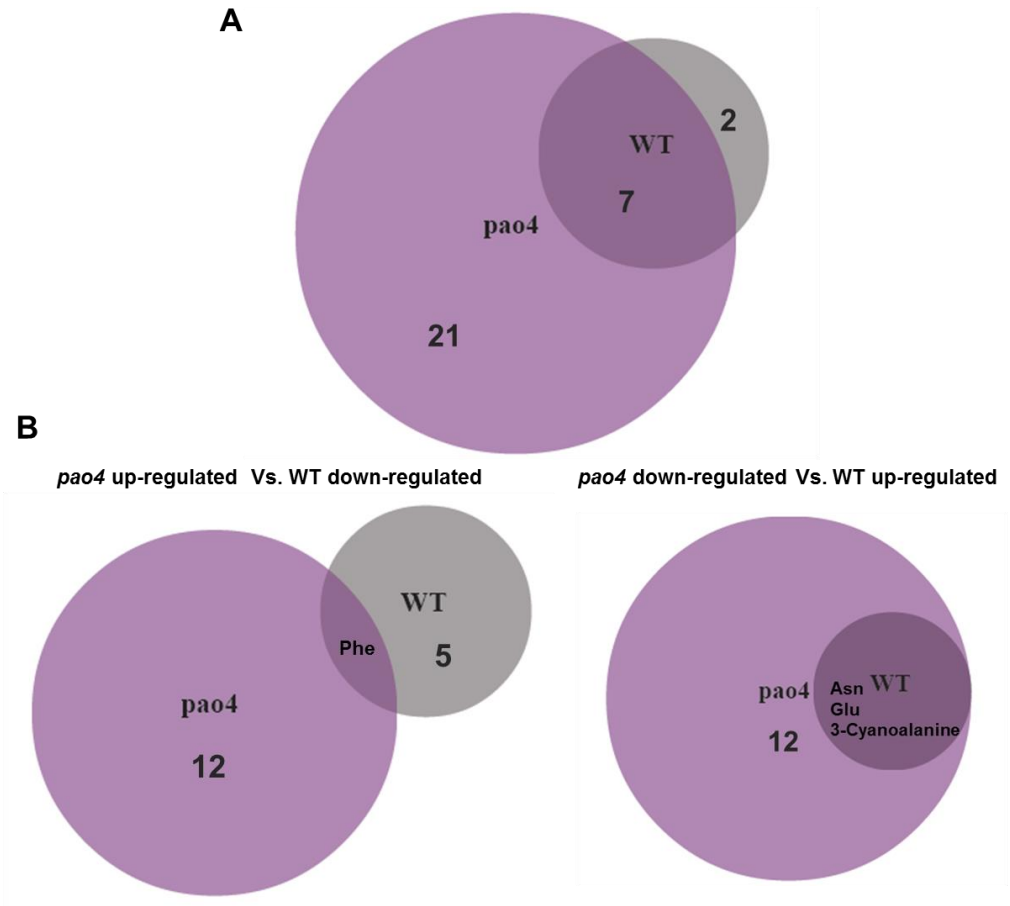

Fig. 23 Proportional Venn diagrams showing the numbers of significantly altered metabolites that are unique or commonly regulated during dark-induced senescence treatment on detached leaves after 4 days dH_2O incubation. **A** Total altered metabolites. **B** Comparison between *pao4* and WT altered metabolites

2. DISCUSSION

2.1 Constitutive enhancement of anti-oxidative capacity was found on *pao4* mutants

The identification of PA down-stream targets and metabolic connections is a necessary step to elucidate the mechanisms underlying PA-mediated stress protection (Shi and Chan, 2014). Here we report that loss-of-function mutations of *PAO4* in *Arabidopsis* plants protect against dark-induced senescence through complex interactions involving other metabolic pathways. The overall metabolic landscape suggests that the PA pathway is intricately connected with amino acid metabolism and anti-oxidative molecules through regulation of precursor molecules of sugar and lipid metabolism (Fig. 24).

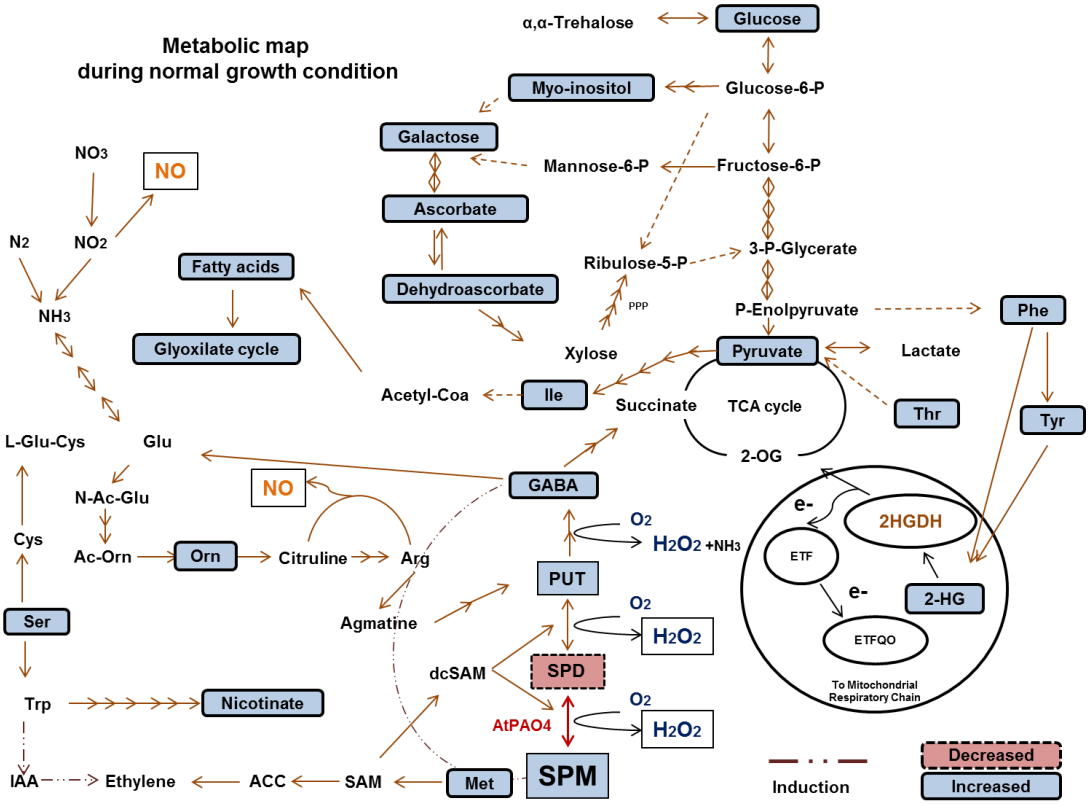

Fig. 24 Metabolic map related to SPM accumulation on *pao4* during normal growth condition. 2-HG, 2-Hydroxyglutarate; 2HGDH, 2-Hydroxyglutarate Dehydrogenase; ETF, Electron-transfer flavoprotein; ETFQO, Electron-transfer flavoprotein: Ubiquinone oxidoreductase; 2-OG, 2-Oxo-glutarate; SAM, S-Adenosylmethionine; dcSAM, Decarboxylated S-Adenosylmethionine; ACC, Aminocyclopropane Carboxilic Acid; IAA, Indole-3-Acetic Acid; N-Ac-Glu; N-Acetyl-L-Glutamate; Ac-Orn, Acetylornithine; L-Glu-Cys, L-Glutamylcysteine.

The high SPM levels in *pao4* mutants (Fig. 18B) are consistent with the higher affinity of the PAO4 enzyme for this PA, and likely underlies PA anti-senescence properties reported herein and previously observed in plants and animals (Pandey *et al.*, 2000; Del Duca *et al.*, 2014; Moschou and Roubelakis-Angelakis, 2014). Pearson's analyses from global metabolomic approaches have revealed the occurrence of significant correlations between the levels of SPM and certain metabolites in non-stressed *pao4* plants (Fig. 19B). Previously, SPM has been reported to reprogram the oxidative status of citrus plants exposed to salt stress and to increase the ascorbate redox state of plants (Tanou *et al.*, 2014). In this study, metabolic profile of *pao4* mutants points to a constitutive enhancement of their anti-oxidative capacity (Table 6), mainly through increases in ASC/DHA, galactose, nicotinate and sinapate. Ascorbate-Glutathione (ASC-GSH) is the most characterized cell anti-oxidative system and has major implications on cell signaling and activation of defense mechanisms upon stress (Foyer and Noctor, 2011). Therefore, its implication is fundamental during the oxidative response (Wang *et al.*, 2010; Gallie, 2012). Moreover, an increase of central metabolic and signaling hubs such as pyruvate and *myo*-Inositol was observed. Loss-of-function of *AtPAO4* also leads to up-regulation of several amino acids and nitrogen-mobilization molecules, such as GABA. The role of GABA is unclear but it has been proposed to be a signaling molecule that coordinates the C:N balance in challenging environments, such as senescence (Buchanan-Wollaston *et al.*, 2005), or a nitrogen-storage related molecule acting on nitro-oxidative stress (Tanou *et al.*, 2012). In summary, metabolic and signaling connections between SPM and precursor molecules from sugar and lipid metabolism were found (Fig. 24), whereby a relationship between SPM and anti-oxidative capacity might be stablished. We suggest that this metabolic condition might confer a prime-like status of *pao4* plants that promotes their stress tolerance.

2.2 Metabolic profiling after dark-induced senescence

To investigate mechanisms underlying the *pao4* anti-senescence phenotype under a metabolic perspective, PA and RONS levels as well as global

metabolite profiles were determined in wild-type and *pao4* mutants after dark-induced senescence. Our findings indicate that SPM remains high in *pao4* after dark-induced senescence (Fig. 20D), while NO increases in correlation with the delayed senescence in agreement with previous works (Niu and Guo, 2012; Liu and Guo, 2013) (Fig. 21B). H_2O_2 levels decrease inversely to ASC-DHA (Fig. 21) thus suggesting that NO induces the oxidative response after stress imposition (Linka and Theodoulou, 2013; Corpas and Barroso, 2014).

Table 7. Metabolic features related with senescence signaling process, according to previous research, on some altered metabolites of *pao4* mutants after dark-induced senescence

METABOLITE	METABOLIC FEATURES
Ascorbate **Dehydroascorbate**	Associated with delay in dark-induced senescence (Hui *et al.*, 2012; Fotopoulos and Kanellis, 2013) Oxidative stress alleviation (Wang *et al.*, 2010)
***myo*-Inositol**	Associated with essential diurnal changes during day/night cycles on *Arabidopsis* (Gibon *et al.*, 2006) Increase with the progression of developmental senescence (Watanabe *et al.*, 2013) Regulatory role on cell-wall synthesis and re-modeling (Krishnamoorthy *et al.*, 2014) Signaling role on plant defense response (Hung *et al.*, 2014) Accumulation associated with general abiotic stress response (Obata and Fernie, 2012)
2-Oxoglutarate	Highly involved in glutamate homeostasis and amino acid formation by the action of aminotransferases (Forde and Lea, 2007) Associated with dark-induced senescence as degradation product of 2-Hydroxyglutarate, which oxidation is increased during extended darkness to provide electron supply to mitochondrial respiratory chain (Araújo *et al.*, 2010; Engqvist *et al.*, 2011)
Glucose	Associated with regulation of gene expression during extended darkness and essential diurnal changes during day/night cycles in *Arabidopsis* (Gibon *et al.*, 2006)
Lactate	Associated with oxidative detoxification of methylglyoxal by glyoxylase II, when a disruption of γ-Glutamyl cycle that maintain increased levels of ASC and glutathione is observed (Tolin *et al.*, 2013) Decrease with the progression of developmental senescence (Watanabe *et al.*, 2013)
Phenylalanine	Associated with regulation of gene expression during extended dark periods in *Arabidopsis* (Gibon, 2006) Associated with essential diurnal changes during day/night cycles in *Arabidopsis* (Gibon, 2006) Associated with ETF complex activity for survival of *Arabidopsis* in extended darkness (Ishizaki, 2006) Associated with coordination of auxin and ethylene levels by its amino group donation to aminotransferase VAS1 in response to light (Zheng, 2013) Alternative electron donor to mitochondrial respiratory chain via 2-Hydroxyglutarate and Isovaleryl-CoA degradation during extended darkness (Araujo, 2011)

METABOLITE	METABOLIC FEATURES
4-Aminobutanoate (GABA)	Associated with regulation of gene expression during extended darkness in *Arabidopsis* (Gibon *et al.*, 2006) Proposed signaling role coordinating C:N balance during leaf senescence (Buchanan-Wollaston *et al.*, 2005) Associated with mitochondrial succinate production to support respiration during extended dark (Araújo *et al.*, 2010) Accumulation associated with general abiotic stress response (Obata and Fernie, 2012) Increased with the progression of senescence (Watanabe *et al.*, 2013)
Glutamate	During extended night periods have the tendency to decrease producing 2-Oxoglutarate during amino acid catabolism (Buchanan-Wollaston *et al.*, 2005; Gibon *et al.*, 2009) Associated with GABA formation via GABA shunt as a possible protective role during senescence (Buchanan-Wollaston *et al.*, 2005) Regulated in response to light by imbalance on sugar metabolites (Gibon *et al.*, 2006) Associated with Glutathion catabolism in *Arabidosis* (Ohkama-Ohtsu *et al.*, 2008)
Glutamine	Its Decrease is associated with efficient N mobilization on late senescence phenotypes (Diaz Céline *et al.*, 2005) Associated with N mobilization role during dark-induced senescence (Buchanan-Wollaston *et al.*, 2005; Soudry *et al.*, 2005)
α,α, Trehalose	Associated with sugar signaling during dark-induced senescence. Genes encoding key enzymes on trehalose metabolism are up-regulated (Buchanan-Wollaston *et al.*, 2005) Its degradation to glucose showed increased tolerance to drought and salinity stress (Van Houtte *et al.*, 2013; Krasensky *et al.*, 2014)
3-Cyanoalanine	First product of cyanide detoxification pathway during ethylene biosynthesis by Hydrogen Cyanide degradation (Piotrowski *et al.*, 2001) Possible role as a defensive compound against herbivory (Mano and Nemoto, 2012)
Asparagine	Product of cyanide detoxification pathway during ethylene biosynthesis by 3-Cyanoalanine degradation (Piotrowski *et al.*, 2001) Its Decrease is associated with efficient N mobilization on late senescence phenotypes (Diaz Céline *et al.*, 2005) Increased on rossete leaves during progression of senescence in *Arabidopsis* (Watanabe *et al.*, 2013) and dark-induced senescence (Buchanan-Wollaston *et al.*, 2005)

From the metabolic profile analyses, we found that most altered metabolites during the stress response belong to oxidative and nitrogen metabolism (Fig. 22). Indeed, a correlation has been found between PAs and amino acids levels during senescence in Arabidopsis (Mattoo *et al.*, 2010; Watanabe *et al.*, 2013). In addition, NO is reported to be involved in the regulation of free amino acid levels by modulation of proteolytic mechanisms such as autophagy and the TOR pathway in *Arabidopsis* and other species (López-Berges *et al.*, 2010; Tripathi *et al.*, 2013). Overall, our data suggest that *pao4* oxidative response relies on the metabolic interaction between SPM with sugar homeostasis and nitrogen metabolism (Fig. 25).

α,α-Trehalose which is decreased in *pao4*, has emerged as a redox signaling molecule with a proposed role on stress, including senescence (Fernandez *et al.*, 2010; Krasensky *et al.*, 2014). Trehalose degradation confers drought tolerance (Van Houtte *et al.*, 2013) producing glucose, which along with *myo*-Inositol (both increased in *pao4*), are precursors for mitochondrial ASC biosynthesis via L-galactose, potentially providing support for the observed increase in ASC content. Furthermore, increased xylose in *pao4* suggests activation of the phosphate-pentose pathway, which is reported to be up-regulated in *Arabidopsis* roots after oxidative stress imposition (Lehmann *et al.*, 2009) as a source of reducing equivalents on peroxisome for GSH biosynthesis (Corpas *et al.*, 2009). Increased lactate was also found, which is consistent with a link between sugar and pyruvate-related amino acid metabolism. This study suggests that AtPAO4 exerts a regulation on essential precursors with a consequence on oxidative cell signaling by anti-oxidative mitochondrial production.

The γ-glutamyl cycle is a NO-induced intracellular pathway (Innocenti *et al.*, 2007), which leads to GSH biosynthesis through amino acid uptake/recycling during the abiotic stress response. Loss-of-function on γ-Glutamyl-Transferase 1, the first enzyme of the pathway, established implication of this cycle on plant adaptation to the environment by modulation of senescence-related metabolites (Tolin *et al.*, 2013). However an alternative mechanism to modulate GSH homeostasis has been described (Ohkama-Ohtsu *et al.*, 2008), in which its catabolism generates Glu in a reversible manner, depending on the nitrogen fixation rate. Recent evidence suggests that increases in nitrogen assimilation favors GSH biosynthesis with a concomitant decrease in pyroglutamate and Glu levels (Paulose *et al.*, 2013). Interestingly, both metabolites are lower after dark-induced senescence. Amino acid content is, to a great extent, lowered after stress imposition. Conversely, NO exhibits 5-fold increase, which suggests an involvement of AtPAO4 on GSH homeostasis. Results here indicate that the SPM-triggered oxidative response might have implication on the maintenance of the redox status throughout modulation of amino acids transport and recycling.

Cross-talk between PAs and hormones such as ET and IAA have been reported, but the molecular nature of such interactions remains elusive (Bitrián *et al.*, 2012). However, metabolic connections between PAs and ET synthesis are one the most characterized (Moschou and Roubelakis-Angelakis, 2014). The final biosynthetic step producing ET is inducible by IAA and produces hydrogen cyanide (Yamagami *et al.*, 2003). This compound is toxic and degrades rapidly through the senescence-responsive cyanide detoxification pathway (Meyer *et al.*, 2003), which involves a series of successive steps that produce 3-Cyanoalanine and Asn (Piotrowski *et al.*, 2001). Because *pao4* mutants display lower levels of 3-cyanoalanine and Asn, it is hypothesized that high SPM levels might also promote delayed entry into dark-induced senescence through inhibition of ET biosynthetic reaction, although this requires further investigation.

Aromatic and branched-chain amino acids have been suggested as alternative electron donors for mitochondrial respiration during the stress response, (Araújo *et al.*, 2010, 2011; Obata *et al.*, 2011) in a process where the hydrolysis of 2-Hydroxyglutarate (2-HG) produces 2-oxoglutarate (2-OG) with concomitant release of electrons that are donated to ubiquinol via ETFQO complex (Ishizaki *et al.*, 2005). Interestingly, Phe, 2-HG and 2-OG are increased in *pao4*, thus suggesting that SPM is involved in this alternative electron donor pathway for mitochondrial respiration (Fig. 9). In support to that , an SPM-induced signaling pathway leading to mitochondrial dysfunction has previously been reported in response to biotic stress in tobacco and Arabidopsis (Takahashi *et al.*, 2004; Mitsuya *et al.*, 2009). Therefore, the possibility that increases in SPM and NO might enhance mitochondrial energy production after dark-induced senescence could not be ruled out.

Overall, we provide a global view of metabolic changes affected by *PAO4* mutation in Arabidopsis, which are associated with delayed entry into dark-induced senescence and SPM accumulation. Our results point to an important role of SPM as a signaling molecule promoting stress protection through

intricate metabolic connections involving ASC-GSH redox state modifications due to changes on sugar and nitrogen metabolism, possible ET biosynthesis inhibition or mitochondrial electron transport chain induction, all of which are highly implicated on the nitro-oxidative response after stress imposition. Moreover, novel metabolic and signaling interactions were identified, suggesting that PAs are able to modulate metabolites like pyruvate and *myo*-Inositol (which also was identified on SPM root signaling) as central metabolic and signaling hubs on several essential pathways.

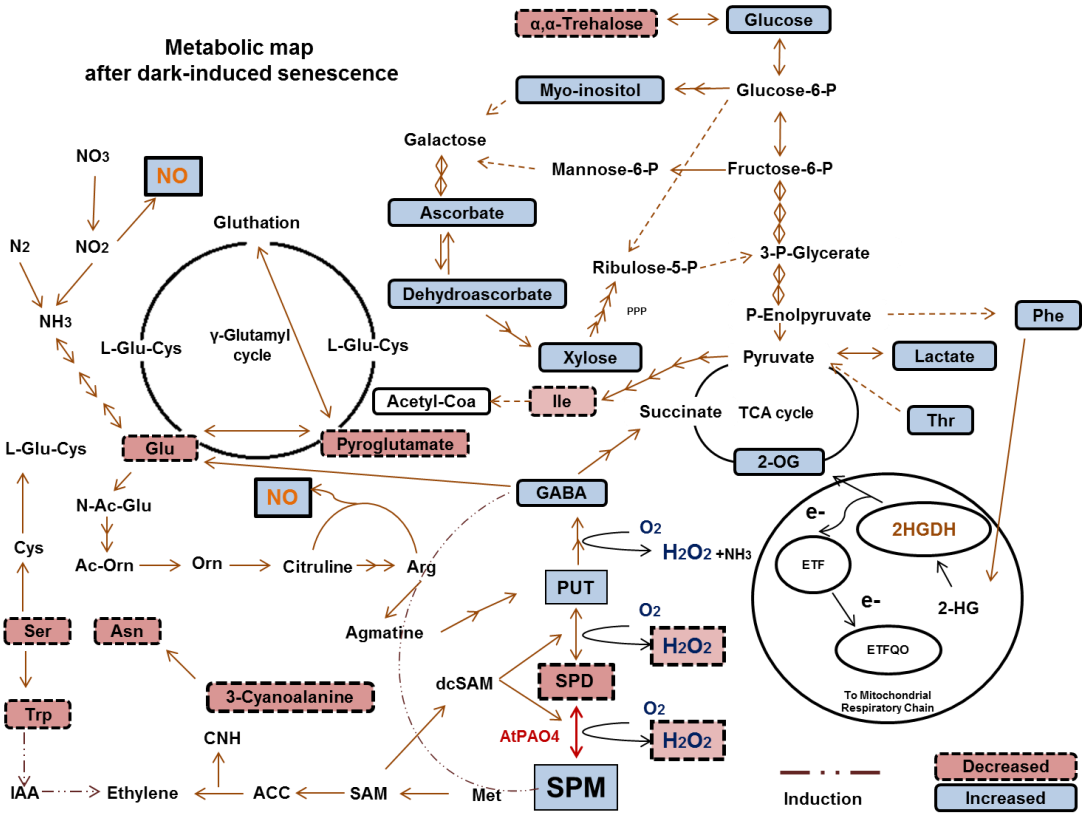

Fig. 25 Metabolic map related to SPM accumulation on *pao4* after dark-induced senescence. 2-HG, 2-Hydroxyglutarate; 2HGDH, 2-Hydroxyglutarate Dehydrogenase; ETF, Electron-transfer flavoprotein; ETFQO, Electron-transfer flavoprotein: Ubiquinone oxidoreductase; 2-OG, 2-Oxo-glutarate; SAM, S-Adenosylmethionine; dcSAM, Decarboxilated S-Adenosylmethionine; ACC, Aminocyclopropane Carboxilic Acid; CNH, Hydrogen Cyanide; IAA, Indole-3-Acetic Acid; SA, Salycilic Acid; JA, Jasmonic acid N-Ac-Glu; N-Acetyl-L-Glutamate; Ac-Orn, Acetylornithine; L-Glu-Cys, L-Glutamylcysteine

CHAPTER III

Loss-of-function of AtPAO4 confers tolerance to water stress

ABSTRACT

Previous chapters have established that SPM homeostasis regulated by AtPAO4 is involved in nitro-oxidative response after stress imposition. Moreover, under control conditions, *pao4* mutants at an adult stage demonstrated an increase in anti-oxidative and primary metabolites (Chapter II). The aim of this chapter was to examine whether this constitutive up-regulation of key defense-related metabolites was effectively priming *pao4* mutants against subsequent abiotic stress factors, a feature that would be highly desirable in light of the increasing yield losses occurring worldwide as a result of climate change. Drought stress was imposed and *pao4* mutants were able to tolerate water loss. SPM homeostasis at an adult stage appears to be closely linked with regulation of stomatal closure.

1. RESULTS

1.1 SPM homeostasis in *pao4* mutants is linked with stomatal dynamics

In this study, we observed that *pao4* mutants did not show morphological differences compared with WT (Fig. 1A). However, increased metabolites related with enhancement of anti-oxidative capacity were observed at both seedling and adult plants under control conditions (discussed in previous chapters). For instance, increase of metabolites such as ASC and DHA was found in adult plants (Chapter II). In consequence, RONS levels were measured in *pao4* mutants, which showed significant increase in H_2O_2 compared with WT and no significant difference in the levels of MDA, suggesting that this ROS increase is not causing deleterious effect on the mutants (Fig. 25B).

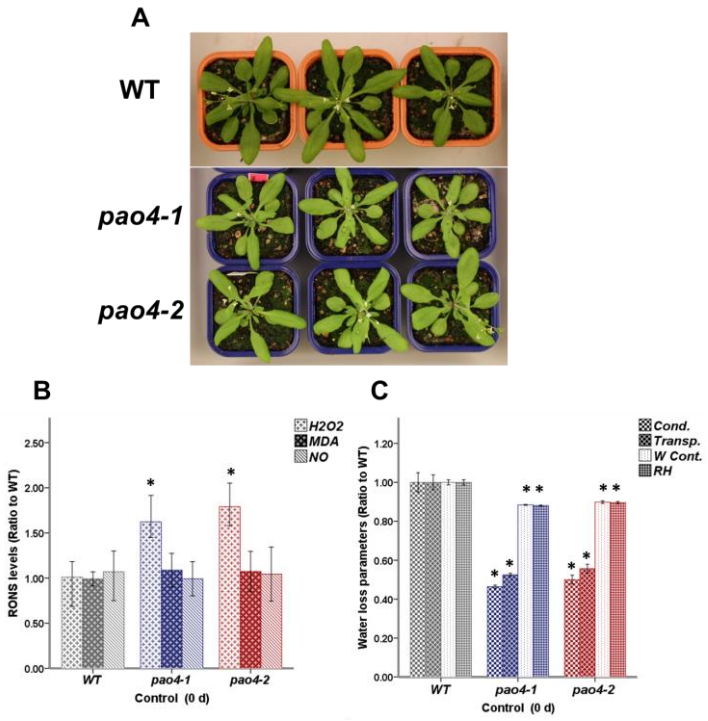

Fig. 26. Physiological parameters on *pao4* mutants during normal growth conditions. **A** Macroscopic observation of *pao4* 4 week-old plants. **B** RONS levels measured in detached leaves. **C** Stomatal conductance (Cond.), Transpiration rate (Transp.), Sample water content (W Cont.) and Relative humidity (RH) were assayed in wild-type plants and in two independent transgenic lines using a Li-6400 portable gas-exchange system (LI-COR), as described. *See materials and methods.* Results are presented as ratios relative to WT Col-0. Each point represents the mean value of at least six independent analyses, error bars refer to standard deviation. Asterisks indicate values which are significantly different from those of the corresponding WT plants, as determined by both one-way ANOVA and Tukey HSD post-hoc tests ($P<0.05$)

1.2 PA and RONS signals confer drought stress tolerance to *pao4* mutants

H_2O_2 production is part of the signaling process required to promote stomatal closure (Gallie, 2012). Moreover, ASC recycling to DHA is an essential element of stomatal dynamics (Chen and Gallie, 2006; Gallie, 2012). Previous reports have related increase on H_2O_2, ASC and DHA with altered stomatal dynamics in tobacco plants (Fotopoulos *et al.*, 2008). Therefore, in order to determine if the increased ROS content was influencing *pao4* stomatal conductance, parameters related with leaf water content were measured using a LI-COR 6400 portable gas analysis system, on 4 week-old plants during normal growth condition (Fig. 25C). Interestingly, stomatal conductance as well as transpiration rate showed significant decrease compared with WT. Conversely, *pao4* mutants showed significant increase in water content suggesting that *pao4* mutants present a high rate of stomatal closure compared with WT during normal growth.

Exogenous addition or endogenous increases of SPM levels have been related with protection against drought stress in *Arabidopsis* and other species (Capell *et al.*, 2004; Yamaguchi *et al.*, 2007; Li *et al.*, 2015). In addition, H_2O_2 endogenous increase has been related with acclimation or induction of prime-like status, preparing plants to tolerate abiotic stresses (Tanou *et al.*, 2009, 2012; Miller *et al.*, 2010). This study demonstrated that *pao4* mutants are able to tolerate nitro-oxidative stress (Chapter I) which is one of the consequences of drought signaling (Filippou *et al.*, 2011; Corpas and Barroso, 2013). Subsequently, water stress was imposed by withholding watering for 10 days. LI-COR measurements were also performed before water recovery to evaluate if the trend observed during normal growth was maintained during the treatment (Fig. 26). As expected, *pao4* mutants were more tolerant to stress condition that WT. Before re-watering, macroscopic observation revealed increased damage indicated by chlorotic leaves on WT plants (Fig. 26A). In accordance, significant decrease in stomatal conductance and transpiration rate was registered, while significant increase in water content was observed in *pao4* mutants (Fig. 26B).

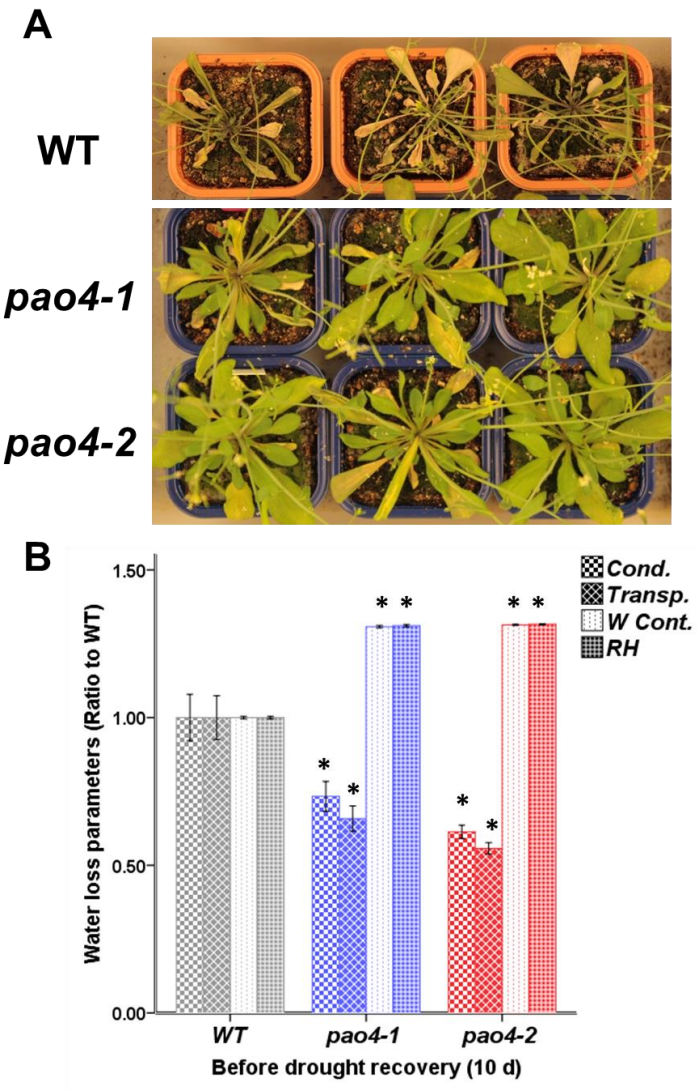

Fig 27. Physiological parameters in *pao4* mutants before drought recovery. **A** Drought tolerance phenotypes of *pao4* mutants compared with WT. A Phenotype of 4-weeks-old plants dehydrated during 10 d. **B** Stomatal conductance (Cond.), Transpiration rate (Transp.), Sample water content (W Cont.) and Relative humidity (RH) were assayed in wild-type plants and in two independent transgenic lines using a Li-6400 portable gas-exchange system (LI-COR), as described. *See materials and methods.* Results are presented as ratios relative to WT Col-0. Each point represents the mean value of at least six independent analyses, error bars refer to standard deviation. Asterisks indicate values which are significantly different from those of the corresponding WT plants, as determined by both one-way ANOVA and Tukey HSD post-hoc tests ($P<0.05$)

RONS molecules are involved in ABA-mediated stomatal closure during stress signaling (Desikan *et al.*, 2004; Neill *et al.*, 2008; Jannat *et al.*, 2011). In order to evaluate the possible implication of RONS signals and PAs on *pao4* tolerant

behavior, the levels of these molecules were determined before re-watering (Fig. 27)

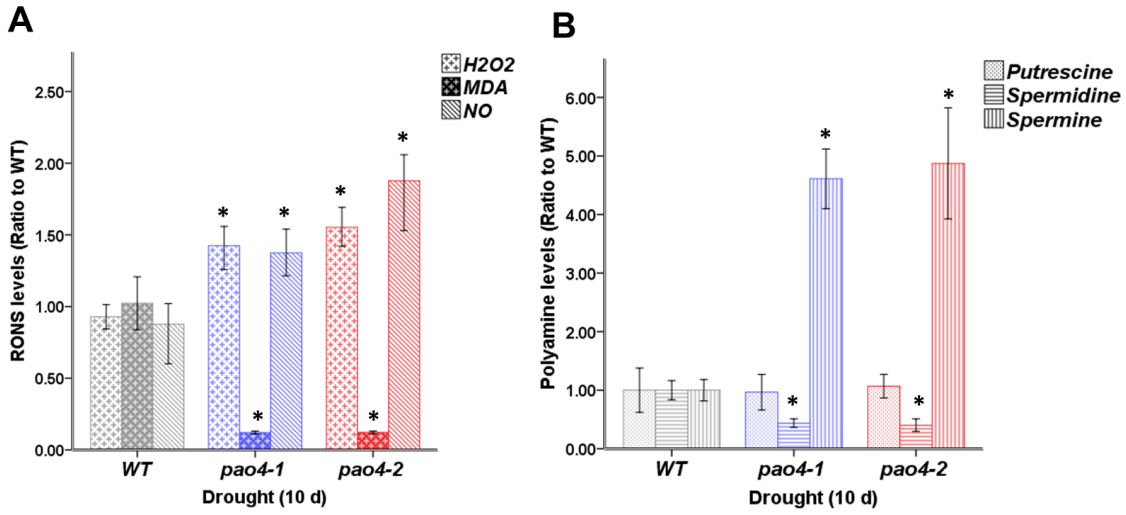

Fig 28. Physiological measurements in *pao4* mutants before drought recovery. **AB** RONS and polyamine levels in *pao4* mutants dehydrated for 10 d. Results are presented as ratios relative to WT Col-0. Each point represents the mean value of at least three independent analyses, error bars refer to standard deviation. Asterisks indicate values which are significantly different from those of the corresponding WT plants, as determined by both one-way ANOVA and Tukey HSD post-hoc tests (P<0.05)

As expected, H_2O_2 presented a significant increase in *pao4* mutants compared with WT, while MDA levels showed marked decrease (Fig 27A), demonstrating a lower extent of damage due to stress imposition. Interestingly, NO showed a significant increase compared with WT (Fig. 28A). In regard with PAs, SPD showed a significant decrease while SPM showed 5-fold increase upon water stress imposition (Fig. 27B).

Re-watered *pao4* plants appeared to recover maintaining turgor after 2 days of re-watering (Fig. 28A). Interestingly, physiological parameters related to water content maintained the trend, which suggests that stomatal conductance and transpiration rate are two features permanently decreased in *pao4* adult plants (Fig. 28B), which might be associated to the significant increase in leaf water content permanently observed in *pao4* mutants (Fig. 28B

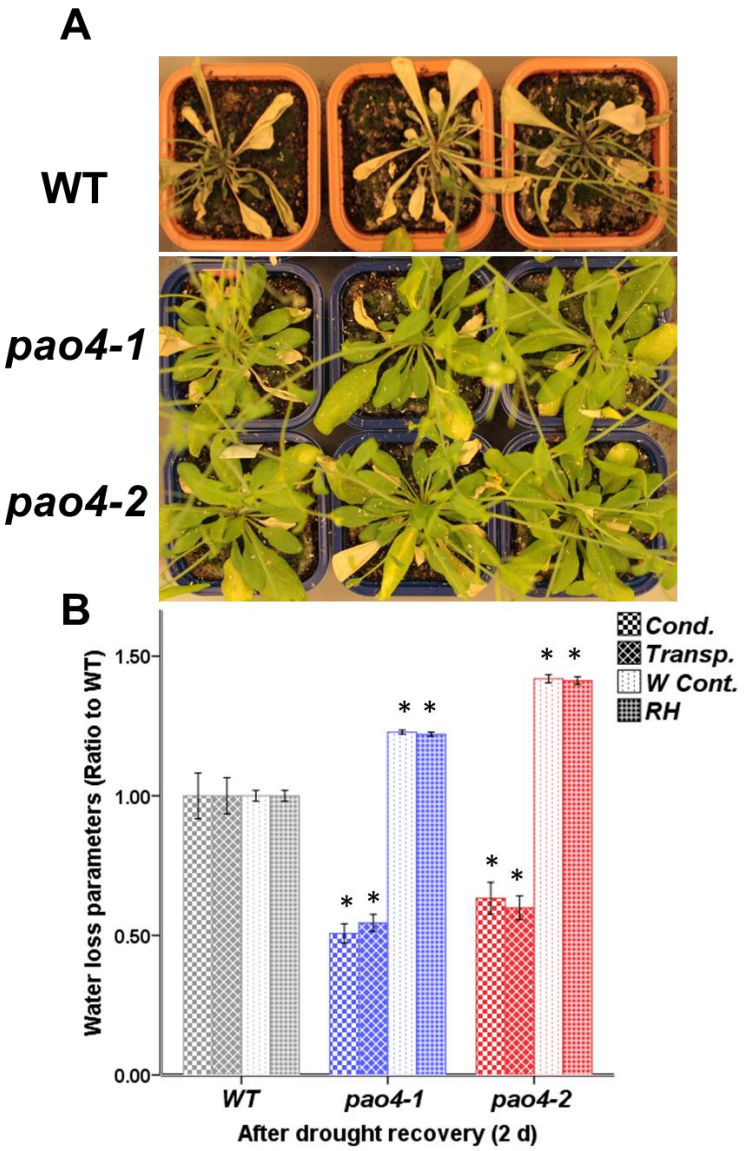

Fig. 29. Physiological parameters in *pao4* mutants 2 d after drought recovery. **A** Drought resistance phenotypes of *pao4* mutants compared with WT. A Phenotype of 4-weeks-old plants 2 d after dehydration during 10 days. **B** Stomatal conductance (Cond.), Transpiration rate (Transp.), Sample water content (W Cont.) and Relative humidity (RH) were assayed in wild-type plants and in two independent transgenic lines using a Li-6400 portable gas-exchange system (LI-COR), as described. *See materials and methods.* Results are presented as ratios relative to WT Col-0. Each point represents the mean value of at least six independent analyses, error bars refer to standard deviation. Asterisks indicate values which are significantly different from those of the corresponding WT plants, as determined by both one-way ANOVA and Tukey HSD post-hoc tests (P<0.05)

In relation with the recovery phenotype showed by the mutants, SPM remained 5-fold increased. Meanwhile, SPD levels maintained a significant decrease compared withWT (Fig. 29B) and PUT levels showed a significant increase after

recovery (Fig. 29B). Interestingly, ROS levels showed a significant decrease while NO maintained 2-fold increase (Fig. 29A). 7 d after recovery, *pao4* mutants showed 50% increase in survival, determined by fully recovered rossetes, suggesting that PA and RONS signals were involved in drought stress tolerance observed in *pao4* mutants (Fig. 29C).

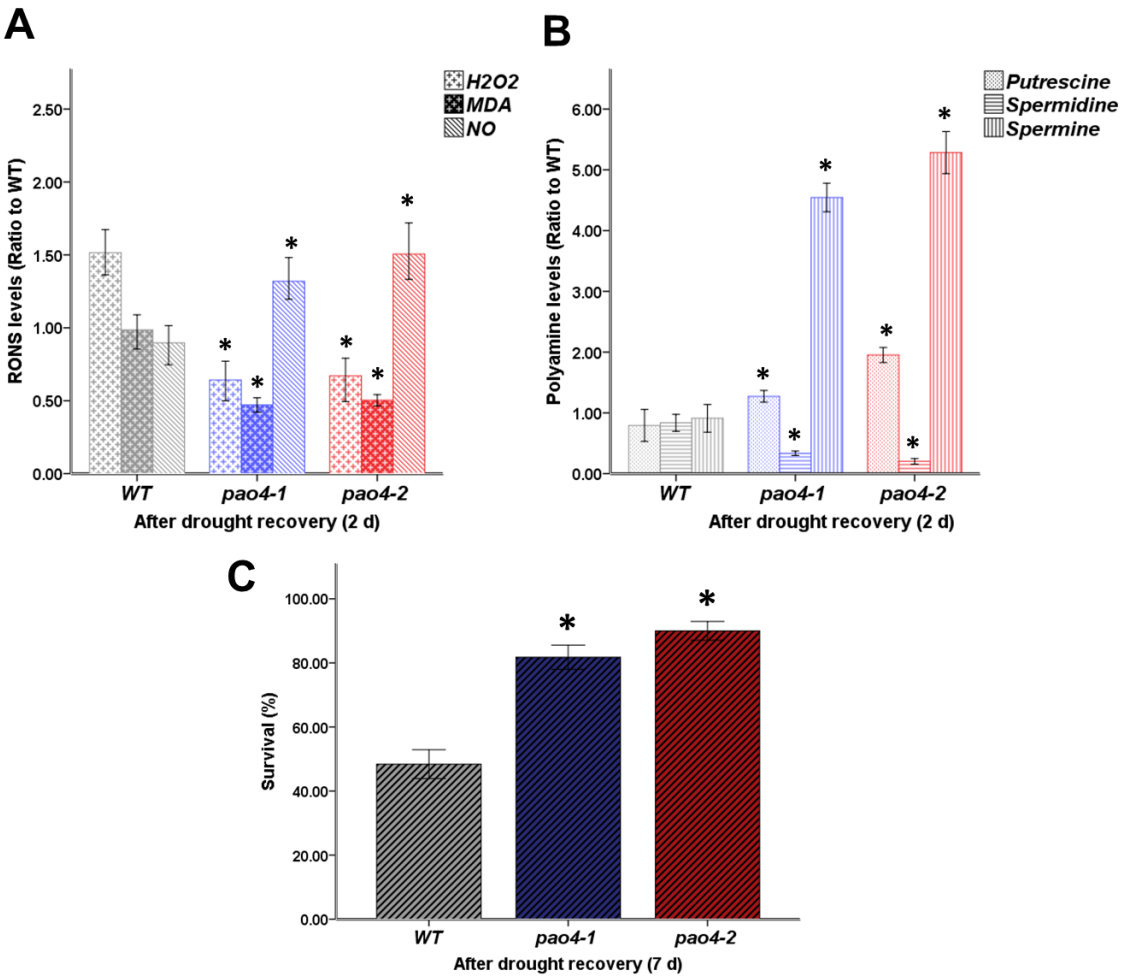

Fig 30. Physiological measurements in *pao4* mutants 2 d after drought recovery. **AB** RONS and PA levels in *pao4* mutants after 2 d of dehydration for 10 d. **C** Survival rate after 7 d of recovery. Results are presented as ratios relative to WT Col-0. Each point represents the mean value of at least three independent analyses, error bars refer to standard deviation. Asterisks indicate values which are significantly different from those of the corresponding WT plants, as determined by both one-way ANOVA and Tukey HSD post-hoc tests (P<0.05)

2. DISCUSSION

Without any doubt, abiotic stress signaling relies on PA metabolism. Protective roles have been described for most abundant PAs in plants in response to several stresses. Manipulation of PA biosynthetic genes to increase PA production has been shown to induce the expression of several genes related with stress signaling (Marco *et al.*, 2011). Nevertheless, this work demonstrates that PA oxidation is also involved in the induction of stress signaling cascades. Regarding stomatal closure, ABA role is undeniable, especially during stress response (Nakashima and Yamaguchi-Shinozaki, 2013). Interestingly, SPMS is an ABA-inducible gene (Rambla *et al.*, 2010), which must be associated with SPM protective role found in *Arabidopsis* during water stress (Yamaguchi *et al.*, 2007). However, PUT is also related with increased drought tolerance (Alcázar *et al.*, 2010*b*), which in our work was not observed. This fact could reinforce the notion that PA oxidation is not just a degradation process, but also has a signaling role. Recent work reported that ABA-mediated stomatal closure implicated a coordinated process where ABA down-stream targets were not just H_2O_2 and NO (Xie *et al.*, 2014). In this report, NO generation was demonstrated to be H_2O_2-dependent, which is not rare since this work showed that ROS and RNS signals are interconnected. The novelty likely relies on hydrogen gas (H_2), which seems to be highly induced by ABA and is a subsequent stimulator of signaling by RONS molecules. The cascade involves activation of an outward/rectifying K^+ channel on the guard cell (GORK) in a ROS/RNS dependent manner (Xie *et al.*, 2014). Interestingly, the connection between ROS and PA signals for activation of plasma membrane cation channels have been established in *Arabidopsis* (Pottosin *et al.*, 2012; Pottosin and Shabala, 2014).

Other recent findings point toward SPM signaling role during drought stress. Lately SPM has been linked with drought stress alleviation in white clover or rice cultivars by influencing sugar metabolism in the first and drought-responsive genes in the latter (Do *et al.*, 2013, 2014; Li *et al.*, 2015). It should

be noted that certain metabolites which demonstrated association with SPM levels either after nitro-oxidative stress or dark-induced senescence, have been also linked with drought tolerance. For instance, ASC-DHA recycling pathway influences ABA-mediated stomatal closure (Chen and Gallie, 2006; Fotopoulos *et al.*, 2008). GSH homeostasis (modulated by SPM according to results found in this study) regulates ABA-induced stomatal closure in *Arabidopsis* (Okuma *et al.*, 2011). Drought tolerant phenotype has been observed after trehalose degradation in *Arabidopsis* (Van Houtte *et al.*, 2013), in fact proposing a redox signaling role for this sugar metabolite.

In any case, further studies are needed to fully elucidate the SPM signaling pathway. SPM homeostasis regulated by PAO4 could be a solid tool for applied research towards generation of stress-tolerant plants.

CONCLUSIONS AND FUTURE PERSPECTIVES

CONCLUSIONS

A Metabolomic approach has been applied for the first time on *Arabidopsis* mutants with affected PA metabolism due to SPM accumulation. The results provide evidence of a PA signaling role during normal growth as well as in response to stressful conditions.

Chapter 1

- SPM homeostasis regulated by AtPAO4 influence RSA by modulating lateral root emergence, thus suggesting a signaling role for higher PAs in this auxin-mediated process.
- Cross-talk between ROS, RNS and PA signals is necessary to restore LR emergence in *pao4* mutants, showing that interaction of these molecules is necessary for RSA modeling.
- SPM homeostasis is involved either in long-term oxidative or short-term nitrosative responses after stress imposition, by modulation of PA biosynthetic precursors, RFOs pathway, *myo*-inositol and glutathione metabolism.

Chapter 2

- Results confirm that AtPAO4 is the main enzyme implicated in SPM homeostasis not only during seedling stage as proposed (Kamada-Nobusada *et al.*, 2008), but also in adult plants.
- SPM homeostasis during normal growth conditions is involved with regulation of central hub metabolites, establishing novel connections between PA metabolism and essential sugar and lipid metabolites.
- Metabolic changes generated by *PAO4* mutation are associated with delayed entry into dark-induced senescence.

Chapter 3

- Metabolic changes generated by *PAO4* mutation, stimulate a protective "housekeeping" condition since *pao4* mutants are tolerant to subsequent water stress. The mutants displayed a phenotype of permanently increased stomatal closure. The *pao4* mutants therefore appear to be in a naturally primed state.
- Results of this work point out that *pao4* mutants might be suitable tools for applied research regarding abiotic stress tolerance.

FUTURE PERSPECTIVES

The increment on natural disasters provoked by climate change is undeniable, with the number of crops affected by dehydration, osmotic shock or extreme temperatures becoming increasingly large. In regard with this matter, the field of plant stress physiology has emerged as a positive and necessary instrument to understand the nature of tolerance responses leading to plant survival.

Lately, it has been observed that perturbation of PA catabolism can either affect important developmental processes such as grape ripening or rice anther formation (Agudelo-Romero *et al.*, 2014; Liu *et al.*, 2014*b*), or the tolerance response to common environmental stresses such as flooding, as in the case of maize (Chen *et al.*, 2014).

The present study provides novel evidence further reinforcing the notion that PA oxidation is a piece of the puzzle regarding PA signaling network. It was demonstrated that loss-of-function of AtPAO4 and subsequent SPM accumulation generates changes in RSA and also leads to delayed senescence, drought stress and nitro-oxidative stress tolerance phenotype, which is interesting since nitrosative/oxidative stress or even the combination of both is a common consequence of several environmental stress factors.

Therefore with the aim to fully decipher the PA signaling pathway, it might be informative to gain deeper insights into the relationship between SPM accumulation of *pao4* mutants and certain aspects of relevance to plant development and survival. Thus, it would be useful to study in the future *pao4* mutants in the context of:

- Expression of auxin, ABA and ET biosynthetic genes as well as responsive elements.
- Nitrosative and oxidative status, by detailed analysis of enzymes directly linked with ROS and RNS homeostasis.
- Glutathione and ascorbate homeostasis and mechanisms involved in the maintenance of adequate levels of these essential redox molecules.

- Essential metabolic pathways on sugar and lipid signaling such as glycolysis, gluconeogenesis, pentose phosphate pathway or TCA cycle.

REFERENCES

A

- **A.I. Saeed, V. Sharov J, White, J. Li, W. Liang N, Bhagabati, J. Braisted M, Klapa, T. Currier MT, A. Sturn MS, A. Rezantsev DP, A. Ryltsov EK, I. Borisovsky, Z. Liu A, Vinsavich, V. Trush A, Quackenbush. J**. 2003. TM4: A Free, Open-Source System for Microarray Data Managment and Analysis. BioTechniques **34**, 374–378.
- **Agudelo-Romero P, Ali K, Choi YH, Sousa L, Verpoorte R, Tiburcio AF, Fortes AM**. 2014. Plant Physiology and Biochemistry Perturbation of polyamine catabolism affects grape ripening of Vitis vinifera cv . Trincadeira. Plant Physiology et Biochemistry **74**, 141–155.
- **Ahou A, Martignago D, Alabdallah O, *et al.*** 2014. A plant spermine oxidase/dehydrogenase regulated by the proteasome and polyamines. Journal of Experimental Botany **65**, 1585–1603.
- **Alcázar R, Altabella T, Marco F, Bortolotti C, Reymond M, Koncz C, Carrasco P, Tiburcio AF**. 2010*a*. Polyamines: Molecules with regulatory functions in plant abiotic stress tolerance. Planta **231**, 1237–1249.
- **Alcázar R, Planas-Portell J, Saxena T, Zarza X, Bortolotti C, Cuevas J, Bitrián M, Tiburcio AF, Altabella T**. 2010*b*. Putrescine accumulation confers drought tolerance in transgenic Arabidopsis plants over-expressing the homologous Arginine decarboxylase 2 gene. Plant Physiology and Biochemistry **48**, 547–552.
- **Alcázar R, Tiburcio AF**. 2014. Plant polyamines in stress and development: an emerging area of research in plant sciences. Frontiers in Plant Science **5**, 1–2.
- **Allwood JW, Erban A, de Koning S, Dunn WB, Luedemann A, Lommen A, Kay L, Löscher R, Kopka J, Goodacre R**. 2009. Inter-laboratory reproducibility of fast gas chromatography-electron impact-time of flight mass spectrometry (GC-EI-TOF/MS) based plant metabolomics. Metabolomics **5**, 479–496.

- **Allwood JW, De Vos RCH, Moing A, Deborde C, Erban A, Kopka J, Goodacre R, Hall RD**. 2011. Plant metabolomics and its potential for systems biology research: Background concepts, technology, and methodology. Methods in Enzymology **500**, 299–336.
- **Alonso JM, Stepanova AN, Leisse TJ, *et al.*** 2003. Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science (New York, N.Y.) **301**, 653–657.
- **Andronis E a, Moschou PN, Toumi I, Roubelakis-Angelakis KA**. 2014. Peroxisomal polyamine oxidase and NADPH-oxidase cross-talk for ROS homeostasis which affects respiration rate in Arabidopsis thaliana. Frontiers in plant science **5**, 132 (1–10).
- **Araújo WL, Ishizaki K, Nunes-Nesi A, *et al.*** 2010. Identification of the 2-hydroxyglutarate and isovaleryl-CoA dehydrogenases as alternative electron donors linking lysine catabolism to the electron transport chain of Arabidopsis mitochondria. The Plant cell **22**, 1549–1563.
- **Araújo WL, Tohge T, Ishizaki K, Leaver CJ, Fernie AR**. 2011. Protein degradation - an alternative respiratory substrate for stressed plants. Trends in Plant Science **16**, 489–498.

B

- **Begara-Morales JC, Chaki M, Sánchez-Calvo B, Mata-Pérez C, Leterrier M, Palma JM, Barroso JB, Corpas FJ**. 2013. Protein tyrosine nitration in pea roots during development and senescence. Journal of Experimental Botany **64**, 1121–1134.
- **Berberich T, Sagor GHM, Kusano T**. 2015. Polyamines in plant stress response. In: Kusano T, Suzuki H, eds. Polyamines. Tokio: Springer, 155–168.
- **Bitrián M, Zarza X, Altabella T, Tiburcio AF, Alcázar R**. 2012. Polyamines under Abiotic Stress: Metabolic Crossroads and Hormonal Crosstalks in Plants. Metabolites **2**, 516–528.

- **Blokhina O, Fagerstedt K V.** 2010. Reactive oxygen species and nitric oxide in plant mitochondria: Origin and redundant regulatory systems. Physiologia Plantarum **138**, 447–462.
- **Buchanan-Wollaston V, Page T, Harrison E, *et al.*** 2005. Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in Arabidopsis. Plant Journal **42**, 567–585.

C

- **Capell T, Bassie L, Christou P**. 2004. Modulation of the polyamine biosynthetic pathway in transgenic rice confers tolerance to drought stress. Proceedings of the National Academy of Sciences of the United States of America **101**, 9909–9914.
- **Chae L, Lee I, Shin J, Rhee SY**. 2012. Towards understanding how molecular networks evolve in plants. Current Opinion in Plant Biology **15**, 177–184.
- **Chen Y, Chen X, Wang H, Bao Y, Zhang W**. 2014. Examination of the leaf proteome during flooding stress and the induction of programmed cell death in maize. Proteome Science **12**, 33 (1–18).
- **Chen Z, Gallie DR**. 2006. Dehydroascorbate reductase affects leaf growth, development, and function. Plant Physiology **142**, 775–787.
- **Cheng L, Sun R, Wang F, Peng Z, Kong F, Wu J, Cao J, Lu G**. 2012. Spermidine affects the transcriptome responses to high temperature stress in ripening tomato fruit. Journal of Zhejiang University SCIENCE B **13**, 283–297.
- **Chiu JC, Brenner ED, DeSalle R, Nitabach MN, Holmes TC, Coruzzi GM**. 2002. Phylogenetic and expression analysis of the glutamate-receptor-like gene family in Arabidopsis thaliana. Molecular biology and evolution **19**, 1066–1082.
- **Choudhury S, Panda P, Sahoo L, Panda SK**. 2013. Reactive oxygen species signaling in plants under abiotic stress. Plant Signaling & Behavior **8**, e23681(1–6).

- **Cona A, Rea G, Angelini R, Federico R, Tavladoraki P**. 2006. Functions of amine oxidases in plant development and defence. Trends in Plant Science **11**, 80–88.
- **Corpas FJ, Barroso JB**. 2013. Nitro-oxidative stress vs oxidative or nitrosative stress in higher plants. New Phytologist **199**, 633–635.
- **Corpas FJ, Barroso JB**. 2014. Peroxynitrite (ONOO-) is endogenously produced in arabidopsis peroxisomes and is overproduced under cadmium stress. Annals of Botany **113**, 87–96.
- **Corpas FJ, Hayashi M, Mano S, Nishimura M, Barroso JB**. 2009. Peroxisomes are required for in vivo nitric oxide accumulation in the cytosol following salinity stress of Arabidopsis plants. Plant Physiology **151**, 2083–2094.
- **Correa-Aragunde N, Foresi N, Delledonne M, Lamattina L**. 2013. Auxin induces redox regulation of ascorbate peroxidase 1 activity by S-nitrosylation/denitrosylation balance resulting in changes of root growth pattern in Arabidopsis. Journal of Experimental Botany **64**, 3339–3349.
- **Correa-Aragunde N, Foresi N, Lamattina L**. 2015. Nitric oxide is an ubiquitous signal for maintaining redox balance in plant cells: regulation of ascorbate peroxidase as a case study. Journal of Experimental Botany **66**, 2913–2921.
- **Cuevas JC, López-Cobollo R, Alcázar R, Zarza X, Koncz C, Altabella T, Salinas J, Tiburcio AF, Ferrando A**. 2008. Putrescine is involved in Arabidopsis freezing tolerance and cold acclimation by regulating abscisic acid levels in response to low temperature. Plant Physiology **148**, 1094–1105.

D

- **Demidchik V, Shabala SN, Davies JM**. 2007. Spatial variation in H2O2 response of Arabidopsis thaliana root epidermal Ca2+ flux and plasma membrane Ca2+ channels. Plant Journal **49**, 377–386.

- **Desikan R, Cheung MK, Bright J, Henson D, Hancock JT, Neill SJ**. 2004. ABA, hydrogen peroxide and nitric oxide signalling in stomatal guard cells. Journal of Experimental Botany **55**, 205–212.
- **Diaz Céline, Purdy S, Christ A, Morot-Gaudry J-F, Wingler A, Masclaux-Daubresse C**. 2005. Characterization of Markers to Determine the Extent and Variability of Leaf Senescence in Arabidopsis . A Metabolic Profiling Approach 1. Plant Physiology **138**, 898–908.
- **Ding Z, De Smet I**. 2013. Localised ABA signalling mediates root growth plasticity. Trends in Plant Science **18**, 533–535.
- **Distéfano AM, Scuffi D, García-Mata C, Lamattina L, Laxalt AM**. 2012. Phospholipase Dδ is involved in nitric oxide-induced stomatal closure. Planta **236**, 1899–1907.
- **Dixon RA, Achnine L, Kota P, Liu CJ, Reddy MSS, Wang L**. 2002. The phenylpropanoid pathway and plant defence - A genomics perspective. Molecular Plant Pathology **3**, 371–390.
- **Dixon RA, Gang DR, Charlton AJ, *et al.*** 2006. Applications of metabolomics in agriculture. Journal of Agricultural and Food Chemistry **54**, 8984–8994.
- **Do PT, Degenkolbe T, Erban A, Heyer AG, Kopka J, Köhl KI, Hincha DK, Zuther E**. 2013. Dissecting Rice Polyamine Metabolism under Controlled Long-Term Drought Stress. PLoS ONE **8**, E60325 (1–14).
- **Do PT, Drechsel O, Heyer AG, Hincha DK, Zuther E**. 2014. Changes in free polyamine levels, expression of polyamine biosynthesis genes, and performance of rice cultivars under salt stress: a comparison with responses to drought. Frontiers in plant science **5**, 182 (1–16).
- **Del Duca S, Serafini-Fracassini D, Cai G**. 2014. Senescence and programmed cell death in plants: polyamine action mediated by transglutaminase. Frontiers in plant science **5**, 120 (1–17).

E

- **Eastmond PJ, Astley HM, Parsley K, Aubry S, Williams BP, Menard GN, Craddock CP, Nunes-Nesi A, Fernie AR, Hibberd JM**. 2015.

Arabidopsis uses two gluconeogenic gateways for organic acids to fuel seedling establishment. Nature Communications **6**, 1–8.

- **Elsayed a. I, Rafudeen MS, Golldack D**. 2014. Physiological aspects of raffinose family oligosaccharides in plants: Protection against abiotic stress. Plant Biology **16**, 1–8.
- **Van den Ende W**. 2013. Multifunctional fructans and raffinose family oligosaccharides. Frontiers in plant science **4**, 247 (1–11).
- **Van den Ende W**. 2014. Sugars take a central position in plant growth, development and, stress responses. A focus on apical dominance. Frontiers in plant science **5**, 1–3.
- **Engqvist MKM, Kuhn A, Wienstroer J, Weber K, Jansen EEW, Jakobs C, Weber APM, Maurino VG**. 2011. Plant D-2-hydroxyglutarate dehydrogenase participates in the catabolism of lysine especially during senescence. Journal of Biological Chemistry **286**, 11382–11390.
- **Erban A, Schauer N, Fernie AR, Kopka J**. 2007. Nonsupervised Construction and Application of Mass Spectral and Retention Time Index Libraries From Time-of-Flight Gas Chromatography-Mass Spectrometry Metabolite Profiles. Methods in Molecular Biology **358**, 19–38.

F

- **Fernandez O, Béthencourt L, Quero A, Sangwan RS, Clément Christophe C**. 2010. Trehalose and plant stress responses: Friend or foe? Trends in Plant Science **15**, 409–417.
- **Fernández-Marcos M, Sanz L, Lorenzo Ó**. 2012. Nitric oxide: An emerging regulator of cell elongation during primary root growth. Plant Signaling & Behavior **7**, 196–200.
- **Fernie AR, Trethewey RN, Krotzky AJ, Willmitzer L**. 2004. Metabolite profiling: from diagnostics to systems biology. Nature reviews **5**, 1–7.
- **Fiehn O, Kopka J, Dörmann P, Altmann T, Trethewey RN, Willmitzer L**. 2000. Metabolite profiling for plant functional genomics. Nature biotechnology **18**, 1157–1161.

- **Filippou P, Antoniou C, Fotopoulos V**. 2011. Effect of drought and rewatering on the cellular status and anti-oxidative response of Medicago truncatula plants. Plant signaling & behavior **6**, 270–277.
- **Filippou P, Antoniou C, Fotopoulos V**. 2013. The nitric oxide donor sodium nitroprusside regulates polyamine and proline metabolism in leaves of Medicago truncatula plants. Free Radical Biology and Medicine **56**, 172–183.
- **Fincato P, Moschou PN, Spedaletti V, Tavazza R, Angelini R, Federico R, Roubelakis-Angelakis K a., Tavladoraki P**. 2011. Functional diversity inside the Arabidopsis polyamine oxidase gene family. Journal of Experimental Botany **62**, 1155–1168.
- **Flores T, Todd CD, Tovar-Mendez A, Dhanoa PK, Correa-Aragunde N, Hoyos ME, Brownfield DM, Mullen RT, Lamattina L, Polacco JC**. 2008. Arginase-negative mutants of Arabidopsis exhibit increased nitric oxide signaling in root development. Plant Physiology **147**, 1936–1946.
- **Forde BG**. 2014. Nitrogen signalling pathways shaping root system architecture: An update. Current Opinion in Plant Biology **21**, 30–36.
- **Forde BG, Lea PJ**. 2007. Glutamate in plants: Metabolism, regulation, and signalling. Journal of Experimental Botany **58**, 2339–2358.
- **Fotopoulos V, Kanellis AK**. 2013. Altered apoplastic ascorbate redox state in tobacco plants via ascorbate oxidase overexpression results in delayed dark-induced senescence in detached leaves. Plant Physiology and Biochemistry **73**, 154–160.
- **Fotopoulos V, De Tullio MC, Barnes J, Kanellis AK**. 2008. Altered stomatal dynamics in ascorbate oxidase over-expressing tobacco plants suggest a role for dehydroascorbate signalling. Journal of Experimental Botany **59**, 729–737.
- **Foyer CH, Noctor G**. 2011. Ascorbate and glutathione: the heart of the redox hub. Plant Physiology **155**, 2–18.
- **Fuell C, Elliott K a., Hanfrey CC, Franceschetti M, Michael AJ**. 2010. Polyamine biosynthetic diversity in plants and algae. Plant Physiology and Biochemistry **48**, 513–520.

- **Fujita M, Fujita Y, Iuchi S, Yamada K, Kobayashi Y, Urano K, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K**. 2012. Natural variation in a polyamine transporter determines paraquat tolerance in Arabidopsis. Proceedings of the National Academy of Sciences **109**, 6343–6347.
- **Fujita M, Shinozaki K**. 2014. Identification of polyamine transporters in plants: Paraquat transport provides crucial clues. Plant and Cell Physiology **55**, 855–861.

G

- **Gallie DR**. 2012. The role of L-ascorbic acid recycling in responding to environmental stress and in promoting plant growth. Journal of Experimental Botany **64**, 433–443.
- **Gao HJ, Yang HQ, Wang JX**. 2009. Arginine metabolism in roots and leaves of apple (Malus domestica Borkh.): The tissue-specific formation of both nitric oxide and polyamines. Scientia Horticulturae **119**, 147–152.
- **Gibon Y, Pyl ET, Sulpice R, Lunn JE, Höhne M, Günther M, Stitt M**. 2009. Adjustment of growth, starch turnover, protein content and central metabolism to a decrease of the carbon supply when Arabidopsis is grown in very short photoperiods. Plant, Cell and Environment **32**, 859–874.
- **Gibon Y, Usadel B, Blaesing OE, Kamlage B, Hoehne M, Trethewey R, Stitt M**. 2006. Integration of metabolite with transcript and enzyme activity profiling during diurnal cycles in Arabidopsis rosettes. Genome biology **7**, R76 (1–23).
- **Gillaspy GE**. 2011. The cellular language of myo-inositol signaling. New Phytologist **192**, 823–839.
- **Golan G, Betzer R, Wolf S**. 2013. Phloem-specific expression of a melon Aux/IAA in tomato plants alters auxin sensitivity and plant development. Frontiers in plant science **4**, 329 (1–10).
- **Van der Graaff E, Schwacke R, Schneider A, Desimone M, Kunze R**. 2006. Transcription Analysis of Arabidopsis Membrane Transporters and

Hormone Pathways during Developmental and Induced Leaf Senescence. Plant Physiology **141**, 776–792.

- **Guo Y, Cai Z, Gan S**. 2004. Transcriptome of Arabidopsis leaf senescence. Plant, Cell and Environment **27**, 521–549.

H

- **Hashida SN, Itami T, Takahashi H, Takahara K, Nagano M, Kawai-Yamada M, Shoji K, Goto F, Yoshihara T, Uchimiya H**. 2010. Nicotinate/nicotinamide mononucleotide adenyltransferase-mediated regulation of NAD biosynthesis protects guard cells from reactive oxygen species in ABA-mediated stomatal movement in Arabidopsis. Journal of Experimental Botany **61**, 3813–3825.
- **He L, Nada K, Kasukabe Y, Tachibana S**. 2002. Enhanced susceptibility of photosynthesis to low-temperature photoinhibition due to interruption of chill-induced increase of S-adenosylmethionine decarboxylase activity in leaves of spinach (Spinacia oleracea L.). Plant & cell physiology **43**, 196–206.
- **Hodges DM, DeLong JM, Forney CF, Prange RK**. 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta **207**, 604–611.
- **Holzmeister C, Gaupels F, Geerlof a., Sarioglu H, Sattler M, Durner J, Lindermayr C**. 2014. Differential inhibition of Arabidopsis superoxide dismutases by peroxynitrite-mediated tyrosine nitration. Journal of Experimental Botany **66**, 989–999.
- **Van Houtte H, Vandesteene L, López-Galvis L, *et al.*** 2013. Overexpression of the trehalase gene AtTRE1 leads to increased drought stress tolerance in Arabidopsis and is involved in abscisic acid-induced stomatal closure. Plant Physiology **161**, 1158–1171.
- **Hui Z, Tian F-X, Wang G, Wang G-P, Wang W**. 2012. The antioxidative defense system is involved in the delayed senescence in a wheat mutant tasg1. Plant cell reports **31**, 1073–1084.

- **Hulsen T, de Vlieg J, Alkema W**. 2008. BioVenn - a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams. BMC genomics **9**, 488–495.
- **Hummel J, Strehmel N, Selbig J, Walther D, Kopka J**. 2010. Decision tree supported substructure prediction of metabolites from GC-MS profiles. Metabolomics **6**, 322–333.
- **Hung C-Y, Aspesi P, Hunter MR, Lomax AW, Perera IY**. 2014. Phosphoinositide-signaling is one component of a robust plant defense response. Frontiers in plant science **5**, 267 (1–15).

I

- **Imai A, Akiyama T, Kato T, Sato S, Tabata S, Yamamoto KT, Takahashi T**. 2004*a*. Spermine is not essential for survival of Arabidopsis. FEBS Letters **556**, 148–152.
- **Imai A, Matsuyama T, Hanzawa Y, Akiyama T, Tamaoki M**. 2004*b*. Spermidine Synthase Genes Are Essential for Survival of Arabidopsis. Plant physiology **135**, 1565–1573.
- **Innocenti G, Pucciariello C, Le Gleuher M, Hopkins J, De Stefano M, Delledonne M, Puppo A, Baudouin E, Frendo P**. 2007. Glutathione synthesis is regulated by nitric oxide in Medicago truncatula roots. Planta **225**, 1597–1602.
- **Ischebeck T, Seiler S, Heilmann I**. 2010. At the poles across kingdoms: Phosphoinositides and polar tip growth. Protoplasma **240**, 13–31.
- **Ishizaki K, Larson TR, Schauer N, Fernie AR, Graham IA, Leaver CJ**. 2005. The critical role of Arabidopsis electron-transfer flavoprotein:ubiquinone oxidoreductase during dark-induced starvation. The Plant Cell **17**, 2587–2600.
- **Ishizaki K, Schauer N, Larson TR, Graham IA, Fernie AR, Leaver CJ**. 2006. The mitochondrial electron transfer flavoprotein complex is essential for survival of Arabidopsis in extended darkness. Plant Journal **47**, 751–760.

J

- **Jannat R, Uraji M, Morofuji M, Islam MM, Bloom RE, Nakamura Y, McClung CR, Schroeder JI, Mori IC, Murata Y**. 2011. Roles of intracellular hydrogen peroxide accumulation in abscisic acid signaling in Arabidopsis guard cells. Journal of Plant Physiology **168**, 1919–1926.

K

- **Kamada-Nobusada T, Hayashi M, Fukazawa M, Sakakibara H, Nishimura M**. 2008. A putative peroxisomal polyamine oxidase, AtPAO4, is involved in polyamine catabolism in Arabidopsis thaliana. Plant and Cell Physiology **49**, 1272–1282.
- **Kanehisa M, Goto S**. 1999. KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Research **27**, 29–34.
- **Kanehisa M, Goto S, Sato Y, Kawashima M, Furumichi M, Tanabe M**. 2014. Data, information, knowledge and principle: Back to metabolism in KEGG. Nucleic Acids Research **42**, 199–205.
- **Kasukabe Y, He LX, Nada K, Misawa S, Ihara I, Tachibana S**. 2004. Overexpression of spermidine synthase enhances tolerance to multiple environmental stresses and up-regulates the expression of various stress regulated genes in transgenic Arabidopsis thaliana. Plant and Cell Physiology **45**, 712–722.
- **Kelly G, David-Schwartz R, Sade N, Moshelion M, Levi A, Alchanatis V, Granot D**. 2012. The Pitfalls of Transgenic Selection and New Roles of AtHXK1: A High Level of AtHXK1 Expression Uncouples Hexokinase1-Dependent Sugar Signaling from Exogenous Sugar. Plant Physiology **159**, 47–51.
- **Khanna-Chopra R**. 2012. Leaf senescence and abiotic stresses share reactive oxygen species-mediated chloroplast degradation. Protoplasma **249**, 469–481.
- **Kim DW, Watanabe K, Murayama C, Izawa S, Niitsu M, Michael AJ, Berberich T, Kusano T**. 2014. Polyamine Oxidase 5 Regulates

Arabidopsis thaliana Growth Through A Thermospermine Oxidase Activity. Plant physiology **165**, 1575–1590.

- **Kolbert Z, Bartha B, Erdei L**. 2008. Exogenous auxin-induced NO synthesis is nitrate reductase-associated in Arabidopsis thaliana root primordia. Journal of Plant Physiology **165**, 967–975.
- **Kopka J, Schauer N, Krueger S, *et al.*** 2005. GMD@CSB.DB: The Golm metabolome database. Bioinformatics **21**, 1635–1638.
- **Kotakis C, Theodoropoulou E, Tassis K, Oustamanolakis C, Ioannidis NE, Kotzabasis K**. 2014. Putrescine, a fast-acting switch for tolerance against osmotic stress. Journal of Plant Physiology **171**, 48–51.
- **Krasensky J, Broyart C, Rabanal F a, Jonak C**. 2014. The Redox-Sensitive Chloroplast Trehalose-6-Phosphate Phosphatase AtTPPD Regulates Salt Stress Tolerance. Anti-oxidatives & redox signaling **21**, 1–16.
- **Krishnamoorthy P, Sanchez-Rodriguez C, Heilmann I, Persson S**. 2014. Regulatory roles of phosphoinositides in membrane trafficking and their potential impact on cell-wall synthesis and re-modelling. Annals of botany **114**, 1049–1057.

L

- **Lehmann M, Schwarzländer M, Obata T, *et al.*** 2009. The metabolic response of Arabidopsis roots to oxidative stress is distinct from that of heterotrophic cells in culture and highlights a complex relationship between the levels of transcripts, metabolites, and flux. Molecular Plant **2**, 390–406.
- **Lemaître T, Gaufichon L, Boutet-Mercey S, Christ A, Masclaux-Daubresse C**. 2008. Enzymatic and metabolic diagnostic of nitrogen deficiency in Arabidopsis thaliana Wassileskija accession. Plant and Cell Physiology **49**, 1056–1065.
- **Leprince A-S, Magalhaes N, De Vos D, Bordenave M, Crilat E, Clément G, Meyer C, Munnik T, Savouré A**. 2015. Involvement of

Phosphatidylinositol 3-kinase in the regulation of proline catabolism in Arabidopsis thaliana. Frontiers in Plant Science **5**, 1–13.

- **Li Z, Jing W, Peng Y, Zhang XQ, Ma X, Huang LK, Yan Y**. 2015. Spermine Alleviates Drought Stress in White Clover with Different Resistance by Influencing Carbohydrate Metabolism and Dehydrins Synthesis. Plos One **10**, e0120708 (1–16).
- **Linka N, Theodoulou FL**. 2013. Peroxisomes and their Key Role in Cellular Signaling and Metabolism. In: Luis A R, ed. Peroxisomes.169–194.
- **Linkohr BI, Williamson LC, Fitter AH, Leyser HMO**. 2002. Nitrate and phosphate availability and distribution have different effects on root system architecture of Arabidopsis. The Plant Journal : for cell and molecular biology **29**, 751–760.
- **Lira-Ruan V, Mendivil SN, Dubrovsky JG**. 2013. Heuristic Aspect of the Lateral Root Initiation Index: A Case Study of the Role of Nitric Oxide in Root Branching. Applications in Plant Sciences **1**, 1300029 (1–8).
- **Lisec J, Schauer N, Kopka J, Willmitzer L, Fernie AR**. 2006. Gas chromatography mass spectrometry-based metabolite profiling in plants. Nature protocols **1**, 387–396.
- **Liu T, Dobashi H, Kim DW, Sagor GHM, Niitsu M, Berberich T, Kusano T**. 2014*a*. Arabidopsis mutant plants with diverse defects in polyamine metabolism show unequal sensitivity to exogenous cadaverine probably based on their spermine content. Physiology and Molecular Biology of Plants **20**, 151–159.
- **Liu F, Guo FQ**. 2013. Nitric Oxide Deficiency Accelerates Chlorophyll Breakdown and Stability Loss of Thylakoid Membranes during Dark-Induced Leaf Senescence in Arabidopsis. PLoS ONE **8**, e56345 (1–12).
- **Liu T, Kim DW, Niitsu M, Maeda S, Watanabe M, Kamio Y, Berberich T, Kusano T**. 2014*b*. Polyamine Oxidase 7 is a Terminal Catabolism-Type Enzyme in Oryza sativa and is Specifically Expressed in Anthers. Plant and Cell Physiology **55**, 1110–1122.

- **Liu JH, Kitashiba H, Wang J, Ban Y, Moriguchi T**. 2007. Polyamines and their ability to provide environmental stress tolerance to plants. Plant Biotechnology **24**, 117–126.
- **Lobet G, Pagès L, Draye X**. 2011. A novel image-analysis toolbox enabling quantitative analysis of root system architecture. Plant Physiology **157**, 29–39.
- **Lombardo MC, Graziano M, Polacco JC, Lamattina L**. 2006. Nitric oxide functions as a positive regulator of root hair development. Plant Signaling & Behavior **1**, 28–33.
- **López-Berges MS, Rispail N, Prados-Rosales RC, Di Pietro A**. 2010. A nitrogen response pathway regulates virulence functions in Fusarium oxysporum via the protein kinase TOR and the bZIP protein MeaB. The Plant Cell **22**, 2459–2475.
- **Luedemann A, Strassburg K, Erban A, Kopka J**. 2008. TagFinder for the quantitative analysis of gas chromatography - Mass spectrometry (GC-MS)-based metabolite profiling experiments. Bioinformatics **24**, 732–737.
- **Luis A R**. 2013. *Peroxisomes and their Key Role in Cellular Signaling and Metabolism* (R Luis A, Ed.). Springer.

M

- **Mano Y, Nemoto K**. 2012. The pathway of auxin biosynthesis in plants. Journal of Experimental Botany **63**, 2853–2872.
- **Manzano C, Pallero-Baena M, Casimiro I, De Rybel B, Orman-Ligeza B, Van Isterdael G, Beeckman T, Draye X, Casero P, Del Pozo JC**. 2014. The Emerging Role of Reactive Oxygen Species Signaling during Lateral Root Development. Plant Physiology **165**, 1105–1119.
- **Marcé M, Brown DS, Capell T, Figueras X, Tiburcio AF**. 1995. Rapid high-performance liquid chromatographic method for the quantitation of polyamines as their dansyl derivatives : application to plant and animal tissues. Journal of Chromatography **666**, 329–335.

- **Marco F, Alcázar R, Tiburcio AF, Carrasco P**. 2011. Interactions between Polyamines and Abiotic Stress Pathway Responses Unraveled by Transcriptome Analysis of Polyamine Overproducers. OMICS: A Journal of Integrative Biology **15**, 775–781.
- **Marina M, Sirera FV, Rambla JL, Gonzalez ME, Blázquez MA, Carbonell J, Pieckenstain FL, Ruiz OA**. 2013. Thermospermine catabolism increases Arabidopsis thaliana resistance to Pseudomonas viridiflava. Journal of Experimental Botany **64**, 1393–1402.
- **Mason MG, Ross JJ, Babst B a., Wienclaw BN, Beveridge CA**. 2014. Sugar demand, not auxin, is the initial regulator of apical dominance. Proceedings of the National Academy of Sciences **111**, 6092–6097.
- **Mattoo AK, Minocha SC, Minocha R, Handa AK**. 2010. Polyamines and cellular metabolism in plants: Transgenic approaches reveal different responses to diamine putrescine versus higher polyamines spermidine and spermine. Amino Acids **38**, 405–413.
- **Meyer T, Burow M, Bauer M, Papenbrock J**. 2003. Arabidopsis sulfurtransferases: investigation of their function during senescence and in cyanide detoxification. Planta **217**, 1–10.
- **Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R**. 2010. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant, Cell and Environment **33**, 453–467.
- **Minocha R, Majumdar R, Minocha SC**. 2014. Polyamines and abiotic stress in plants: a complex relationship. Frontiers in Plant Science **5**, 175 (11–17).
- **Mitsuya Y, Takahashi Y, Berberich T, Miyazaki A, Matsumura H, Takahashi H, Terauchi R, Kusano T**. 2009. Spermine signaling plays a significant role in the defense response of Arabidopsis thaliana to cucumber mosaic virus. Journal of Plant Physiology **166**, 626–643.
- **Molassiotis A, Fotopoulos V**. 2011. Oxidative and nitrosative signaling in plants: two branches in the same tree? Plant Signaling & Behavior **6**, 210–214.

- **Møller SG, McPherson MJ**. 1998. Developmental expression and biochemical analysis of the Arabidopsis atao1 gene encoding an H2O2-generating diamine oxidase. Plant Journal **13**, 781–791.
- **Moschou PN, Delis ID, Paschalidis KA, Roubelakis-Angelakis KA**. 2008*a*. Transgenic tobacco plants overexpressing polyamine oxidase are not able to cope with oxidative burst generated by abiotic factors. Physiologia Plantarum **133**, 140–156.
- **Moschou PN, Paschalidis KA, Delis ID, Andriopoulou AH, Lagiotis GD, Yakoumakis DI, Roubelakis-Angelakis KA**. 2008*b*. Spermidine exodus and oxidation in the apoplast induced by abiotic stress is responsible for H2O2 signatures that direct tolerance responses in tobacco. The Plant Cell **20**, 1708–1724.
- **Moschou PN, Paschalidis KA, Roubelakis-Angelakis KA**. 2008*c*. Plant polyamine catabolism: The state of the art. Plant Signaling & Behavior **3**, 1061–1066.
- **Moschou PN, Roubelakis-Angelakis KA**. 2014. Polyamines and programmed cell death. Journal of Experimental Botany **65**, 1285–1296.
- **Moschou PN, Wu J, Cona A, Tavladoraki P, Angelini R, Roubelakis-Angelakis KA**. 2012*a*. The polyamines and their catabolic products are significant players in the turnover of nitrogenous molecules in plants. Journal of Experimental Botany **63**, 5003–5015.
- **Moschou PN, Wu J, Cona A, Tavladoraki R, Angelini R, Roubelakis-Angelakis KA**. 2012*b*. The polyamines and their catabolic products are significant players in the turnover of nitrogenous molecules in plants. Journal of Experimental Botany **63**, 5003–5015.
- **Mueller LA, Zhang P, Rhee SY**. 2003. AraCyc: a biochemical pathway database for Arabidopsis. Plant Physiology **132**, 453–460.
- **Mulangi V, Phuntumart V, Aouida M, Ramotar D, Morris P**. 2012. Functional analysis of OsPUT1, a rice polyamine uptake transporter. Planta **235**, 1–11.

N

- **Nakabayashi R, Yonekura-Sakakibara K, Urano K, *et al.*** 2014. Enhancement of oxidative and drought tolerance in Arabidopsis by overaccumulation of anti-oxidative flavonoids. Plant Journal **77**, 367–379.
- **Nakashima K, Yamaguchi-Shinozaki K**. 2013. ABA signaling in stress-response and seed development. Plant Cell Reports **32**, 959–970.
- **Neill S, Barros R, Bright J, Desikan R, Hancock J, Harrison J, Morris P, Ribeiro D, Wilson I**. 2008. Nitric oxide, stomatal closure, and abiotic stress. Journal of Experimental Botany **59**, 165–176.
- **Niu YH, Guo FQ**. 2012. Nitric Oxide Regulates Dark-Induced Leaf Senescence Through EIN2 in Arabidopsis. Journal of Integrative Plant Biology **54**, 516–525.
- **Noctor G, Arisi ACM, Jouanin L, Valadier MH, Roux Y, Foyer CH**. 1997. Light-dependent modulation of foliar glutathione synthesis and associated amino acid metabolism in poplar overexpressing γ-glutamylcysteine synthetase. Planta **202**, 357–369.

O

- **O'Gorman A, Brennan L**. 2015. Metabolomic applications in nutritional research: a perspective. Journal of the Science of Food and Agriculture, 1–4.
- **O'Hara LE, Paul MJ, Wingler A**. 2013. How do sugars regulate plant growth and development? new insight into the role of trehalose-6-phosphate. Molecular Plant **6**, 261–274.
- **Obata T, Fernie AR**. 2012. The use of metabolomics to dissect plant responses to abiotic stresses. Cellular and Molecular Life Sciences **69**, 3225–3243.
- **Obata T, Matthes A, Koszior S, Lehmann M, Araújo WL, Bock R, Sweetlove LJ, Fernie AR**. 2011. Alteration of mitochondrial protein complexes in relation to metabolic regulation under short-term oxidative stress in Arabidopsis seedlings. Phytochemistry **72**, 1081–1091.
- **Ohkama-Ohtsu N, Oikawa A, Zhao P, Xiang C, Saito K, Oliver DJ**. 2008. A gamma-glutamyl transpeptidase-independent pathway of

glutathione catabolism to glutamate via 5-oxoproline in Arabidopsis. Plant Physiology **148**, 1603–1613.

- **Ohkama-Ohtsu N, Zhao P, Xiang C, Oliver DJ**. 2007. Glutathione conjugates in the vacuole are degraded by gamma-glutamyl transpeptidase GGT3 in Arabidopsis. The Plant Journal : for cell and molecular biology **49**, 878–888.
- **Okuma E, Jahan MS, Munemasa S, *et al.*** 2011. Negative regulation of abscisic acid-induced stomatal closure by glutathione in Arabidopsis. Journal of Plant Physiology **168**, 2048–2055.

P

- **Pagnussat GC, Simontacchi M, Puntarulo S, Lamattina L**. 2002. Nitric oxide is required for root organogenesis. Plant physiology **129**, 954–956.
- **Pál M, Szalai G, Janda T**. 2015. Speculation: Polyamines are important in abiotic stress signaling. Plant Science **237**, 16–23.
- **Pandey S, Ranade S a, Nagar PK, Kumar N**. 2000. Role of polyamines and ethylene as modulators of plant senescence. Journal of Biosciences **25**, 291–299.
- **Park SY, Yu JW, Park JS, *et al.*** 2007. The senescence-induced staygreen protein regulates chlorophyll degradation. The Plant cell **19**, 1649–1664.
- **Parvin S, Lee OR, Sathiyaraj G, Khorolragchaa A, Kim YJ, Yang DC**. 2014. Spermidine alleviates the growth of saline-stressed ginseng seedlings through antioxidative defense system. Gene **537**, 70–78.
- **Pathak MR, Teixeira da Silva JA, Wani SH**. 2014. Polyamines in response to abiotic stress tolerance through transgenic approaches. GM Crops & Food **5**, 87–96.
- **Paulose B, Chhikara S, Coomey J, Jung H Il, Vatamaniuk O, Dhankher OP**. 2013. A γ-Glutamyl Cyclotransferase Protects Arabidopsis Plants from Heavy Metal Toxicity by Recycling Glutamate to Maintain Glutathione Homeostasis. The Plant cell **25**, 4580–4595.

- **Perez-Amador MA, Leon J, Green PJ, Carbonell J**. 2002. Induction of the arginine decarboxylase ADC2 gene provides evidence for the involvement of polyamines in the wound response in Arabidopsis. Plant Physiology **130**, 1454–1463.
- **Petřivalský M, Brauner F, Luhová L, Gagneul D, Šebela M**. 2007. Aminoaldehyde dehydrogenase activity during wound healing of mechanically injured pea seedlings. Journal of Plant Physiology **164**, 1410–1418.
- **Piotrowski M, Schönfelder S, Weiler EW**. 2001. The Arabidopsis thaliana Isogene NIT4 and Its Orthologs in Tobacco Encode β-Cyano-L-alanine Hydratase/Nitrilase. Journal of Biological Chemistry **276**, 2616–2621.
- **Planas-Portell J, Gallart M, Tiburcio AF, Altabella T**. 2013. Copper-containing amine oxidases contribute to terminal polyamine oxidation in peroxisomes and apoplast of Arabidopsis thaliana. BMC Plant Biology **13**, 109 (1–13).
- **Pottosin I, Shabala S**. 2014. Polyamines control of cation transport across plant membranes: implications for ion homeostasis and abiotic stress signaling. Frontiers in plant science **5**, 154 (1–16).
- **Pottosin I, Velarde-Buendía AM, Bose J, Zepeda-Jazo I, Shabala S, Dobrovinskaya O**. 2014. Cross-talk between reactive oxygen species and polyamines in regulation of ion transport across the plasma membrane: Implications for plant adaptive responses. Journal of Experimental Botany **65**, 1271–1283.
- **Pottosin I, Velarde-Buendía A-M, Zepeda-Jazo I, Dobrovinskaya O, Shabala S**. 2012. Synergism between polyamines and ROS in the induction of Ca2+ and K+ fluxes in roots. Plant Signaling & Behavior **7**, 1084–1087.

R

- **Rambla JL, Vera-Sirera F, Blázquez MA, Carbonell J, Granell A**. 2010. Quantitation of biogenic tetraamines in Arabidopsis thaliana. Analytical Biochemistry **397**, 208–211.
- **Rangan P, Subramani R, Kumar R, Singh AK, Singh R**. 2014. Recent Advances in Polyamine Metabolism and Abiotic Stress Tolerance. BioMed Research International **2014**, 239621 (1–9).
- **Reumann S, Quan S, Aung K, *et al.*** 2009. In-depth proteome analysis of Arabidopsis leaf peroxisomes combined with in vivo subcellular targeting verification indicates novel metabolic and regulatory functions of peroxisomes. Plant Physiology **150**, 125–143.
- **Richardson AD, Duigan SP, Berlyn GP**. 2002. An evaluation of noninvasive methods to estimate foliar chlorophyll content. New Phytologist **153**, 185–194.
- **Rigas S, Ditengou FA, Ljung K, Daras G, Tietz O, Palme K, Hatzopoulos P**. 2013. Root gravitropism and root hair development constitute coupled developmental responses regulated by auxin homeostasis in the Arabidopsis root apex. New Phytologist **197**, 1130–1141.
- **Del Rio LA**. 2015. ROS and RNS in plant physiology: an overview. Journal of Experimental Botany **66**, 2827–2837.
- **Roessner U, Wagner C, Kopka J, Trethewey RN, Willmitzer L**. 2000. Simultaneous analysis of metabolites in potato tuber by gas chromatography ± mass spectrometry. The Plant Journal **23**, 131–142.

S

- **Sagor GHM, Takahashi H, Niitsu M, Takahashi Y, Berberich T, Kusano T**. 2012. Exogenous thermospermine has an activity to induce a subset of the defense genes and restrict cucumber mosaic virus multiplication in Arabidopsis thaliana. Plant Cell Reports **31**, 1227–1232.
- **Sandalio LM, Romero-Puertas MC**. 2015. Peroxisomes sense and respond to environmental cues by regulating ROS and RNS signalling networks. Annals of Botany, 1–11.

- **Dos Santos R, Vergauwen R, Pacolet P, Lescrinier E, Van Den Ende W**. 2013. Manninotriose is a major carbohydrate in red deadnettle (Lamium purpureum, Lamiaceae). Annals of Botany **111**, 385–393.
- **Sato EM, Hijazi H, Bennett MJ, Vissenberg K, Swarup R**. 2014. New insights into root gravitropic signalling. Journal of Experimental Botany **66**, 2155–2165.
- **Schlicht M, Ludwig-Müller J, Burbach C, Volkmann D, Baluska F**. 2013. Indole-3-butyric acid induces lateral root formation via peroxisome-derived indole-3-acetic acid and nitric oxide. New Phytologist **200**, 473–482.
- **Shelp BJ, Bozzo GG, Trobacher CP, Zarei A, Deyman KL, Brikis CJ**. 2012. Hypothesis/review: Contribution of putrescine to 4-aminobutyrate (GABA) production in response to abiotic stress. Plant Science **193-194**, 130–135.
- **Shi H, Chan Z**. 2014. Improvement of plant abiotic stress tolerance through modulation of the polyamine pathway. Journal of Integrative Plant Biology **56**, 114–121.
- **Sims DA, Gamon JA**. 2002. Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. Remote Sensing of Environment **81**, 337–354.
- **De Smet I, Signora L, Beeckman T, Inzé D, Foyer CH, Zhang H**. 2003. An abscisic acid-sensitive checkpoint in lateral root development of Arabidopsis. Plant Journal **33**, 543–555.
- **Soudry E, Ulitzur S, Gepstein S**. 2005. Accumulation and remobilization of amino acids during senescence of detached and attached leaves: In planta analysis of tryptophan levels by recombinant luminescent bacteria. Journal of Experimental Botany **56**, 695–702.
- **Soyka S, Heyer AG**. 1999. Arabidopsis knockout mutation of ADC2 gene reveals inducibility by osmotic stress. FEBS Letters **458**, 219–223.

- **Stitt M, Krapp A**. 1999. The interaction between elevated carbon dioxide and nitrogen nutrition : the physiological and molecular background. Plant, Cell and Environment **22**, 583–621.
- **Strehmel N, Hummel J, Erban A, Strassburg K, Kopka J**. 2008. Retention index thresholds for compound matching in GC-MS metabolite profiling. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences **871**, 182–190.

T

- **Takahashi Y, Berberich T, Miyazaki A, Seo S, Ohashi Y, Kusano T**. 2003. Spermine signalling in tobacco: Activation of mitogen-activated protein kinases by spermine is mediated through mitochondrial dysfunction. Plant Journal **36**, 820–829.
- **Takahashi Y, Cong R, Sagor GHM, Niitsu M, Berberich T, Kusano T**. 2010. Characterization of five polyamine oxidase isoforms in Arabidopsis thaliana. Plant Cell Reports **29**, 955–965.
- **Takahashi T, Kakehi JI**. 2010. Polyamines: Ubiquitous polycations with unique roles in growth and stress responses. Annals of Botany **105**, 1–6.
- **Takahashi Y, Uehara Y, Berberich T, Ito A, Saitoh H, Miyazaki A, Terauchi R, Kusano T**. 2004. A subset of hypersensitive response marker genes, including HSR203J, is the down-stream target of a spermine signal transduction pathway in tobacco. Plant Journal **40**, 586–595.
- **Takano A, Kakehi JI, Takahashi T**. 2012. Thermospermine is not a minor polyamine in the plant kingdom. Plant and Cell Physiology **53**, 606–616.
- **Tan X, Calderon-Villalobos LI, Sharon M, Zheng C, Robinson C V., Estelle M, Zheng N**. 2007. Mechanism of auxin perception by the TIR1 ubiquitin ligase. Nature **446**, 640–645.
- **Tanou G, Filippou P, Belghazi M, Job D, Diamantidis G, Fotopoulos V, Molassiotis A**. 2012. Oxidative and nitrosative-based signaling and

associated post-translational modifications orchestrate the acclimation of citrus plants to salinity stress. Plant Journal **72**, 585–599.

- **Tanou G, Molassiotis A, Diamantidis G**. 2009. Hydrogen peroxide- and nitric oxide-induced systemic anti-oxidative prime-like activity under NaCl-stress and stress-free conditions in citrus plants. Journal of Plant Physiology **166**, 1904–1913.
- **Tanou G, Ziogas V, Belghazi M, Christou A, Filippou P, Job D, Fotopoulos V, Molassiotis A**. 2014. Polyamines reprogram oxidative and nitrosative status and the proteome of citrus plants exposed to salinity stress. Plant, Cell and Environment **37**, 864–885.
- **Tavladoraki P, Cona A, Federico R, Tempera G, Viceconte N, Saccoccio S, Battaglia V, Toninello A, Agostinelli E**. 2012. Polyamine catabolism: Target for antiproliferative therapies in animals and stress tolerance strategies in plants. Amino Acids **42**, 411–426.
- **Tian H, De Smet I, Ding Z**. 2014. Shaping a root system: Regulating lateral versus primary root growth. Trends in Plant Science **19**, 426–431.
- **Tiburcio AF, Altabella T, Bitrián M, Alcázar R**. 2014. The roles of polyamines during the lifespan of plants: From development to stress. Planta **240**, 1–18.
- **Tisi A, Federico R, Moreno S, Lucretti S, Moschou PN, Roubelakis-Angelakis KA, Angelini R, Cona A**. 2011. Perturbation of polyamine catabolism can strongly affect root development and xylem differentiation. Plant Physiology **157**, 200–215.
- **Tolin S, Arrigoni G, Trentin AR, Veljovic-Jovanovic S, Pivato M, Zechman B, Masi A**. 2013. Biochemical and quantitative proteomics investigations in Arabidopsis ggt1 mutant leaves reveal a role for the gamma-glutamyl cycle in plant's adaptation to environment. Proteomics **13**, 2031–2045.
- **Tong W, Yoshimoto K, Kakehi JI, Motose H, Niitsu M, Takahashi T**. 2014. Thermospermine modulates expression of auxin-related genes in Arabidopsis. Frontiers in Plant Science **5**, 94 (1–10).

- **Töpfer N, Scossa F, Fernie AR, Nikoloski Z**. 2014. Variability of Metabolite Levels Is Linked to Differential Metabolic Pathways in Arabidopsis's Responses to Abiotic Stresses. PLoS Computational Biology **10**, e1003656 (1–11).
- **Tripathi DN, Chowdhury R, Trudel LJ, Tee AR, Slack RS, Walker CL, Wogan GN**. 2013. Reactive nitrogen species regulate autophagy through ATM-AMPK-TSC2-mediated suppression of mTORC1. Proceedings of the National Academy of Sciences of the United States of America **110**, E2950 (1–8).
- **Tsukagoshi H, Busch W, Benfey PN**. 2010. Transcriptional regulation of ROS controls transition from proliferation to differentiation in the root. Cell **143**, 606–616.
- **Tun NN, Santa-Catarina C, Begum T, Silveira V, Handro W, Segal Floh EI, Scherer GFE**. 2006. Polyamines induce rapid biosynthesis of nitric oxide (NO) in Arabidopsis thaliana seedlings. Plant and Cell Physiology **47**, 346–354.
- **Urano K, Yoshiba Y, Nanjo T, Ito T, Yamaguchi-Shinozaki K, Shinozaki K**. 2004. Arabidopsis stress-inducible gene for arginine decarboxylase AtADC2 is required for accumulation of putrescine in salt tolerance. Biochemical and Biophysical Research Communications **313**, 369–375.

V

- **Valpuesta V, Botella MA**. 2004. Biosynthesis of L-ascorbic acid in plants: New pathways for an old anti-oxidative. Trends in Plant Science **9**, 573–577.
- **Velikova V, Yordanov I, Edreva a**. 2000. Oxidative stress and some anti-oxidative systems in acid rain-treated bean plants. Plant Science **151**, 59–66.
- **Vilches-Barro A, Maizel A**. 2015. Talking through walls: mechanisms of lateral root emergence in Arabidopsis thaliana. Current Opinion in Plant Biology **23**, 31–38.

- **Vincill ED, Clarin AE, Molenda JN, Spalding EP**. 2013. Interacting glutamate receptor-like proteins in Phloem regulate lateral root initiation in Arabidopsis. The Plant cell **25**, 1304–1313.
- **Vlot AC, Dempsey DA, Klessig DF**. 2009. Salicylic Acid, a multifaceted hormone to combat disease. Annual Review of Phytopathology **47**, 177–206.

W

- **Wagner C, Sefkow M, Kopka J**. 2003. Construction and application of a mass spectral and retention time index database generated from plant GC/EI-TOF-MS metabolite profiles. Phytochemistry **62**, 887–900.
- **Walch-Liu P, Liu LH, Remans T, Tester M, Forde BG**. 2006. Evidence that L-glutamate can act as an exogenous signal to modulate root growth and branching in Arabidopsis thaliana. Plant and Cell Physiology **47**, 1045–1057.
- **Wang Y, Loake GJ, Chu C**. 2013. Cross-talk of nitric oxide and reactive oxygen species in plant programed cell death. Frontiers in Plant Science **4**, 314 (1–7).
- **Wang W, Vignani R, Scali M, Cresti M**. 2006. A universal and rapid protocol for protein extraction from recalcitrant plant tissues for proteomic analysis. Electrophoresis **27**, 2782–2786.
- **Wang Z, Xiao Y, Chen W, Tang K, Zhang L**. 2010. Increased vitamin C content accompanied by an enhanced recycling pathway confers oxidative stress tolerance in Arabidopsis. Journal of Integrative Plant Biology **52**, 400–409.
- **Watanabe M, Balazadeh S, Tohge T, Erban A, Giavalisco P, Kopka J, Mueller-Roeber B, Fernie AR, Hoefgen R**. 2013. Comprehensive dissection of spatiotemporal metabolic shifts in primary, secondary, and lipid metabolism during developmental senescence in Arabidopsis. Plant Physiology **162**, 1290–1310.

- **Weckwerth W**. 2011. Green systems biology - From single genomes, proteomes and metabolomes to ecosystems research and biotechnology. Journal of Proteomics **75**, 284–305.
- **Wi SJ, Kim WT, Park KY**. 2006. Overexpression of carnation S-adenosylmethionine decarboxylase gene generates a broad-spectrum tolerance to abiotic stresses in transgenic tobacco plants. Plant Cell Reports **25**, 1111–1121.
- **Williams SP, Gillaspy GE, Perera IY**. 2015. Biosynthesis and possible functions of inositol pyrophosphates in plants. Frontiers in Plant Science **6**, 1–12.
- **Wimalasekera R, Tebartz F, Scherer GFE**. 2011*a*. Polyamines, polyamine oxidases and nitric oxide in development, abiotic and biotic stresses. Plant Science **181**, 593–603.
- **Wimalasekera R, Villar C, Begum T, Scherer GFE**. 2011*b*. COPPER AMINE OXIDASE1 (CuAO1) of arabidopsis thaliana contributes to abscisic acid-and polyamine-induced nitric oxide biosynthesis and abscisic acid signal transduction. Molecular Plant **4**, 663–678.

X

- **Xie Y, Mao Y, Zhang W, Lai D, Wang Q, Shen W**. 2014. Reactive Oxygen Species-Dependent Nitric Oxide Production Contributes to Hydrogen-Promoted Stomatal Closure in Arabidopsis. Plant Physiology **165**, 759–773.
- **Xie Y, Xu D, Cui W, Shen W**. 2012. Mutation of Arabidopsis HY1 causes UV-C hypersensitivity by impairing carotenoid and flavonoid biosynthesis and the down-regulation of anti-oxidative defence. Journal of Experimental Botany **63**, 3869–3884.

Y

- **Yamagami T, Tsuchisaka A, Yamada K, Haddon WF, Harden L a., Theologis A**. 2003. Biochemical diversity among the 1-amino-

cyclopropane-1-carboxylate synthase isozymes encoded by the Arabidopsis gene family. The Journal of Biological Chemistry **278**, 49102–49112.

- **Yamaguchi K, Takahashi Y, Berberich T, Imai A, Takahashi T, Michael AJ, Kusano T**. 2007. A protective role for the polyamine spermine against drought stress in Arabidopsis. Biochemical and Biophysical Research Communications **352**, 486–490.

Z

- **Zhang H, Han W, De Smet I, Talboys P, Loya R, Hassan A, Rong H, Jürgens G, Paul Knox J, Wang MH**. 2010. ABA promotes quiescence of the quiescent centre and suppresses stem cell differentiation in the Arabidopsis primary root meristem. Plant Journal **64**, 764–774.
- **Zhang H, Rong H, Pilbeam D**. 2007*a*. Signalling mechanisms underlying the morphological responses of the root system to nitrogen in Arabidopsis thaliana. Journal of Experimental Botany **58**, 2329–2338.
- **Zhang ZB, Yang G, Arana F, Chen Z, Li Y, Xia HJ**. 2007*b*. Arabidopsis inositol polyphosphate 6-/3-kinase (AtIpk2beta) is involved in axillary shoot branching via auxin signaling. Plant Physiology **144**, 942–951.
- **Zhao Y**. 2014. Auxin Biosynthesis. The Arabidopsis Book **12**, e0173 (1–14).
- **Zheng Z, Guo Y, Novák O, Dai X, Zhao Y, Ljung K, Noel JP, Chory J**. 2013. Coordination of auxin and ethylene biosynthesis by the aminotransferase VAS1. Nature Chemical Biology **9**, 244–248.
- **Zhou B, Guo Z, Xing J, Huang B**. 2005. Nitric oxide is involved in abscisic acid-induced anti-oxidative activities in Stylosanthes guianensis. Journal of Experimental Botany **56**, 3223–3228.

ANNEX

ANNEX 1

Detected metabolites after oxidative stress with no significant differences between WT and *pao4* mutants. Compounds abundance is presented as log_2-transformed fold-changes realtive to the mean of WT-Col 0 samples (P<0.05).

	DETECTED METABOLITES AFTER OXIDATIVE STRESS DEMONSTRATING NO SIGNIFICANT DIFFERENCE RESPECT TO WT					
	WT		***pao4-1***		***pao4-2***	
	Mean	**Std. Deviation**	**Mean**	**Std. Deviation**	**Mean**	**Std. Deviation**
Arabinonic acid	-.068	.494	-.161	.637	-.576	.183
Benzoic acid	-.582	2.191	-1.233	1.132	-1.011	.995
Butanoic acid, 2,4-dihydroxy-	-.039	.375	-.343	.347	-.329	.177
Citric acid	-.115	.635	.067	.555	-.239	.590
Glutaric acid, 2-hydroxy-	-.051	.417	-.723	.478	-.410	.358
Glutaric acid, 3-hydroxy-3-methyl-	-.053	.442	-.292	.759	-.622	.270
Glycolic acid	-.030	.328	-.492	.385	-.375	.461
Malic acid	-.064	.466	-.515	.576	-.759	.463
Malonic acid	-.093	.608	-.030	.333	-.537	.481
Aspartic acid	-.066	.496	-.305	.860	-.909	.492
Glycine	-.102	.587	-.379	.676	-.148	.456
Histidine	-.754	1.713	.814	1.098	-.985	.565
Ornithine-1,5-lactam	-.025	.327	-.647	.070	-.131	.393
Serine	-.647	1.811	-.305	.835	-.551	.778
Threonine	-.532	1.610	.322	.560	-.371	.501
Tryptophan	-.049	.536	.465	.714		
Agmatine	-.022	.278	-.044	.360	-.322	.499
Ethanolamine	-.547	1.657	-.586	.523	-.763	.380
Glucose-6-phosphate	-.083	.518	-.029	.352	-.470	.433
Glyceric acid-3-phosphate	-.007	.153	-.041	.316	-.446	.676
Glycerol-3-phosphate	-.049	.434	-.380	.558	-.799	.507
Phosphoric acid	-.247	.815	-.396	.115	-.439	.090
Ascorbic acid	.000		-1.562	.196	-1.410	
Erythronic acid	-.082	.534	-.115	.516	-.461	.385
Erythronic acid-1,4-lactone	-.557	2.420	-.623	1.412	-1.101	.638
Galactaric acid	-.043	.409	-.111	.409	-.482	.242
Galactonic acid	-.026	.304	-.152	.332	-.229	.340
Ribonic acid	-.161	.787	-.419	.544	-.894	.510
Erythritol	-.038	.373	-.136	.337	-.373	.464
Ribitol	-.025	.299	-.414	.363	-.547	.596
Threitol	-.105	.636	.150	.383	-.309	.688
Salicylic acid-glucopyranoside	-.086	.561	.141	.400	-.119	.309
Fructose	-.022	.284	-.070	.436	-.431	.250
Glucose	-.060	.464	-.353	.594	-.671	.347

	DETECTED METABOLITES AFTER OXIDATIVE STRESS DEMONSTRATING NO SIGNIFICANT DIFFERENCE RESPECT TO WT					
	WT		***pao4-1***		***pao4-2***	
	Mean	**Std. Deviation**	**Mean**	**Std. Deviation**	**Mean**	**Std. Deviation**
Lyxose	-.088	.609	.076	.548	-.074	
Maltose	-.115	.650	-.840	1.147	-.267	.869
Psicose	-.036	.364	-.187	.395	-.612	.457
Sucrose	-.180	.707	-.340	.117	-.344	.061
Xylose	-.088	.580	.023	.385	-.631	.247
A112003	1.545	1.363	.784		-.894	2.458
A116201	-.167	.728	-.110	.432	-.007	.321
A147011	-.149	.785	-.301	.618	-.703	.901
A148006	-.028	.321	-.252	.401	-.085	.400
A159003	-.029	.318	-.537	.367	-.342	.361
A167004	-.169	.791	-.029	1.093	-.492	.592
A172005	-.095	.589	.010	1.220	-.836	.846
A174001	-.048	.403	-.806	.519	-.710	.313
A176001	-.046	.396	.041	.400	-.441	.330
A180012	-.007	.173	-.029	.243	-.092	.008
A196004	-.067	.460	.198	.377	-.240	.367
A211001	-.029	.321	-.608	.418	-.362	.328
A213001	-.077	.517	-.500	.565	-.830	.307
A228001	-.083	.530	-.074	.595	-.383	.310
A250001	-.027	.307	-.230	.293	-.282	.391
A254002	-.014	.220	-.264	.224	-.203	.227
A274014	-.258	.910	-.198	.782	-.211	.788
A278005	-.005	.155	-.241	.213	-.448	.458
A313001	-.284	1.056	-.819	1.559	-.768	.989
A324001	-.096	.615	-.057	1.050	-.497	.660

ANNEX 2

Detected metabolites after nitrosative stress with no significant differences between WT and pao4 mutants. Compounds abundance is presented as log_2-transformed fold-changes realtive to the mean of WT-Col 0 samples (P<0.05).

	DETECTED METABOLITES AFTER NITROSATIVE STRESS DEMONSTRATING NO SIGNIFICANT DIFFERENCES RESPECT TO WT					
	WT		***pao4-1***		***pao4-2***	
	Mean	**Std. Deviation**	**Mean**	**Std. Deviation**	**Mean**	**Std. Deviation**
Arabinonic acid	-.094	.579	-.403	.633	.030	
Benzoic acid	-.200	.819	.854	1.130	-1.265	1.203
Butanoic acid, 2,4-dihydroxy-	-.050	.413	-.288	.355	-.323	.579
Fumaric acid	-.114	.675	-.443	.675	-.387	.737
Glutaric acid, 2-hydroxy-	-.054	.451	-.176	.310	-.509	.557
Glutaric acid, 3-hydroxy-3-methyl-	-.094	.571	-.468	.686	-.228	.689
Lactic acid	-.212	1.030	-.134	.994	-.527	1.015
Malic acid	-.017	.241	-.339	.470	.291	.466
Oxalic acid	-.124	.669	-.590	.725	-.009	.534
Succinic acid	-.008	.164	-.235	.263	.089	.580
Alanine	.219	.438	-.001	.722	-.276	1.064
Alanine, 3-cyano-	-.049	.456	-.460	.597	-.437	.613
Glycine	-.156	.720	-.404	.517	-1.018	.685
Histidine	-.551	1.303	-.817	1.790	-.481	
Isoleucine	-.077	.530	-.516	.647	-.503	.424
Leucine	-.020	.265	-.489	.488	-.333	.392
Phenylalanine	.479	.921	-.474	1.581	-.839	2.194
Threonine	-.173	.827	-.975	2.888	-.001	.829
Tyrosine	-.477	1.473	-1.285	2.678	-.864	3.115
Valine	-.145	.770	-.735	.992	-.466	.806
Agmatine	-.194	.889	-.372	1.292	-.357	1.076
Carbodiimide	.086	.873	.400	1.909	-2.108	1.085
Pyridine, 2-hydroxy-	-.218	.848	-.187	1.452	-.851	.820
Uridine	-.360	1.025	-.701	.639	-.095	1.702
Sinapic acid, cis-	-.039	.356	-.415	.924	-.189	.702
Glucose-6-phosphate	-.009	.174	-.287	.457	.152	.765
Glycerol-3-phosphate	-.044	.391	.054	.448	.054	.714
Erythronic acid	-.017	.242	-.422	.238	-.270	.500
Galactaric acid	-.038	.362	-.101	.681	.000	.517
Galactonic acid	-.005	.137	-.215	.315	.062	.292
Glyceric acid	-.092	.564	-.388	.361	-.417	.757
Ribonic acid	-.052	.444	-.467	.436	-.233	1.486
Threonic acid	-.036	.358	-.357	.342	.136	.663
Threonic acid-1,4-lactone	-.071	.508	-.308	.248	-.094	.624
Erythritol	-.064	.488	-.316	.424	-.492	.749

	DETECTED METABOLITES AFTER NITROSATIVE STRESS DEMONSTRATING NO SIGNIFICANT DIFFERENCES RESPECT TO WT					
	WT		***pao4-1***		***pao4-2***	
	Mean	**Std. Deviation**	**Mean**	**Std. Deviation**	**Mean**	**Std. Deviation**
Glycerol	-.122	.658	-.212	.683	-1.188	.422
Ribitol	-.061	.472	-.470	.586	-.842	.848
Threitol	-.098	.601	-.396	.450	-.826	.820
Salicylic acid-glucopyranoside	-.319	1.160	-.050	1.028	-.128	.193
Fructose	-.019	.248	-.104	.265	-.400	.332
Glucose	-.011	.196	-.176	.367	.108	.450
Lyxose	-.069	.507	-.209	.451	-.405	.553
Maltose	-.542	1.292	-.799	1.003	.137	.845
Psicose	-.012	.266	.081	.123	.222	.444
Raffinose	-.193	.800	-.408	.571	.125	.638
Xylose	-.042	.401	-.181	.463	-.391	.601
A147011	-.070	.513	-.448	.214	-.429	.671
A148006	-.169	.801	-.392	.563	-.015	.527
A155004	-.005	.148	-.092	.505	.214	
A159003	-.039	.371	-.296	.499	-.799	.640
A167004	-.041	.378	-.348	1.366	-.328	.877
A172005	-.379	1.137	-.612	1.901	-.826	1.085
A174001	-.026	.303	-.362	.398	-.595	.458
A176001	-.020	.269	-.120	.350	-.162	.434
A194007	-.131	.692	-.347	.505	.043	.762
A203003	-.181	.823	-.790	1.186	.096	1.361
A211001	-.027	.313	-.267	.471	-.382	.664
A214003	-.062	.463	-.632	.551	-.435	.448
A217004	-.042	.391	-.239	.437	.014	.923
A228001	-.008	.166	-.173	.382	.173	.520
A250001	-.059	.454	-.434	.484	-.568	.808
A254002	-.014	.222	-.258	.407	-.406	.482
A274014	-.205	.912	-.747	.675	.030	.569
A278005	-.138	.775	.426	.793	-.266	1.200
A289002	-.107	.592	-.129	.512	.225	.284
A308003	-.032	.343	-.474	.037	.701	.628
A311002	-.086	.533	-.345	.265	-.202	.463
A324001	-.071	.499	-.749	.472	.455	.860

ANNEX 3

Pearson correlation of altered metabolites after oxidative stress imposition (*P≤0.05)*

	Oxalic acid	Arginine	Glutamate	Isoleucine
Oxalate	**1.000**	.623*	.525*	0.513
Arginine	.623*	**1.000**	0.454	0.531
Glutamate	.525*	0.454	**1.000**	0.450
Isoleucine	0.513	0.531	0.450	**1.000**
Leucine	.527*	0.352	.677**	.696**
Ornithine	0.413	.649*	.540*	0.341
Pyroglutamate	0.374	0.224	.588*	0.462
Tyrosine	0.475	.752*	.767**	.676*
Valine	.569*	0.444	.592*	.572*
Carbodiimide	0.463	0.561	0.286	0.362
Putrescine	0.151	0.081	.591*	0.296
Uridine	.664**	.635*	0.531	0.531
Glycerophosphoglycerol	.692**	0.478	.645**	0.502
Threonate	0.152	0.066	.563*	0.347
Threonate-1,4-Lactone	0.260	0.078	0.364	.592*
Galactose	0.044	0.261	0.406	-0.081
Raffinose	0.195	-0.156	0.597	0.432
A194007	.611*	0.437	.632**	.622*
A217004	.725**	0.506	.622*	.606*
A290003	0.469	0.240	0.250	.764**
A296004	.697**	0.275	0.195	0.069
A302001	.594*	0.549	0.304	.662**
A308003	.775*	.866*	-0.074	0.385
Fumarate	-0.069	-0.129	-0.238	-0.083
Proline	0.058	-0.092	-0.208	0.186
Succinate	-0.073	-0.163	-0.268	-0.136
Asparagine	-0.293	-0.256	-0.077	0.138
Glutamine	-0.306	-0.391	0.184	0.109
Phenylalanine	-0.209	-0.099	0.107	0.218
Palmitate	-0.350	-0.146	0.311	-0.221
Estearate	-0.101	0.167	-0.443	-0.370
Sinapate	-0.209	-0.268	0.008	-0.036
Glycerate	-0.089	-0.257	-0.091	0.132
Glycerol	-0.116	0.318	-0.415	-0.027
Myo-Inositol	0.051	-0.436	0.476	-0.297
Galactinol	0.105	-0.248	-0.040	-0.031
Ribose	-0.332	-0.650	0.065	0.070
A143002	-0.164	0.425	-0.018	0.260
A145016	-0.376	-0.067	0.282	0.083
A203003	-0.042	0.009	0.190	-0.137
A207003	-0.226	-0.380	-0.324	-0.055
A214003	-0.070	-0.172	-0.222	0.021
A260482	-0.141	0.476	0.194	0.134
A278931	-0.350	0.409	-0.404	0.220
A311002	-0.016	-0.350	0.025	-0.115

ANNEX 3 (Cont.)

	Leucine	Ornithine	Pyroglutamate	Tyrosine	Valine
Oxalate	.527*	0.413	0.374	0.475	.569*
Arginine	0.352	.649*	0.224	.752*	0.444
Glutamate	.677**	.540*	.588*	.767**	.592*
Isoleucine	.696**	0.341	0.462	.676*	.572*
Leucine	**1.000**	0.266	.713**	.769**	.884**
Ornithine	0.266	**1.000**	0.252	.742**	0.382
Pyroglutamate	.713**	0.252	**1.000**	.858**	.744**
Tyrosine	.769**	.742**	.858**	**1.000**	.841**
Valine	.884**	0.382	.744**	.841**	**1.000**
Carbodiimide	-0.182	0.437	-0.224	0.209	-0.301
Putrescine	0.472	0.392	.904**	.859**	.534*
Uridine	.577*	.694**	.566*	.868**	.646*
Glycerophosphoglycerol	.769**	.584*	.761**	.850**	.796**
Threonate	.664**	0.370	.640**	.844**	.634**
Threonate-1,4-Lactone	.572*	0.033	0.208	0.399	0.243
Galactose	-0.116	0.417	0.267	0.083	-0.024
Raffinose	.635*	-0.178	0.550	0.159	0.379
A194007	.829**	0.368	.812**	.874**	.785**
A217004	.712**	0.486	.624**	.839**	.622**
A290003	.720**	0.001	.571*	0.580	.617*
A296004	0.122	0.325	0.162	-0.048	0.349
A302001	.785**	0.284	.548*	.736*	.725**
A308003	0.316	0.612	0.157	0.525	0.295
Fumarate	-0.009	-0.341	-0.034	-0.091	0.162
Proline	.579*	-0.464	0.324	0.169	0.445
Succinate	0.036	-0.377	-0.055	-0.133	0.180
Asparagine	0.166	-.623*	0.138	-0.104	-0.019
Glutamine	0.310	-0.537	0.336	0.715	0.106
Phenylalanine	0.364	-0.393	0.243	0.211	0.000
Palmitate	-0.303	0.190	-.676*	0.450	-0.349
Estearate	-.687*	0.167	-.895**	-0.149	-.750**
Sinapate	0.069	-0.281	-0.144	0.284	-0.064
Glycerate	0.233	-0.303	0.067	0.070	0.292
Glycerol	-0.443	0.258	-.870**	-0.186	-0.411
Myo-Inositol	0.501	-0.556	0.307	0.102	0.332
Galactinol	0.311	-0.472	0.149	0.016	0.308
Ribose	0.469	-.748*	0.459	-0.141	-0.128
A143002	-0.491	0.260	-.802**	0.222	-.589*
A145016	-0.464	-0.314	-.690*	0.454	-.650*
A203003	0.284	-0.309	0.119	0.300	0.156
A207003	0.010	-.727**	0.125	-0.335	-0.061
A214003	0.141	-0.437	0.354	0.024	0.245
A260482	-.637*	.668*	-.887**	0.455	-.637*
A278931	-.647*	-0.089	-.880**	-0.165	-.695*
A311002	0.317	-0.498	0.397	-0.085	0.324

ANNEX 3 (Cont.)

	Carbodiimide	Putrescine	Uridine	Glycero phosphoglycerol	Threonic acid
Oxalate	0.463	0.151	.664**	.692**	0.152
Arginine	0.561	0.081	.635*	0.478	0.066
Glutamate	0.286	.591*	0.531	.645**	.563*
Isoleucine	0.362	0.296	0.531	0.502	0.347
Leucine	-0.182	0.472	.577*	.769**	.664**
Ornithine	0.437	0.392	.694**	.584*	0.370
Pyroglutamate	-0.224	.904**	.566*	.761**	.640**
Tyrosine	0.209	.859**	.868**	.850**	.844**
Valine	-0.301	.534*	.646*	.796**	.634**
Carbodiimide	**1.000**	0.050	0.521	0.172	-0.175
Putrescine	0.050	**1.000**	0.375	.552*	.567*
Uridine	0.521	0.375	**1.000**	.867**	.578*
Glycerophosphoglycerol	0.172	.552*	.867**	**1.000**	.635**
Threonate	-0.175	.567*	.578*	.635**	**1.000**
Threonate-1,4-Lactone	0.320	0.233	0.377	0.204	0.428
Galactose	0.492	0.403	0.140	0.281	0.077
Raffinose	0.187	.632*	0.216	0.419	0.573
A194007	0.234	.586*	.823**	.928**	.665**
A217004	0.526	0.449	.931**	.913**	.585*
A290003	-0.096	0.141	.550*	.581*	0.404
A296004	0.314	-0.066	0.372	.532*	-0.270
A302001	0.044	0.198	.679*	.764**	0.360
A308003	0.583	-0.242	.755*	0.671	0.157
Fumarate	-0.435	-0.217	-0.025	-0.039	0.007
Proline	-0.453	-0.191	0.166	0.222	0.199
Succinate	-.593*	-0.226	-0.111	-0.097	0.040
Asparagine	-0.164	0.048	-0.374	-0.280	-0.076
Glutamine	-0.225	0.274	-0.173	0.037	0.349
Phenylalanine	-0.005	0.021	-0.214	-0.094	0.123
Palmitate	0.072	-.688*	0.201	-0.405	0.066
Estearate	0.485	-.889**	0.483	-.643*	-0.460
Sinapate	-0.393	-0.245	0.115	-0.118	0.307
Glycerate	-.733**	-0.165	-0.091	0.044	0.178
Glycerol	0.032	-.926**	0.119	-0.504	-0.464
Myo-Inositol	-0.435	0.128	0.138	0.375	0.491
Galactinol	-0.405	-0.116	-0.101	0.121	0.007
Ribose	-0.186	0.094	-0.443	0.046	0.266
A143002	0.556	-.711**	0.227	-.688*	-0.233
A145016	0.515	-.612*	-0.152	-.761**	-0.161
A203003	-0.321	-0.107	0.219	0.141	0.287
A207003	-0.204	-0.011	-0.343	-0.239	-0.172
A214003	-0.347	-0.032	0.022	0.124	0.122
A260482	0.615	-.827**	0.298	-.724*	-0.283
A278931	0.641	-.831**	-0.078	-.800**	-0.492
A311002	-.699*	-0.167	-0.255	0.162	-0.012

ANNEX 3 (Cont.)

	Threonic acid-1,4-lactone	Galactose	Raffinose	A194007	A217004
Oxalate	0.260	0.044	0.195	.611*	.725**
Arginine	0.078	0.261	-0.156	0.437	0.506
Glutamate	0.364	0.406	0.597	.632**	.622*
Isoleucine	.592*	-0.081	0.432	.622*	.606*
Leucine	.572*	-0.116	.635*	.829**	.712**
Ornithine	0.033	0.417	-0.178	0.368	0.486
Pyroglutamate	0.208	0.267	0.550	.812**	.624**
Tyrosine	0.399	0.083	0.159	.874**	.839**
Valine	0.243	-0.024	0.379	.785**	.622**
Carbodiimide	0.320	0.492	0.187	0.234	0.526
Putrescine	0.233	0.403	.632*	.586*	0.449
Uridine	0.377	0.140	0.216	.823**	.931**
Glycerophosphoglycerol	0.204	0.281	0.419	.928**	.913**
Threonate	0.428	0.077	0.573	.665**	.585*
Threonate-1,4-Lactone	**1.000**	-0.337	.851**	0.355	0.422
Galactose	-0.337	**1.000**	0.050	0.175	0.227
Raffinose	.851**	0.050	**1.000**	0.542	0.570
A194007	0.355	0.175	0.542	**1.000**	.918**
A217004	0.422	0.227	0.570	.918**	**1.000**
A290003	0.489	-0.229	0.332	.684**	.608*
A296004	-0.247	0.370	-0.546	0.320	0.378
A302001	0.202	-0.149	-0.077	.831**	.733**
A308003	0.145	-0.599	-0.328	0.581	.716*
Fumarate	-0.105	-0.520	-0.083	0.012	-0.154
Proline	0.193	-.850**	0.275	0.466	0.257
Succinate	-0.041	-.625*	-0.024	-0.087	-0.227
Asparagine	0.350	-0.142	0.361	-0.009	-0.179
Glutamine	0.304	-0.049	0.602	0.223	0.107
Phenylalanine	.608*	-0.551	0.541	0.279	0.127
Palmitate	0.454	-.724*	-0.485	-0.378	-0.310
Estearate	0.166	-0.276	-0.577	-.667*	-0.372
Sinapate	0.253	-.609*	0.265	-0.049	0.010
Glycerate	0.006	-0.526	0.005	0.039	-0.125
Glycerol	-0.266	-0.372	-.650*	-.584*	-0.465
Myo-Inositol	0.272	-0.434	0.532	0.419	0.309
Galactinol	0.121	-.733**	0.117	0.250	0.063
Ribose	0.547	-0.536	0.347	0.472	0.277
A143002	.650*	-.719*	-0.313	-.672*	-0.495
A145016	0.541	-0.540	-0.171	-0.527	-0.352
A203003	0.122	-0.439	0.358	0.230	0.187
A207003	0.139	-0.294	0.238	-0.013	-0.157
A214003	-0.050	-.577*	-0.003	0.323	0.059
A260482	0.364	-.838**	-0.486	-.715*	-0.591
A278931	0.372	-0.230	-0.505	-.763**	-.700*
A311002	-0.012	-.583*	0.058	0.262	-0.005

ANNEX 3 (Cont.)

	A290003	A296004	A302001	A308003	Fumarate
Oxalate	0.469	.697**	.594*	.775*	-0.069
Arginine	0.240	0.275	0.549	.866*	-0.129
Glutamate	0.250	0.195	0.304	-0.074	-0.238
Isoleucine	.764**	0.069	.662**	0.385	-0.083
Leucine	.720**	0.122	.785**	0.316	-0.009
Ornithine	0.001	0.325	0.284	0.612	-0.341
Pyroglutamate	.571*	0.162	.548*	0.157	-0.034
Tyrosine	0.580	-0.048	.736*	0.525	-0.091
Valine	.617*	0.349	.725**	0.295	0.162
Carbodiimide	-0.096	0.314	0.044	0.583	-0.435
Putrescine	0.141	-0.066	0.198	-0.242	-0.217
Uridine	.550*	0.372	.679*	.755*	-0.025
Glycerophosphoglycerol	.581*	.532*	.764**	0.671	-0.039
Threonate	0.404	-0.270	0.360	0.157	0.007
Threonate-1,4-Lactone	0.489	-0.247	0.202	0.145	-0.105
Galactose	-0.229	0.370	-0.149	-0.599	-0.520
Raffinose	0.332	-0.546	-0.077	-0.328	-0.083
A194007	.684**	0.320	.831**	0.581	0.012
A217004	.608*	0.378	.733**	.716*	-0.154
A290003	**1.000**	0.134	.846**	0.504	0.181
A296004	0.134	**1.000**	0.339	0.459	0.068
A302001	.846**	0.339	**1.000**	.897**	0.068
A308003	0.504	0.459	.897**	**1.000**	0.076
Fumarate	0.181	0.068	0.068	0.076	**1.000**
Proline	0.491	-0.261	0.492	0.558	.601*
Succinate	0.176	-0.072	0.044	-0.015	.937**
Asparagine	0.230	-0.330	-0.129	-.857**	0.141
Glutamine	0.230	-0.374	-0.228	-.768*	0.152
Phenylalanine	0.182	-.569*	0.126	-0.594	0.160
Palmitate	-0.204	-0.268	-0.356	-0.342	0.242
Estearate	-0.149	-0.131	-0.364	0.547	-0.146
Sinapate	0.206	-.700**	-0.092	-0.278	0.271
Glycerate	0.389	-0.163	0.180	-0.149	.777**
Glycerol	-0.108	0.262	-0.209	0.368	0.318
Myo-Inositol	0.279	-0.029	0.193	-0.023	0.596
Galactinol	0.338	0.152	0.276	-0.152	.712**
Ribose	0.248	-0.499	0.232	-0.372	0.085
A143002	-0.037	-0.561	-0.443	0.007	-0.055
A145016	-0.211	-0.591	-0.573	-0.634	-0.184
A203003	0.231	-0.370	0.175	0.161	0.390
A207003	0.202	-0.094	-0.095	-.785*	0.269
A214003	0.367	0.042	0.278	0.250	.789**
A260482	-0.341	-0.432	-0.483	-0.157	0.033
A278931	0.020	-0.300	-0.438	-0.219	0.148
A311002	0.201	0.098	0.208	-0.510	.748**

ANNEX 3 (Cont.)

	Proline	Succinate	Asparagine	Glutamine	Phenylalanine
Oxalate	0.058	-0.073	-0.293	-0.306	-0.209
Arginine	-0.092	-0.163	-0.256	-0.391	-0.099
Glutamate	-0.208	-0.268	-0.077	0.184	0.107
Isoleucine	0.186	-0.136	0.138	0.109	0.218
Leucine	.579*	0.036	0.166	0.310	0.364
Ornithine	-0.464	-0.377	-.623*	-0.537	-0.393
Pyroglutamate	0.324	-0.055	0.138	0.336	0.243
Tyrosine	0.169	-0.133	-0.104	0.715	0.211
Valine	0.445	0.180	-0.019	0.106	0.000
Carbodiimide	-0.453	-.593*	-0.164	-0.225	-0.005
Putrescine	-0.191	-0.226	0.048	0.274	0.021
Uridine	0.166	-0.111	-0.374	-0.173	-0.214
Glycerophosphoglycerol	0.222	-0.097	-0.280	0.037	-0.094
Threonate	0.199	0.040	-0.076	0.349	0.123
Threonate-1,4-Lactone	0.193	-0.041	0.350	0.304	.608*
Galactose	-.850**	-.625*	-0.142	-0.049	-0.551
Raffinose	0.275	-0.024	0.361	0.602	0.541
A194007	0.466	-0.087	-0.009	0.223	0.279
A217004	0.257	-0.227	-0.179	0.107	0.127
A290003	0.491	0.176	0.230	0.230	0.182
A296004	-0.261	-0.072	-0.330	-0.374	-.569*
A302001	0.492	0.044	-0.129	-0.228	0.126
A308003	0.558	-0.015	-.857**	-.768*	-0.594
Fumarate	.601*	.937**	0.141	0.152	0.160
Proline	**1.000**	.635*	0.178	0.253	0.489
Succinate	.635*	**1.000**	0.109	0.146	0.146
Asparagine	0.178	0.109	**1.000**	.967**	.715**
Glutamine	0.253	0.146	.967**	**1.000**	.753*
Phenylalanine	0.489	0.146	.715**	.753*	**1.000**
Palmitate	-0.175	0.223	0.508	0.086	.742*
Estearate	-0.021	-0.119	-0.241	-0.308	-0.028
Sinapate	.732**	0.433	0.134	0.482	0.477
Glycerate	.580*	.867**	0.127	0.219	0.148
Glycerol	0.077	0.248	-0.169	-0.208	-0.142
Myo-Inositol	.707*	0.559	0.197	0.384	0.403
Galactinol	.739**	.671**	0.408	0.452	.561*
Ribose	0.552	0.072	0.457	0.503	.753*
A143002	0.001	-0.019	0.318	-0.121	0.574
A145016	-0.069	-0.185	.754**	0.344	.749*
A203003	.726**	0.471	0.264	.624*	0.520
A207003	0.344	0.221	.881**	.904**	.550*
A214003	.745**	.641**	0.333	0.375	0.379
A260482	-0.143	0.051	0.227	-0.094	.807*
A278931	-0.184	0.070	.627*	-0.111	0.455
A311002	.623*	.732**	0.405	0.478	0.480

ANNEX 3 (Cont.)

	Palmitate	Estearate	Sinapate	Glycerate	Glycerol
Oxalate	-0.350	-0.101	-0.209	-0.089	-0.116
Arginine	-0.146	0.167	-0.268	-0.257	0.318
Glutamate	0.311	-0.443	0.008	-0.091	-0.415
Isoleucine	-0.221	-0.370	-0.036	0.132	-0.027
Leucine	-0.303	-.687*	0.069	0.233	-0.443
Ornithine	0.190	0.167	-0.281	-0.303	0.258
Pyroglutamate	-.676*	-.895**	-0.144	0.067	-.870**
Tyrosine	0.450	-0.149	0.284	0.070	-0.186
Valine	-0.349	-.750**	-0.064	0.292	-0.411
Carbodiimide	0.072	0.485	-0.393	-.733**	0.032
Putrescine	-.688*	-.889**	-0.245	-0.165	-.926**
Uridine	0.201	0.483	0.115	-0.091	0.119
Glycerophosphoglycerol	-0.405	-.643*	-0.118	0.044	-0.504
Threonate	0.066	-0.460	0.307	0.178	-0.464
Threonate-1,4-Lactone	0.454	0.166	0.253	0.006	-0.266
Galactose	-.724*	-0.276	-.609*	-0.526	-0.372
Raffinose	-0.485	-0.577	0.265	0.005	-.650*
A194007	-0.378	-.667*	-0.049	0.039	-.584*
A217004	-0.310	-0.372	0.010	-0.125	-0.465
A290003	-0.204	-0.149	0.206	0.389	-0.108
A296004	-0.268	-0.131	-.700**	-0.163	0.262
A302001	-0.356	-0.364	-0.092	0.180	-0.209
A308003	-0.342	0.547	-0.278	-0.149	0.368
Fumarate	0.242	-0.146	0.271	.777**	0.318
Proline	-0.175	-0.021	.732**	.580*	0.077
Succinate	0.223	-0.119	0.433	.867**	0.248
Asparagine	0.508	-0.241	0.134	0.127	-0.169
Glutamine	0.086	-0.308	0.482	0.219	-0.208
Phenylalanine	.742*	-0.028	0.477	0.148	-0.142
Palmitate	**1.000**	.876**	0.498	0.056	.813**
Estearate	.876**	**1.000**	0.321	-0.200	.863**
Sinapate	0.498	0.321	**1.000**	0.495	0.210
Glycerate	0.056	-0.200	0.495	**1.000**	0.174
Glycerol	.813**	.863**	0.210	0.174	**1.000**
Myo-Inositol	0.107	-0.151	0.424	0.546	-0.268
Galactinol	0.188	-0.254	0.289	.642**	0.118
Ribose	0.087	-0.058	0.521	0.281	-.672*
A143002	.842**	.870**	0.468	-0.151	.822**
A145016	.894**	.727*	.732*	-0.214	.616*
A203003	0.279	0.136	.814**	0.457	0.036
A207003	0.376	-0.232	0.147	0.142	-0.071
A214003	-0.064	-0.485	0.218	.547*	0.224
A260482	.876**	.905**	0.592	-0.033	.926**
A278931	.906**	.909**	0.085	-0.059	.895**
A311002	-0.053	-0.545	0.345	.730**	-0.015

ANNEX 3 (Cont.)

	myo-Inositol	Galactinol	Ribose	A143002	A145016
Oxalate	0.051	0.105	-0.332	-0.164	-0.376
Arginine	-0.436	-0.248	-0.650	0.425	-0.067
Glutamate	0.476	-0.040	0.065	-0.018	0.282
Isoleucine	-0.297	-0.031	0.070	0.260	0.083
Leucine	0.501	0.311	0.469	-0.491	-0.464
Ornithine	-0.556	-0.472	-.748*	0.260	-0.314
Pyroglutamate	0.307	0.149	0.459	-.802**	-.690*
Tyrosine	0.102	0.016	-0.141	0.222	0.454
Valine	0.332	0.308	-0.128	-.589*	-.650*
Carbodiimide	-0.435	-0.405	-0.186	0.556	0.515
Putrescine	0.128	-0.116	0.094	-.711**	-.612*
Uridine	0.138	-0.101	-0.443	0.227	-0.152
Glycerophosphoglycerol	0.375	0.121	0.046	-.688*	-.761**
Threonate	0.491	0.007	0.266	-0.233	-0.161
Threonate-1,4-Lactone	0.272	0.121	0.547	.650*	0.541
Galactose	-0.434	-.733**	-0.536	-.719*	-0.540
Raffinose	0.532	0.117	0.347	-0.313	-0.171
A194007	0.419	0.250	0.472	-.672*	-0.527
A217004	0.309	0.063	0.277	-0.495	-0.352
A290003	0.279	0.338	0.248	-0.037	-0.211
A296004	-0.029	0.152	-0.499	-0.561	-0.591
A302001	0.193	0.276	0.232	-0.443	-0.573
A308003	-0.023	-0.152	-0.372	0.007	-0.634
Fumarate	0.596	.712**	0.085	-0.055	-0.184
Proline	.707*	.739**	0.552	0.001	-0.069
Succinate	0.559	.671**	0.072	-0.019	-0.185
Asparagine	0.197	0.408	0.457	0.318	.754**
Glutamine	0.384	0.452	0.503	-0.121	0.344
Phenylalanine	0.403	.561*	.753*	0.574	.749*
Palmitate	0.107	0.188	0.087	.842**	.894**
Estearate	-0.151	-0.254	-0.058	.870**	.727*
Sinapate	0.424	0.289	0.521	0.468	.732*
Glycerate	0.546	.642**	0.281	-0.151	-0.214
Glycerol	-0.268	0.118	-.672*	.822**	.616*
Myo-Inositol	**1.000**	.848**	.676*	-0.424	-0.053
Galactinol	.848**	**1.000**	.746*	-0.143	-0.038
Ribose	.676*	.746*	**1.000**	-0.536	0.345
A143002	-0.424	-0.143	-0.536	**1.000**	.897**
A145016	-0.053	-0.038	0.345	.897**	**1.000**
A203003	.687*	0.461	0.429	0.031	0.338
A207003	0.385	.550*	0.589	0.165	.650*
A214003	.742*	.789**	0.583	-0.255	-0.299
A260482	-0.304	-0.035	-0.581	.960**	.908**
A278931	-0.478	-0.058	-0.693	.927**	.912**
A311002	.836**	.957**	.693*	-0.534	-0.191

ANNEX 3 (Cont.)

	A203003	A207003	A214003	A260482	A278931
Oxalate	-0.042	-0.226	-0.070	-0.141	-0.350
Arginine	0.009	-0.380	-0.172	0.476	0.409
Glutamate	0.190	-0.324	-0.222	0.194	-0.404
Isoleucine	-0.137	-0.055	0.021	0.134	0.220
Leucine	0.284	0.010	0.141	-.637*	-.647*
Ornithine	-0.309	-.727**	-0.437	.668*	-0.089
Pyroglutamate	0.119	0.125	0.354	-.887**	-.880**
Tyrosine	0.300	-0.335	0.024	0.455	-0.165
Valine	0.156	-0.061	0.245	-.637*	-.695*
Carbodiimide	-0.321	-0.204	-0.347	0.615	0.641
Putrescine	-0.107	-0.011	-0.032	-.827**	-.831**
Uridine	0.219	-0.343	0.022	0.298	-0.078
Glycerophosphoglycerol	0.141	-0.239	0.124	-.724*	-.800**
Threonate	0.287	-0.172	0.122	-0.283	-0.492
Threonate-1,4-Lactone	0.122	0.139	-0.050	0.364	0.372
Galactose	-0.439	-0.294	-.577*	-.838**	-0.230
Raffinose	0.358	0.238	-0.003	-0.486	-0.505
A194007	0.230	-0.013	0.323	-.715*	-.763**
A217004	0.187	-0.157	0.059	-0.591	-.700*
A290003	0.231	0.202	0.367	-0.341	0.020
A296004	-0.370	-0.094	0.042	-0.432	-0.300
A302001	0.175	-0.095	0.278	-0.483	-0.438
A308003	0.161	-.785*	0.250	-0.157	-0.219
Fumarate	0.390	0.269	.789**	0.033	0.148
Proline	.726**	0.344	.745**	-0.143	-0.184
Succinate	0.471	0.221	.641**	0.051	0.070
Asparagine	0.264	.881**	0.333	0.227	.627*
Glutamine	.624*	.904**	0.375	-0.094	-0.111
Phenylalanine	0.520	.550*	0.379	.807*	0.455
Palmitate	0.279	0.376	-0.064	.876**	.906**
Estearate	0.136	-0.232	-0.485	.905**	.909**
Sinapate	.814**	0.147	0.218	0.592	0.085
Glycerate	0.457	0.142	.547*	-0.033	-0.059
Glycerol	0.036	-0.071	0.224	.926**	.895**
Myo-Inositol	.687*	0.385	.742*	-0.304	-0.478
Galactinol	0.461	.550*	.789**	-0.035	-0.058
Ribose	0.429	0.589	0.583	-0.581	-0.693
A143002	0.031	0.165	-0.255	.960**	.927**
A145016	0.338	.650*	-0.299	.908**	.912**
A203003	**1.000**	0.254	0.375	0.133	-0.087
A207003	0.254	**1.000**	.523*	0.102	0.495
A214003	0.375	.523*	**1.000**	-0.468	-0.037
A260482	0.133	0.102	-0.468	**1.000**	.986**
A278931	-0.087	0.495	-0.037	.986**	**1.000**
A311002	.563*	0.492	.708**	-0.677	-0.320

ANNEX 3 (Cont.)

	A311002
Oxalate	-0.016
Arginine	-0.350
Glutamate	0.025
Isoleucine	-0.115
Leucine	0.317
Ornithine	-0.498
Pyroglutamate	0.397
Tyrosine	-0.085
Valine	0.324
Carbodiimide	-.699*
Putrescine	-0.167
Uridine	-0.255
Glycerophosphoglycerol	0.162
Threonate	-0.012
Threonate-1,4-Lactone	-0.012
Galactose	-.583*
Raffinose	0.058
A194007	0.262
A217004	-0.005
A290003	0.201
A296004	0.098
A302001	0.208
A308003	-0.510
Fumarate	.748**
Proline	.623*
Succinate	.732**
Asparagine	0.405
Glutamine	0.478
Phenylalanine	0.480
Palmitate	-0.053
Estearate	-0.545
Sinapate	0.345
Glycerate	.730**
Glycerol	-0.015
Myo-Inositol	.836**
Galactinol	.957**
Ribose	.693*
A143002	-0.534
A145016	-0.191
A203003	.563*
A207003	0.492
A214003	.708**
A260482	-0.677
A278931	-0.320
A311002	**1.000**

ANNEX 4

Pearson correlation of altered metabolites after nitrosative stress imposition (*P≤0.05)*

	Citrate	Proline	Aspartate	Ethanolamine	Putrescine
Citrate	**1.000**	-0.016	0.178	0.137	0.350
Proline	-0.016	**1.000**	.740**	-0.509	.547*
Aspartate	0.178	.740**	**1.000**	0.027	.568*
Ethanolamine	0.137	-0.509	0.027	**1.000**	0.014
Putrescine	0.350	.547*	.568*	0.014	**1.000**
Glycerate-3-Phosphate	.654*	0.427	0.434	0.221	0.456
Glycerophosphoglycerol	.853**	0.084	0.443	0.258	0.386
Phosphoric acid	-0.061	0.201	0.152	0.367	-0.198
myo-Inositol	.552*	0.293	.538*	-0.128	0.107
Galactinol	.717**	0.010	0.242	0.121	0.158
Sucrose	0.231	-.664**	0.175	.803**	-0.339
A112003	0.061	-0.374	0.122	-0.213	-0.493
A116201	0.153	-.745**	0.287	.762**	-0.386
A196004	.626*	0.435	0.402	-0.009	0.454
A278931	0.142	0.189	0.074	0.077	-0.003
A290003	.614*	0.418	.608*	0.233	.636**
A296004	.624*	0.050	0.193	0.284	0.334
A302001	0.425	.576*	.562*	0.019	0.301
A313001	.736**	0.150	0.344	0.384	0.426
Glycolate	0.515	0.220	0.164	-0.335	0.500
Arginine	-0.021	0.377	0.294	-0.478	0.520
Asparagine	0.260	0.241	0.246	-0.220	0.543
GABA	-0.190	0.084	-0.049	-0.269	0.274
Glutamate	0.279	-0.101	-0.017	-0.264	0.025
Ornithine	0.021	0.132	-0.087	-0.292	0.119
Pyroglutamate	0.235	0.092	-0.195	-0.400	0.268
Serine	0.203	-0.073	-0.057	-0.188	0.324
Pyridine, 2,3-dihydroxy-	-0.039	-0.065	-0.184	-0.462	-0.020
Pyridine, 3-hydroxy-	-0.442	0.346	-0.272	-.713*	-0.042
Erythronate-1,4-lactone	0.126	-0.157	-0.014	-0.529	0.271
Gluconate	0.011	0.013	-0.251	-0.443	0.012
Galactose	-0.007	0.057	-0.035	-0.394	0.350
Ribose	-0.040	0.025	-0.189	-0.354	0.051
A124002	-0.072	0.002	-0.155	-0.446	0.051
A207003	0.047	-0.143	-0.063	-0.080	0.227

ANNEX 4 (Cont.)

	Glycerate-3-Phosphate	Glycero phosphoglycerol	Phosphoric acid	*myo*-Inositol	Galactinol
Citrate	.654*	.853**	-0.061	.552*	.717**
Proline	0.427	0.084	0.201	0.293	0.010
Aspartate	0.434	0.443	0.152	.538*	0.242
Ethanolamine	0.221	0.258	0.367	-0.128	0.121
Putrescine	0.456	0.386	-0.198	0.107	0.158
Glycerate-3-Phosphate	**1.000**	.703**	0.389	.581*	0.467
Glycerophosphoglycerol	.703**	**1.000**	0.164	.653**	.726**
Phosphoric acid	0.389	0.164	**1.000**	.472*	-0.041
myo-Inositol	.581*	.653**	.472*	**1.000**	.566*
Galactinol	0.467	.726**	-0.041	.566*	**1.000**
Sucrose	0.048	0.364	.615**	0.431	0.196
A112003	-0.391	-0.009	0.281	0.475	0.399
A116201	-0.187	0.259	0.325	0.269	0.163
A196004	.846**	.695**	0.312	.583*	.513*
A278931	0.263	0.262	0.514	0.306	-0.219
A290003	.570*	.682**	-0.132	0.450	0.460
A296004	0.443	.697**	0.011	0.423	.812**
A302001	.672**	.542*	0.236	.718**	.575*
A313001	0.320	.531*	0.170	.592*	.502*
Glycolate	0.150	0.411	-0.141	0.259	0.111
Arginine	0.168	0.011	-0.085	0.081	-0.261
Asparagine	0.237	0.191	-0.112	0.101	-0.181
GABA	-0.266	-0.291	-0.466	-0.301	-0.312
Glutamate	0.078	0.350	-0.481	0.237	0.089
Ornithine	0.003	0.015	-0.226	-0.140	-0.343
Pyroglutamate	-0.160	-0.006	-.593*	-0.168	-0.131
Serine	0.027	0.189	-.500*	-0.035	-0.145
Pyridine, 2,3-dihydroxy-	-0.292	-0.048	0.010	-0.025	-0.261
Pyridine, 3-hydroxy-	-0.154	-0.227	-0.128	0.026	-0.307
Erythronate-1,4-lactone	-0.348	-0.022	-.589*	-0.004	-0.086
Gluconate	-0.200	-0.301	-0.333	-0.216	-0.336
Galactose	-0.303	-0.222	-0.432	-0.259	-0.324
Ribose	-0.286	-0.260	-0.269	-0.159	-0.324
A124002	-0.245	-0.135	-0.339	-0.157	-0.319
A207003	0.125	0.168	-.600*	-0.119	-0.060

ANNEX 4 (Cont.)

	Sucrose	A112003	A116201	A196004	A278931
Citrate	0.231	0.061	0.153	.626*	0.142
Proline	-.664**	-0.374	-.745**	0.435	0.189
Aspartate	0.175	0.122	0.287	0.402	0.074
Ethanolamine	.803**	-0.213	.762**	-0.009	0.077
Putrescine	-0.339	-0.493	-0.386	0.454	-0.003
Glycerate-3-Phosphate	0.048	-0.391	-0.187	.846**	0.263
Glycerophosphoglycerol	0.364	-0.009	0.259	.695**	0.262
Phosphoric acid	.615**	0.281	0.325	0.312	0.514
myo-Inositol	0.431	0.475	0.269	.583*	0.306
Galactinol	0.196	0.399	0.163	.513*	-0.219
Sucrose	**1.000**	0.528	.924**	0.026	0.361
A112003	0.528	**1.000**	0.507	-0.184	-0.314
A116201	.924**	0.507	**1.000**	-0.238	0.064
A196004	0.026	-0.184	-0.238	**1.000**	0.254
A278931	0.361	-0.314	0.064	0.254	**1.000**
A290003	-0.072	-0.428	-0.023	0.463	0.261
A296004	0.220	0.247	-0.023	.519*	-0.034
A302001	0.012	0.096	-0.034	.615**	0.156
A313001	0.127	0.129	0.054	0.430	0.171
Glycolate	-0.151	-0.447	-0.306	0.410	0.023
Arginine	-.580*	-0.689	-.587*	0.212	-0.339
Asparagine	-0.357	-.876*	-0.315	0.191	-0.407
GABA	-0.384	-0.495	-0.332	-0.310	-.613*
Glutamate	-0.147	-0.433	-0.057	0.142	0.090
Ornithine	-0.213	-.767**	-0.162	-0.046	0.094
Pyroglutamate	-.518*	-0.602	-0.413	-0.055	-0.485
Serine	-0.394	-0.601	-0.265	0.057	0.003
Pyridine, 2,3-dihydroxy-	-0.054	-0.066	-0.363	-0.003	0.108
Pyridine, 3-hydroxy-	-0.426	-0.288	-.662*	-0.031	0.256
Erythronate-1,4-lactone	-0.379	-0.234	-0.202	-0.209	-0.490
Gluconate	-0.414	-0.501	-0.447	0.004	-0.526
Galactose	-0.378	-0.463	-0.361	-0.248	-0.479
Ribose	-0.250	-0.390	-0.257	-0.287	-0.464
A124002	-0.245	-0.284	-0.276	-0.271	-0.321
A207003	-0.351	-0.609	-0.188	0.043	-0.328

ANNEX 4 (Cont.)

	A290003	A296004	A302001	A313001	Glycolate
Citrate	.614*	.624*	0.425	.736**	0.515
Proline	0.418	0.050	.576*	0.150	0.220
Aspartate	.608*	0.193	.562*	0.344	0.164
Ethanolamine	0.233	0.284	0.019	0.384	-0.335
Putrescine	.636**	0.334	0.301	0.426	0.500
Glycerate-3-Phosphate	.570*	0.443	.672**	0.320	0.150
Glycerophosphoglycerol	.682**	.697**	.542*	.531*	0.411
Phosphoric acid	-0.132	0.011	0.236	0.170	-0.141
myo-Inositol	0.450	0.423	.718**	.592*	0.259
Galactinol	0.460	.812**	.575*	.502*	0.111
Sucrose	-0.072	0.220	0.012	0.127	-0.151
A112003	-0.428	0.247	0.096	0.129	-0.447
A116201	-0.023	-0.023	-0.034	0.054	-0.306
A196004	0.463	.519*	.615**	0.430	0.410
A278931	0.261	-0.034	0.156	0.171	0.023
A290003	**1.000**	0.379	.645**	.507*	0.245
A296004	0.379	**1.000**	0.291	.597*	0.151
A302001	.645**	0.291	**1.000**	.543*	0.086
A313001	.507*	.597*	.543*	**1.000**	0.212
Glycolate	0.245	0.151	0.086	0.212	**1.000**
Arginine	-0.126	-0.118	-0.128	-0.053	.733*
Asparagine	0.087	-0.224	0.041	0.106	.759*
GABA	0.097	-0.323	-0.190	-0.208	.680*
Glutamate	0.285	-0.026	0.091	0.004	0.509
Ornithine	0.145	-0.427	-0.046	-0.284	.575*
Pyroglutamate	0.208	-0.265	0.004	0.038	.670*
Serine	0.226	-0.097	-0.129	0.118	.594*
Pyridine, 2,3-dihydroxy-	-0.337	0.052	-0.504	-0.275	.634*
Pyridine, 3-hydroxy-	-0.360	-0.218	-0.122	-0.419	0.600
Erythronate-1,4-lactone	-0.076	-0.154	-0.260	-0.018	.747**
Gluconate	-0.221	-0.565	-0.248	-0.242	0.584
Galactose	0.027	-0.360	-0.271	-0.197	.813**
Ribose	-0.033	-0.362	-0.218	-0.156	.686**
A124002	-0.022	-0.415	-0.256	-0.308	.786**
A207003	0.329	-0.156	0.028	-0.096	0.338

ANNEX 4 (Cont.)

	Arginine	Asparagine	GABA	Glutamate	Ornithine
Citrate	-0.021	0.260	-0.190	0.279	0.021
Proline	0.377	0.241	0.084	-0.101	0.132
Aspartate	0.294	0.246	-0.049	-0.017	-0.087
Ethanolamine	-0.478	-0.220	-0.269	-0.264	-0.292
Putrescine	0.520	0.543	0.274	0.025	0.119
Glycerate-3-Phosphate	0.168	0.237	-0.266	0.078	0.003
Glycerophosphoglycerol	0.011	0.191	-0.291	0.350	0.015
Phosphoric acid	-0.085	-0.112	-0.466	-0.481	-0.226
myo-Inositol	0.081	0.101	-0.301	0.237	-0.140
Galactinol	-0.261	-0.181	-0.312	0.089	-0.343
Sucrose	-.580*	-0.357	-0.384	-0.147	-0.213
A112003	-0.689	-.876*	-0.495	-0.433	-.767**
A116201	-.587*	-0.315	-0.332	-0.057	-0.162
A196004	0.212	0.191	-0.310	0.142	-0.046
A278931	-0.339	-0.407	-.613*	0.090	0.094
A290003	-0.126	0.087	0.097	0.285	0.145
A296004	-0.118	-0.224	-0.323	-0.026	-0.427
A302001	-0.128	0.041	-0.190	0.091	-0.046
A313001	-0.053	0.106	-0.208	0.004	-0.284
Glycolate	.733*	.759*	.680*	0.509	.575*
Arginine	**1.000**	.787**	0.481	0.444	0.528
Asparagine	.787**	**1.000**	.714**	.736**	.849**
GABA	0.481	.714**	**1.000**	0.427	.608*
Glutamate	0.444	.736**	0.427	**1.000**	.668**
Ornithine	0.528	.849**	.608*	.668**	**1.000**
Pyroglutamate	0.533	.852**	.723**	.755**	.845**
Serine	.694**	.775**	0.495	.845**	.608*
Pyridine, 2,3-dihydroxy-	.617*	0.185	0.237	0.197	0.043
Pyridine, 3-hydroxy-	.765*	0.315	0.420	0.397	0.418
Erythronate-1,4-lactone	.600*	.675*	.631**	.569*	.596*
Gluconate	.739*	0.636	.869**	0.552	.645*
Galactose	.650*	.715*	.904**	0.376	.639*
Ribose	.596*	.748**	.901**	0.488	.719**
A124002	.655*	.778**	.846**	0.511	.606*
A207003	0.408	.752**	.562*	.875**	.596*

ANNEX 4 (Cont.)

	Pyroglutamate	Serine	Pyridine, 2,3-dihydroxy-	Pyridine, 3-hydroxy-	Erythronate-1,4-lactone
Citrate	0.235	0.203	-0.039	-0.442	0.126
Proline	0.092	-0.073	-0.065	0.346	-0.157
Aspartate	-0.195	-0.057	-0.184	-0.272	-0.014
Ethanolamine	-0.400	-0.188	-0.462	-.713*	-0.529
Putrescine	0.268	0.324	-0.020	-0.042	0.271
Glycerate-3-Phosphate	-0.160	0.027	-0.292	-0.154	-0.348
Glycerophosphoglycerol	-0.006	0.189	-0.048	-0.227	-0.022
Phosphoric acid	-.593*	-.500*	0.010	-0.128	-.589*
myo-Inositol	-0.168	-0.035	-0.025	0.026	-0.004
Galactinol	-0.131	-0.145	-0.261	-0.307	-0.086
Sucrose	-.518*	-0.394	-0.054	-0.426	-0.379
A112003	-0.602	-0.601	-0.066	-0.288	-0.234
A116201	-0.413	-0.265	-0.363	-.662*	-0.202
A196004	-0.055	0.057	-0.003	-0.031	-0.209
A278931	-0.485	0.003	0.108	0.256	-0.490
A290003	0.208	0.226	-0.337	-0.360	-0.076
A296004	-0.265	-0.097	0.052	-0.218	-0.154
A302001	0.004	-0.129	-0.504	-0.122	-0.260
A313001	0.038	0.118	-0.275	-0.419	-0.018
Glycolate	.670*	.594*	.634*	0.600	.747**
Arginine	0.533	.694**	.617*	.765*	.600*
Asparagine	.852**	.775**	0.185	0.315	.675*
GABA	.723**	0.495	0.237	0.420	.631**
Glutamate	.755**	.845**	0.197	0.397	.569*
Ornithine	.845**	.608*	0.043	0.418	.596*
Pyroglutamate	**1.000**	.754**	0.105	0.362	.764**
Serine	.754**	**1.000**	0.347	0.530	.714**
Pyridine, 2,3-dihydroxy-	0.105	0.347	**1.000**	.693*	0.505
Pyridine, 3-hydroxy-	0.362	0.530	.693*	**1.000**	.625*
Erythronate-1,4-lactone	.764**	.714**	0.505	.625*	**1.000**
Gluconate	.728*	.636*	0.265	0.583	.783**
Galactose	.732**	.561*	0.490	.623*	.808**
Ribose	.715**	.517*	0.358	0.462	.681**
A124002	.617*	.590*	.616*	.907**	.614*
A207003	.730**	.818**	-0.044	0.227	0.474

ANNEX 4 (Cont.)

	Gluconate	Galactose	Ribose	A124002	A207003
Citrate	0.011	-0.007	-0.040	-0.072	0.047
Proline	0.013	0.057	0.025	0.002	-0.143
Aspartate	-0.251	-0.035	-0.189	-0.155	-0.063
Ethanolamine	-0.443	-0.394	-0.354	-0.446	-0.080
Putrescine	0.012	0.350	0.051	0.051	0.227
Glycerate-3-Phosphate	-0.200	-0.303	-0.286	-0.245	0.125
Glycerophosphoglycerol	-0.301	-0.222	-0.260	-0.135	0.168
Phosphoric acid	-0.333	-0.432	-0.269	-0.339	-.600*
myo-Inositol	-0.216	-0.259	-0.159	-0.157	-0.119
Galactinol	-0.336	-0.324	-0.324	-0.319	-0.060
Sucrose	-0.414	-0.378	-0.250	-0.245	-0.351
A112003	-0.501	-0.463	-0.390	-0.284	-0.609
A116201	-0.447	-0.361	-0.257	-0.276	-0.188
A196004	0.004	-0.248	-0.287	-0.271	0.043
A278931	-0.526	-0.479	-0.464	-0.321	-0.328
A290003	-0.221	0.027	-0.033	-0.022	0.329
A296004	-0.565	-0.360	-0.362	-0.415	-0.156
A302001	-0.248	-0.271	-0.218	-0.256	0.028
A313001	-0.242	-0.197	-0.156	-0.308	-0.096
Glycolate	0.584	.813**	.686**	.786**	0.338
Arginine	.739*	.650*	.596*	.655*	0.408
Asparagine	0.636	.715*	.748**	.778**	.752**
GABA	.869**	.904**	.901**	.846**	.562*
Glutamate	0.552	0.376	0.488	0.511	.875**
Ornithine	.645*	.639*	.719**	.606*	.596*
Pyroglutamate	.728*	.732**	.715**	.617*	.730**
Serine	.636*	.561*	.517*	.590*	.818**
Pyridine, 2,3-dihydroxy-	0.265	0.490	0.358	.616*	-0.044
Pyridine, 3-hydroxy-	0.583	.623*	0.462	.907**	0.227
Erythronate-1,4-lactone	.783**	.808**	.681**	.614*	0.474
Gluconate	**1.000**	.822**	.794**	.614*	0.529
Galactose	.822**	**1.000**	.892**	.849**	0.415
Ribose	.794**	.892**	**1.000**	.874**	0.431
A124002	.614*	.849**	.874**	**1.000**	.537*
A207003	0.529	0.415	0.431	.537*	**1.000**

ANNEX 5

Pearson correlation between altered metabolites after oxidative/nitrossative imposition (*P≤0.05)*

	Proline	Arginine	Asparagine	Glutamate
Proline	**1.000**	-0.073	0.113	-0.156
Arginine	-0.073	**1.000**	.608**	.508**
Asparagine	0.113	.608**	**1.000**	.418**
Glutamate	-0.156	.508**	.418**	**1.000**
Ornithine	-0.211	.827**	.393**	.629**
Pyroglutamate	-0.207	.570**	.535**	.763**
Putrescine	0.190	.570**	.416**	0.150
Glycerophosphoglycerol	0.008	-0.018	-0.145	.389**
myo-Inositol	.447**	-0.327	-0.038	-0.015
Galactinol	.409**	-.516**	-0.039	-0.219
Galactose	-.382*	.639**	0.299	.563**
Ribose	.374*	-0.015	.397*	0.194
A207003	0.050	0.169	.688**	.566**
A278931	0.251	-.464**	0.073	-0.198
A290003	0.050	-0.084	-0.047	0.120
A296004	-0.133	-.291*	-.379**	-0.029
A302001	0.024	-0.266	-0.204	0.064

	Ornithine	Pyroglutamate	Putrescine	Glycero phosphoglycerol
Proline	-0.211	-0.207	0.190	0.008
Arginine	.827**	.570**	.570**	-0.018
Asparagine	.393**	.535**	.416**	-0.145
Glutamate	.629**	.763**	0.150	.389**
Ornithine	**1.000**	.745**	.500**	0.085
Pyroglutamate	.745**	**1.000**	.399**	.273*
Putrescine	.500**	.399**	**1.000**	0.122
Glycerophosphoglycerol	0.085	.273*	0.122	**1.000**
myo-Inositol	-.447**	-0.142	0.005	.398**
Galactinol	-.646**	-0.250	-0.165	0.222
Galactose	.735**	.669**	.386*	.300*
Ribose	-0.019	0.290	-0.005	-0.140
A207003	0.117	.497**	0.055	-0.014
A278931	-.457**	-.655**	-0.245	-0.247
A290003	0.016	0.220	0.120	.520**
A296004	-.280*	-0.068	-0.199	.572**
A302001	-0.076	0.102	-0.044	.503**

ANNEX 5 (Cont.)

	myo-Inositol	Galactinol	Galactose	Ribose
Proline	.447**	.409**	-.382*	.374*
Arginine	-0.327	-.516**	.639**	-0.015
Asparagine	-0.038	-0.039	0.299	.397*
Glutamate	-0.015	-0.219	.563**	0.194
Ornithine	-.447**	-.646**	.735**	-0.019
Pyroglutamate	-0.142	-0.250	.669**	0.290
Putrescine	0.005	-0.165	.386*	-0.005
Glycerophosphoglycerol	.398**	0.222	.300*	-0.140
myo-Inositol	**1.000**	.767**	-.422**	.319*
Galactinol	.767**	**1.000**	-.684**	0.187
Galactose	-.422**	-.684**	**1.000**	0.086
Ribose	.319*	0.187	0.086	**1.000**
A207003	-0.073	0.139	0.157	.367*
A278931	0.100	0.285	-.551**	-0.308
A290003	0.131	.262*	-0.048	-0.155
A296004	0.191	.390**	-0.159	-.459**
A302001	.357*	.325**	-0.226	-0.279

	A207003	A278931	A290003	A296004	A302001
Proline	0.050	0.251	0.050	-0.133	0.024
Arginine	0.169	-.464**	-0.084	-.291*	-0.266
Asparagine	.688**	0.073	-0.047	-.379**	-0.204
Glutamate	.566**	-0.198	0.120	-0.029	0.064
Ornithine	0.117	-.457**	0.016	-.280*	-0.076
Pyroglutamate	.497**	-.655**	0.220	-0.068	0.102
Putrescine	0.055	-0.245	0.120	-0.199	-0.044
Glycerophosphoglycerol	-0.014	-0.247	.520**	.572**	.503**
myo-Inositol	-0.073	0.100	0.131	0.191	.357*
Galactinol	0.139	0.285	.262*	.390**	.325**
Galactose	0.157	-.551**	-0.048	-0.159	-0.226
Ribose	.367*	-0.308	-0.155	-.459**	-0.279
A207003	**1.000**	0.003	0.036	-0.148	-0.059
A278931	0.003	**1.000**	0.029	-0.017	-0.127
A290003	0.036	0.029	**1.000**	.475**	.736**
A296004	-0.148	-0.017	.475**	**1.000**	.566**
A302001	-0.059	-0.127	.736**	.566**	**1.000**

ANNEX 6

Detected metabolites during normal growth condition with no significant differences between WT and *pao4* mutants. Compounds abundance is presented as log_2-transformed fold-changes realtive to the mean of WT-Col 0 samples (P<0.05).

NORMAL GROWTH CONDITION									
	DETECTED METABOLITES WITH NO SIGNIFICANT DIFERENCE BETWEEN WT AND *pao4* MUTANTS								
	WT			*pao*4-1			*pao*4-2		
	Mean	N	Std. Deviation	Mean	N	Std. Deviation	Mean	N	Std. Deviation
Aconitic acid, cis-	-1.2822	3	0.96	-0.3377	4	0.82	0.5807	3	0.40
Citrate	-0.8282	4	1.11	-0.4624	5	0.48	-0.1785	6	0.49
Fumarate	-0.8734	4	0.84	-0.6981	5	0.52	-0.2968	6	0.20
Itaconate	-1.0694	2	0.09	0.1983	3	0.79	0.1881	3	0.68
Lactate	-0.2974	4	0.86	0.2348	5	1.13	-0.0400	6	0.51
Maleate	-1.1853	2	0.73	0.1238	3	0.94	-0.2665	5	0.84
Malate	-0.9449	4	0.97	-0.7879	5	0.37	-0.1089	6	0.40
Succinate	-0.6294	4	0.81	-0.3381	5	0.67	0.1395	6	0.15
Alanine	-0.2421	4	0.54	0.0109	5	0.48	0.0508	6	0.33
Glycine	-0.9759	4	0.36	-0.9174	5	0.64	-0.3539	6	0.55
Tetradecanoic acid	-0.2562	4	0.46	-0.0163	5	0.34	0.1058	6	0.42
Squalene, all-trans-	-0.2586	4	1.24	-0.7917	5	0.20	-0.3511	6	1.10
Ethanolamine	-0.2883	4	0.45	0.1838	5	0.33	0.2019	6	0.27
Erythronate	-1.0298	3	0.59	-0.5637	2	0.56	-0.0526	5	0.35
Galactonate	0.0384	4	1.06	-0.1652	4	0.99	-0.1102	6	0.74
Glycerate	-0.9001	4	0.89	-0.6281	5	0.57	-0.2735	6	0.31
Threonate	-0.3531	4	0.68	-0.7577	5	0.49	-0.2718	6	0.38
Threonate-1,4-lactone	0.0790	4	0.27	-0.2209	5	0.21	0.0578	6	0.23
Erythritol	-0.3908	4	0.57	0.0182	5	0.28	0.3247	6	0.33
Glycerol	-0.2869	4	0.49	0.1868	5	0.30	0.1765	6	0.27
Glucopyranose	-0.3724	4	0.72	-1.0717	5	0.82	-0.4261	6	0.45

NORMAL GROWTH CONDITION									
	DETECTED METABOLITES WITH NO SIGNIFICANT DIFERENCE BETWEEN WT AND *pao4* MUTANTS								
	WT			*pao*4-1			*pao*4-2		
	Mean	N	Std. Deviation	Mean	N	Std. Deviation	Mean	N	Std. Deviation
Glucopyranose	-0.5829	4	1.07	-0.1630	5	0.48	0.1574	6	0.38
Glucose	-0.2893	4	0.34	-0.8070	5	0.26	-0.5639	6	0.56
Galactosylglycerol	-0.6938	2	0.01	-0.2761	2	2.02	0.2673	5	0.39
A143003	-0.3310	4	0.50	0.2097	5	0.52	0.2215	6	0.36
A147001	-0.3585	4	0.30	0.2258	5	0.39	0.2807	6	0.43
A159003	-0.8449	4	0.75	-0.3823	4	0.51	-0.0575	6	0.42
A175008	-0.1373	4	0.27	-0.1098	5	0.35	0.0084	6	0.17
A187005	-0.6972	4	1.10	-0.3374	4	0.50	-0.0088	6	0.36
A155405	-0.3899	4	0.94	0.4573	5	0.53	0.3752	6	0.35
A260482	-0.3617	4	0.11	0.1378	5	0.52	0.2117	6	0.40
A278931	-0.1908	4	0.28	0.1528	5	0.35	0.2286	6	0.28
A145015	-0.3907	4	0.24	0.1925	5	0.41	0.1595	6	0.44
A170001	-0.0821	4	0.51	0.3038	5	0.47	0.5865	6	0.34
A176001	-1.0968	4	1.86	-0.6890	5	0.83	0.0276	6	0.30
A211001	-0.8631	4	0.57	-0.0888	4	0.59	0.1217	6	0.44
Salicin	-1.1355	3	1.79	-0.1441	2	1.44	-0.1295	6	0.77

ANNEX 7

Down-regulated metabolites on *pao4* mutants under normal growth conditions. Results are expressed on means of log2 transformation to WT. Altered metabolites were detected with MeV tool V.4.9 by rank product statistical test (*P*<0.05).

NORMAL GROWTH CONDITION									
	DECREASED METABOLITES ON *pao*4 MUTANTS								
	WT			*pao*4-1			*pao*4-2		
	Mean	N	Std. Deviation	Mean	N	Std. Deviation	Mean	N	Std. Deviation
Sucrose	1.2864	3	.28	-.6878	3	.15	-.4030	6	.10
A112003	.3135	3	.06	.1915	4	.02	-.0604	6	.05
A115002	.7625	4	.06	.3099	5	.13	.1859	6	.07
A148006	.0421	3	.04	-.6809	3	.04	-.3137	3	.03
A174001	.8850	4	.09	-.8164	5	.10	-.1891	6	.04
A236005	.7132	2	.03	-.5566	3	.07	-.4382	6	.05
A116201	.3205	4	.05	.1437	5	.04	.1844	6	.03
Indole-3-acetonitrile	.1001	4	.06	-.1243	3	.10	-.1853	4	.05
A145016	.6049	4	.03	-.1095	5	.02	-.1030	6	.03
A147011	.7023	4	.04	-.2706	5	.03	-.0659	6	.03

ANNEX 8

Metabolic pathways associated with some up-regulated metabolites of *pao4* mutants under normal growth conditions according to public metabolic databases or original publications.

METABOLITE	PATHWAYS
Ascorbate Dehydroascorbate	Aminosugar and nucleotide sugar metabolism (KEGG) (Valpuesta and Botella, 2004) Ascorbate and aldarate metabolism (KEGG) Ascorbate-Glutathion Pathway (AraCyc) (Foyer and Noctor, 2011)
Nicotinate	Pyridine nucleotide cycling (AraCyc) Nicotinate and nicotinamide metabolism (KEGG) Alanine, Aspartate and Glutamate metabolism (KEGG)
4-Aminobutanoate (GABA)	Alanine, aspartate and glutamate metabolism (KEGG) Glutamate, arginine and putrescine degradation (AraCyc) GABA shunt (AraCyc) activation during senescence (Buchanan-Wollaston *et al.*, 2005)
Palmitate	Fatty acid metabolism (KEGG) Cutine, suberine and wax biosynthesis (KEGG) Peroxisome fatty acid β-Oxidation (AraCyc)
2-hydroxiglutarate	Butanoate metabolism (KEGG) C5-Branched dibasic acid metabolism (KEGG) Lysine catabolism (Araújo *et al.*, 2010; Engqvist *et al.*, 2011)
***myo*-Inositol**	Inositol phosphate metabolism (KEGG) Phosphatidylinositol signaling system (KEGG) Galactose metabolism (KEGG) Plant ascorbate biosynthesis (Lorence, 2004)
Sinapate	Phenylpropanoid biosynthesis (KEGG, AraCyc) Ferulate and Sinapate biosynthesis (AraCyc) Flavonoid biosynthesis (Dixon *et al.*, 2002)
Pyruvate	Glycolisis/Gluconeogenesis (KEGG,AraCyc) Biosynthesis of amino acids (KEGG) TCA cycle (KEGG,AraCyc) Pentose Phosphate Pathway (KEGG) Glutathione-mediated detoxification (AraCyc) Phenylpropanoid biosynthetic pathway on plant defence response in *Arabidopsis* (Dixon *et al.*, 2002)
1,6-Anhydro-β-D-Glucose	Glycolisis/Gluconeogenesis (KEGG,AraCyc) Carbon metabolism (KEGG) Pentose Phosphate Pathway (KEGG) Starch and sucrose metabolism (KEGG,AraCyc)
Galactose	Galactose metabolism (KEGG) Amino sugar and nucleotide sugar metabolism (KEGG) Ascorbate and aldarate metabolism (KEGG)
Threonine	Glycine, serine and threonine metabolism (KEGG,AraCyc) Cysteine and methionine metabolism (KEGG) Valine, leucine and isoleucine biosynthesis from pyruvate (KEGG) γ-Glutamyl cycle (AraCyc)
Serine	Glycine, serine and threonine metabolism (KEGG,AraCyc) Cysteine and methionine metabolism (KEGG) Valine, leucine and isoleucine biosynthesis from pyruvate (KEGG) γ-Glutamyl cycle (AraCyc) Sphingolipid biosynthesis (AraCyc)

METABOLITE	PATHWAYS
Tyrosine	Tyrosine metabolism (KEGG) Phenylalanine, tyrosine and tryptophan biosynthesis (KEGG) Regulatory role of anti-oxidative enzymes by it peroxinitrate-mediated nitration of residues on active sites (Holzmeister *et al.*, 2014) γ-Glutamyl cycle (AraCyc)
Phenylalanine	Phenylalanine metabolism (KEGG) Phenylalanine, tyrosine and tryptophan biosynthesis (KEGG) Phenylpropanoid biosynthesis (KEGG, AraCyc) Biosynthesis of Salycilic acid (KEGG) Flavonoids biosynthesis (Dixon *et al.*, 2002; Xie *et al.*, 2012) Cyanoamino acid metabolism (KEGG) γ-Glutamyl cycle (AraCyc)
Isoleucine	Valine, leucine and isoleucine metabolism (KEGG) γ-Glutamyl cycle (AraCyc)
Valine	Valine, leucine and isoleucine metabolism (KEGG) γ-Glutamyl cycle (AraCyc) Pyruvate metabolism (KEGG) TCA cycle (KEGG)
Methionine	Cysteine and methionine metabolism (KEGG) Arginine and proline metabolism (KEGG) γ-Glutamyl cycle (AraCyc) S-adenosyl-L-methionine cycle (AraCyc) Biosynthesis of Ethylene (KEGG)

ANNEX 9

Detected metabolites after dark induced senescence with no significant differences between WT and *pao4* mutants. Compounds abundance is presented as log_2-transformed fold-changes realtive to the mean of WT-Col 0 samples (P<0.05).

AFTER DARK INDUCED SENESCENCE									
	DETECTED METABOLITES WITH NO SIGNIFICANT DIFFERENCE BETWEEN WT AND pao4 MUTANTS								
	WT			*pao*4-1			*pao*4-2		
	Mean	N	Std. Deviation	Mean	N	Std. Deviation	Mean	N	Std. Deviation
2-Aminoadipic-acid	-0.1104	2	0.81	0.0087	6	0.76	-0.1505	4	0.72
Aconitic acid, cis-	-0.0897	3	0.61	-0.2834	6	0.76	-0.3696	5	0.68
Benzoic acid	-0.0982	4	0.65	0.2063	6	0.53	0.3331	5	0.42
Butanoic acid, 2,4-dihydroxy-	-0.7866	4	2.25	0.1308	6	0.75	0.1262	5	0.43
Butanoic acid, 2-oxo-	-0.1007	4	0.68	0.1617	6	0.67	0.6247	5	0.33
Citric acid	-0.9520	4	2.63	0.4576	6	0.66	0.2343	5	0.53
Fumaric acid	-1.4687	4	3.39	0.2798	6	0.70	0.4063	5	0.76
Glutaric acid	0.0121	4	0.34	-0.0019	5	0.47	0.0662	5	0.20
Glutaric acid, 2-hydroxy-	-0.9685	4	2.59	0.2138	6	0.77	0.1219	5	0.51
Glutaric acid, 3-hydroxy-3-methyl-	-0.0398	4	0.39	-0.1163	5	0.51	-0.2658	5	0.31
Itaconic acid	0.0348	4	0.61	0.1694	6	0.54	0.2514	5	0.45
Malic acid	-1.2938	4	3.19	0.3633	6	0.76	0.3024	5	0.75
Malonic acid, Methyl	-0.2955	3	1.09	-0.1035	6	0.76	-0.0287	4	0.92
Pyruvic acid	-0.0871	4	0.62	0.2340	6	0.62	-0.3723	5	2.01
Succinic acid	-1.1180	4	2.67	-0.2115	6	0.61	0.0658	5	0.86
Alanine	-0.4512	4	1.43	-0.1952	6	0.58	0.1456	5	1.15
Glycine	-0.2335	4	1.02	-0.0253	6	0.42	0.1238	5	0.41
Lysine	-0.0026	2	0.12	0.2659	6	0.54	-0.4685	5	1.61
Methionine	-0.3860	4	1.36	0.2570	6	0.62	0.4593	5	0.51
Ornithine (Arginine, Citrulline)	0.0000	4	0.01	0.4817	4	1.02	0.7279	4	0.60
Serine, N-acetyl-	-0.7655	4	2.07	0.5288	6	0.88	0.4702	5	0.79

AFTER DARK INDUCED SENESCENCE									
	DETECTED METABOLITES WITH NO SIGNIFICANT DIFFERENCE BETWEEN WT AND pao4 MUTANTS								
	WT			*pao*4-1			*pao*4-2		
	Mean	N	Std. Deviation	Mean	N	Std. Deviation	Mean	N	Std. Deviation
Serine, O-acetyl-	-0.8584	2	2.45	-0.8520	2	0.61	0.7678	2	1.51
Tyrosine	-0.1470	3	0.86	0.3214	6	1.09	-0.9394	5	1.05
Valine	-0.8161	4	2.17	0.3143	6	0.76	0.0679	5	0.74
Hexadecanoic acid	-0.0651	4	0.51	0.2763	6	0.48	0.4630	5	0.49
Octadecanoic acid	-0.1011	4	0.64	0.3031	6	0.43	0.3743	5	0.38
Tetradecanoic acid	-0.0918	4	0.60	0.3145	6	0.48	0.6852	5	0.85
Squalene, all-trans-	-0.3111	4	1.23	0.1859	6	1.06	0.5192	5	0.63
Ethanolamine	-0.0717	4	0.54	0.3148	6	0.47	0.2348	5	0.46
Nicotinic acid	-0.1390	4	0.76	0.0087	6	0.53	0.0349	5	0.37
Pyridine, 2-hydroxy-	-0.1687	4	0.85	0.3476	6	0.66	-0.3667	5	1.72
Glucose-6-phosphate	-0.2935	4	0.66	0.4318	6	1.20	-0.2772	5	0.75
Phosphoric acid	-0.5568	4	1.59	0.1377	6	0.43	-0.0033	5	0.45
Phosphoric acid monomethyl ester	-0.3283	4	1.22	0.2971	6	0.70	0.8275	5	1.96
Erythronic acid	-0.0999	3	0.64	0.0762	6	0.69	-0.1613	5	0.46
Galactonic acid	-0.1675	3	0.88	-0.1757	6	0.80	-0.3095	5	0.67
Glyceric acid	-0.3810	4	1.24	0.1404	6	0.52	0.3367	5	0.70
Threonic acid	-0.1636	3	0.82	-0.2417	6	0.77	0.0122	5	0.71
Erythritol	-0.1981	3	0.96	-0.0614	6	0.86	-0.0274	5	0.59
Glycerol	-0.1208	4	0.72	0.1941	6	0.30	0.5312	5	0.41
Galactose	-0.5913	4	0.78	0.0898	6	0.40	0.4968	5	0.80
Glucopyranose	-0.1465	3	0.77	-0.2559	6	0.95	0.5820	5	0.81
Glucopyranose [-H20]	-0.5674	4	1.67	0.1876	6	0.81	0.0297	5	0.55
Glucose, 1,6-anhydro	-0.3137	4	1.17	0.2600	6	0.67	-0.0038	5	0.48
Maltose	-0.1588	4	0.80	0.0057	5	0.88	0.5940	3	1.61
Sucrose	-0.0061	4	0.49	0.1480	5	0.73	-0.2681	5	0.60

AFTER DARK INDUCED SENESCENCE									
	DETECTED METABOLITES WITH NO SIGNIFICANT DIFFERENCE BETWEEN WT AND pao4 MUTANTS								
	WT			*pao*4-1			*pao*4-2		
	Mean	N	Std. Deviation	Mean	N	Std. Deviation	Mean	N	Std. Deviation
Galactosylglycerol	-0.6465	4	1.95	0.7669	6	0.56	0.2864	5	0.64
A112003	-0.0315	4	0.36	-0.0197	6	0.39	0.7862	5	1.53
A115002	-0.6897	4	1.98	0.1363	6	0.62	-0.1789	5	1.41
A136010	-0.2884	4	0.74	-0.1816	6	0.84	0.1710	5	0.77
A143003	-0.1575	4	0.83	0.2364	6	0.47	0.2806	5	0.40
A145008	-0.1686	4	0.82	-0.6349	2	0.33	-0.6898	1	.
A147001	-0.1259	4	0.74	0.2183	6	0.51	0.2627	5	0.47
A147005	-0.0564	4	0.47	0.0060	2	0.19	-0.5451	2	0.25
A159003	0.0735	4	0.54	-0.0985	6	0.75	0.0815	5	0.47
A174001	0.0429	4	0.46	-0.0076	6	0.65	0.0458	5	0.50
A175008	0.0793	4	0.46	0.0943	6	0.61	0.0162	5	0.50
A187005	0.0841	4	0.63	0.0448	6	0.45	-0.0568	5	0.45
A214003	0.0398	4	0.16	0.2426	6	0.82	-0.1432	5	0.28
A228001	-0.0589	4	0.48	-0.1221	5	0.62	-0.3485	3	0.65
A236005	0.0575	4	0.57	0.1923	6	0.67	0.2775	5	0.52
A250001	0.0680	4	0.70	-0.1132	6	0.92	-0.2275	5	1.20
A251003	0.0467	4	0.61	-0.2688	6	1.07	-0.3212	5	1.01
A254002	0.0682	4	0.82	-0.0158	6	0.81	-0.3533	5	1.12
A116201	-0.1039	4	0.63	0.2553	6	0.60	0.1004	5	0.69
A155405	-0.2816	4	1.19	0.0743	6	0.76	0.1724	5	0.65
A260482	-0.1317	4	0.75	0.4344	6	0.48	0.3460	5	0.26
A278931	-0.0760	4	0.56	0.3841	6	0.44	0.2960	5	0.22
A145015	-0.1398	4	0.78	0.3117	6	0.47	0.2423	5	0.53
A145016	-0.1329	4	0.75	0.2996	6	0.50	0.3848	5	0.36
A170001	-0.2137	4	0.63	0.1224	6	0.82	-0.0050	5	0.60
A176001	-0.6976	4	2.06	0.3177	6	0.58	0.4583	5	0.49

AFTER DARK INDUCED SENESCENCE									
	DETECTED METABOLITES WITH NO SIGNIFICANT DIFFERENCE BETWEEN WT AND pao4 MUTANTS								
	WT			*pao*4-1			*pao*4-2		
	Mean	N	Std. Deviation	Mean	N	Std. Deviation	Mean	N	Std. Deviation
A213001	-0.0237	4	0.21	0.1213	6	0.74	-0.1904	5	0.32
A143002	-0.0867	4	0.60	0.2100	6	0.49	0.2343	5	0.39
A114002	-0.0319	4	0.36	0.0204	6	0.40	0.1312	5	0.27

APPENDIX

ACTIVITIES DURING PRE-DOCTORAL PERIOD

Meetings

07/2015	**Phenotyping at plant and cellular level.** Poster COST Action FA1306 meeting
05/2015	**VIII *Jornadas de la Facultad de Farmacia*** *Oral communication* *University of Barcelona*
07/2014	**VIII Congréso de la Federación Española de Biotecnológos.** **Biotech Annual Congress 2014**. Oral communication Federación Española de Biotecnología
11/2011	**Plant Abiotic Stress: From Systems Biology to Sustainable Agriculture** COST Action FA0605 meeting. Limassol-Cyprus
05/2010	**Plant Abiotic Stress: from signaling to crop improvement** COST Action FA0605 meeting. Valencia-España

Short-terms

09/2013-11/2013	**Laboratory of Applied Metabolome Analysis.** **Max Planck Institute of Molecular Plant Physiology** Supervisor: Dr. Joachim Kopka Potsdam-Golm, Germany
04/2011-08/2011	**Laboratory of Plant Stress Physiology**. **Cyprus University of Technology** Supervisor: Dr. Vasileios Fotopoulos Limassol, Cyprus

Courses and trainings

07/2014	**Introducction to Project Management**. Associació Biotecnòlegs de Catalunya
05/2014	**Curs Introductori a la Metabolòmica**. Associació Biotecnòlegs de Catalunya

05/2014	**Claus per a Bioempendre** Fundació Escola de Emprenedors
03/2014-06/2014	**Research yourself-UB: Servei de coaching per a personal investigador predoctoral** Alumni UB. Universitat de Barcelona
09/2010-12/2010	**Executive and Bussines Coaching** **First Coaching Training** Coaching Corporation