



UNIVERSITAT DE
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Applied biotechnology to improve Mediterranean rice varieties

Biotecnologia aplicada a la millora de varietats d'arròs mediterrànies

Xavier Serrat Gurrera

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APPLIED BIOTECHNOLOGY TO IMPROVE MEDITERRANEAN RICE VARIETIES

BIOTECNOLOGIA APLICADA A LA MILLORA DE VARIETATS D'ARRÒS MEDITERRÀNIES

Memòria presentada per Xavier Serrat Gurrera per optar al títol de Doctor per la Universitat de Barcelona. Aquest treball s'emmarca dins el programa de doctorat "Biotecnologia Molecular", corresponent al bienni 2009/2011.

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Doctorand

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Resum (summary in Catalan)

L'arròs (*Oryza sativa*) és l'aliment bàsic més àmpliament consumit per una gran part de la població mundial. És el segon cereal en termes de producció mundial (730 milions de tones l'any), per davant del blat i per darrera del blat de moro [1]. Si tenim en compte que l'arròs produeix més calories per superfície que el blat [2] i que la producció d'arròs es destina principalment al consum humà, a diferència del de la canya de sucre que s'utilitza per bioenergia, i el del blat de moro que es dedica en gran mesura a consum animal, a la producció de biocombustible i producció de midons industrials [3], podem concloure que l'arròs és el cultiu més important en quant a la contribució de calories en l'alimentació humana [4].

La població mundial actual és de més de 7.400 milions i s'espera que superi els 9.000 milions al 2040 [1]. Això provocarà un increment de la demanda d'aliments d'un 70%, en un marc en el que l'àrea total mundial cultivable i la disponibilitat d'aigua estan minvant [3]. La salinització dels terrenys cultivables és un problema que afecta més de 100 països al món i que s'agreuja any rere any afectant sovint els deltes on hi ha les principals zones de producció arrossera [5]. Diversos factors com la contaminació, l'escalfament global i la progressiva salinització dels sòls han contribuït a aquesta degradació mediambiental global i a la disminució de la disponibilitat hídrica. D'altra banda, l'alt cost de la elevada dependència energètica en la producció de fertilitzants i l'ús de maquinària agrícola, junt amb l'impacte mediambiental dels fitosanitaris i de l'activitat humana, han promogut la recerca biotecnològica en cereals [7,8].

L'any 2014, la producció d'arròs a la Unió Europea es va estimar en 2.871.874 tones, ocupant una àrea cultivada de 431.062 hectàrees, principalment situades a la conca mediterrània [1]. Els majors productors d'arròs a Europa són Itàlia (50,3%), Espanya (33,6%), Grècia (6,6%), Portugal (5,2%) i França (4,4%) [1]. Curiosament, un 74,6% del gra que es produeix a Europa és rodó o mitjà (tipus *japonica*) [6], en contraposició a la producció mundial que majoritàriament és de gra llarg (tipus *indica*).

Tanmateix, la principal característica del cultiu d'arròs a Europa és que la majoria dels arrossars es localitzen en la proximitat de zones protegides i espais naturals de gran interès ecològic. Per exemple, en la zona mediterrània trobem cultius d'arròs al Delta de l'Ebre, l'Albufera de València les Marismes de Doñana, la conca del Po a Itàlia i a la Camarga francesa.

Actualment existeixen legislacions adaptades a les zones arrosseres protegides d'Europa, per tal d'evitar la contaminació dels ecosistemes limítrofs. Tanmateix, hi ha alguna diferència en criteris de permissibilitat entre els diferents països. Per exemple, a Catalunya s'apliquen des de 1998 els mètodes de producció agrària compatibles amb la protecció mediambiental de zones humides incloses en el llistat de la convenció de RAMSAR [7]. Així es prioritza la substitució i reducció de tractaments químics en la lluita contra plagues, amb la finalitat de protegir les aus i les espècies aquàtiques [8].

La gran importància mundial del cultiu d'arròs l'ha convertit en una espècie vegetal especialment tractada per la ciència. A més, l'arròs és la planta model de recerca genètica en cereals donada la petita mida del seu genoma (450 Mb, el més petit dels cereals), seqüenciat des del desembre del 2004 [9]. La constant millora varietal de l'arròs inclou aspectes com augmentar el rendiment i la qualitat del gra, la resistència a insectes i malalties, així com una major tolerància a l'estrès causat per factors com la sequera, la salinitat, el fred i la deficiència de nutrients [10]. En aquest sentit, la biotecnologia es considera una gran eina per aconseguir-ho.

La biotecnologia en l'arròs passa per la utilització de tècniques de cultiu de teixits i de biologia molecular. El cultiu de teixits com ara son les cèl·lules en suspensió, protoplasts i calls derivats de diferents explants permeten incrementar i accelerar la selecció d'encreuaments varietals, establint més ràpidament les noves línies. Per altra banda, la utilització de marcadors moleculars i altres eines biotecnològiques ajuden a accelerar el programes tradicionals de millora genètica.

Una altra de les contribucions en el camp de la biotecnologia es basa en l'addició de gens aliens al genoma de l'arròs per enginyeria genètica. Un gran nombre de gens que confereixen una ampla varietat de trets han sigut transferits amb èxit a diferents varietats d'arròs. Entre el 1998 i 2008 s'han dut a terme 35 assajos de camp oficials emprant varietats d'arròs modificat genèticament a la Comunitat Europea, mentre que als Estats Units en varen ser 264 entre l'any 1990 i el 2010 [11].

Donada la importància econòmica i ecològica de l'arròs arreu del món, el flux genètic des de l'arròs modificat genèticament cap a les varietats convencionals o varietats salvatges és una de les qüestions més importants pels ecologistes degut al risc associat a la sortida comercial de varietats d'arròs transgèniques [12]. Cal estudiar en profunditat el flux genètic de transgens via pol·len en arròs abans que se n'autoritzi el cultiu de transgènic per evitar problemes de convivència amb cultius no transgènics, així com l'efecte de l'arròs salvatge en la transmissió d'aquests transgens.

La variabilitat natural havia sigut la font de variabilitat en la genètica clàssica, més recentment es va obtenir una font de variabilitat amb el desenvolupament de les tècniques de cultiu *in vitro* i, sobretot, amb el procés de cultiu de calls derivats d'explants vegetals. Però és la producció de poblacions de mutants mitjançant agents químics la més potent i actual. La variabilitat en poblacions mutants o naturals, combinada amb sistemes *High-Throughput* de detecció enzimàtica de mutacions, han permès la identificació de nous al·lels mutants. Gràcies a això, la genòmica funcional ha avançat molt ja que permet estudiar la funció de molts gens orfes identificats per la seqüenciació del genoma de l'arròs. Però també s'han obtingut d'al·lels mutants que es poden emprar per la millora de varietats ja que afecten caràcters tant importants com el rendiment, la qualitat del gra, resistències a estressos abiòtics o biòtics, barreres reproductives, epigenètica i temps de floració entre d'altres [13]. Com hem comentat abans, la producció d'arròs a Europa està associada a zones protegides i zones humides de gran

interès ecològic, però les diferents legislacions no fan restriccions en l'ús de cultius millorats mitjançant mutagènesi. Un exemple són les varietats *Clearfield* resistents a herbicides obtingudes mitjançant la introgressió d'al·lels mutants, aquests al·lels mutants han estat obtinguts mitjançant la selecció amb herbicida sobre poblacions d'arròs mutants que s'han obtingut per mutagènesi química. Per tant, aquestes varietats mutants resistents a herbicides no són considerades organismes modificats genèticament. La Cooperativa de Productores de Semillas de Arroz, S.C.L. (Cosemar, SCL) està produint i comercialitzant varietats mutants tolerants a herbicida sota llicència per la distribució a l'estat Espanyol. La resistència a l'Imazamox d'aquestes varietats Clearfield es transmet ràpidament a l'arròs vermell o "assalvatjat" segons s'ha detectat a diferents països [14-16]. És una paradoxa que aquest sistema que s'empra per eliminar males herbes i en especial l'arròs "assalvatjat", crea en poc temps plantes d'arròs "assalvatjat" resistent a l'herbicida.

Els actuals mutàgens químics són capaços de causar petites deleccions i canvis de base útils per l'estudi de la genòmica funcional, i han permès la generació de col·leccions de mutants saturades en les quals hi ha individus amb al·lels mutants per gairebé tots els gens del genoma de les plantes models. S'han millorat molt les tècniques per detectar mutants en gens d'interès, però és la detecció enzimàtica de falsos aparellaments la que ha promogut, junt amb els garbellats *High-Throughput*, la detecció dels individus que contenen aquestes mutacions d'interès [17-20].

Un cop es disposa de variabilitat genètica d'origen natural o artificial, el següent pas per la millora de varietats és la selecció i estabilització dels caràcters desitjats. A l'encreuament o material genètic subjecte a mutacions se li aplica una selecció que pot basar-se en diferents mètodes [10]. El sistema tradicional es autofecundar o retrocreuar híbrids i seleccionar-ne els caràcters desitjats durant cinc a sis anys fins que s'elimina la heterozigosi residual i s'obté una línia estable i homogènia [21]. L'arròs és una planta autògama i tradicionalment se'n cultiven

línies estables seleccionades que presenten un grau mínim d'heterozigosi residual. Existeix una llei espanyola que protegeix el dret dels agricultors de poder sembrar llavor descendent de la llavor certificada [10]. Els milloradors i els programes de producció de llavor certificada treballen per garantir aquesta puresa genètica. Tot i així, quan una varietat d'arròs es cultiva durant anys, els trets agronòmics poden començar a diferir dels originals com a resultats de mutacions naturals i creuaments espontanis entre varietats deguts a contaminació durant la recol·lecció amb segadores mecàniques, el transport, l'assecat i l'emmagatzemament de llavors, entre d'altres factors [22].

El principal objectiu de la Tesi Doctoral és l'estudi de diferents eines biotecnològiques i la seva aplicació a la millora de varietats mediterrànies d'arròs. Per tal d'assolir aquest objectiu la tesi recull alguns dels resultats obtinguts al llarg de molts anys d'investigació i desenvolupament biotecnològic aplicat a la millora de varietats hispanes d'arròs com ara son la tecnologia transgènica, el cultiu d'anteres i l'obtenció i detecció molecular de mutants.

Aquesta Tesi Doctoral es divideix en cinc apartats que inclouen: Introducció General, Objectius, Resultats, Discussió General i Conclusions. A la Introducció General es descriuen les bases teòriques i els coneixements actuals associats als diferents camps d'estudi. L'apartat de Resultats inclou tres capítols, els quals han estat publicats a revistes internacionals especialitzades.

El Capítol 1 estudià el flux de gens entre l'arròs vermell, arròs resistent a herbicides i arròs convencional per determinar-ne les taxes de flux genètic en relació a les distàncies i les orientacions espaials. D'aquí se'n poden extreure les distàncies mínimes de seguretat en un hipotètic marc de convivència entre camps convencionals i transgènics en el Delta de l'Ebre. Per aconseguir els objectius d'aquest primer capítol es van dissenyar dos assajos de camp amb plantes transgèniques resistents a herbicides, plantes convencionals i arròs vermell o "assalvatjat". La llavor obtinguda es va analitzar mitjançant tractament amb herbicida per així

detectar i estimar un valor de flux genètic a diferents distàncies i orientació espacial. A més es va estudiar la incidència del flux genètic en l'aparició de plantes d'arròs "assalvatjat" transgèniques.

El Capítol 2 estudia i aplica un protocol ràpid i eficient per a la millora d'una varietat tradicional d'arròs del Delta de l'Ebre mitjançant cultiu d'anteres *in vitro*. S'obtenen línies dihaploids estables i es seleccionen, primer en hivernacle i, posteriorment, en assajos de camp a petita, mitjana i gran escala. Se n'obtenen línies noves d'arròs de les que actualment se n'han registrat quatre i se n'estan comercialitzant dues a diferents zones arrosseres peninsulars.

Capítol 3 desenvolupa un protocol ràpid per a l'obtenció de mutants amb interès agronòmic en una varietat comú que es cultiva a Sevilla. Es tracta d'un protocol de mutagènesis sobre call derivat de llavors madures que permet estalviar temps en la obtenció de poblacions de mutants saturades, i obtenir-ne mutants d'interès mitjançant un garbellat molecular.

Els resultats d'aquesta Tesi Doctoral van detectar que les distàncies de seguretat establertes per altres investigadors d'arreu del món en assajos de camp sobre flux genètic en arròs, menystenien l'efecte de l'arròs "assalvatjat", el qual és un factor de propagació de transgens en quant que ràpidament apareixen individus transgènics "assalvatjats" que comprometen la possible convivència entre cultius transgènics i convencionals (Capítol 1).

El protocol desenvolupat per obtenir línies dihaploids millorades a partir de varietats tradicionals ha generat el registre de 4 noves línies millorades, dues d'elles comercialitzades (Capítol 2).

El protocol de mutagènesi de call derivat de llavors madures ha permès escurçar en més de vuit mesos la obtenció de poblacions mutants i ha permès identificar individus mutants en gens d'interès que han estat assajats en camp (Capítol 3).

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
AFLP	Amplified Fragment Length Polymorphism
BB	Bacterial Blight
BCE	Before Common Era
BOE	<i>Boletín Oficial del Estado</i>
bp	Base pair
Bt	<i>Bacillus thuringiensis</i>
CAPS	Cleaved Amplified Polymorphic Sequences
CE	Common Era
CL	Clearfield
CMS	Cytoplasmatic Male Sterility
CTB	Cholera Toxin B
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAF	DNA Amplification Fingerprint marker
DALP	Direct Amplification of Length Polymorphisms
DARP	<i>Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural</i>
DEB	Diepoxybutane
DH	Doubled Haploid, Dihaploid or Haplodiploid
DNA	Deoxyribonucleic Acid
EAS	<i>Estación Arrocera de Sueca</i>
EEE	<i>Estació Experimental de l'Ebre</i>
EFSA	European Food Security Authority
EGMS	Environmental Genetic Male Sterility
EMS	ethyl methyl sulfonate
ESTs	Expressed Sequence Tags
EU	European Union

F1	First filial generation
FAO	Food and Agriculture Organization of the United Nations
FCE	Fennel Crude Extract
GBS	Genotyping-by-Sequencing
GM	Genetically Modified
GMO	Genetically Modified Organism
GNA	<i>Galanthus nivalis</i> Agglutinin
GUS	β -Glucuronidase
HI	Harvest Index
HR	Herbicide Resistant
HYVs	High-yielding Varieties
IAEA	International Atomic Energy Agency
INIA	<i>Instituto Nacional de Investigaciones Agronómicas</i>
IRIC	International Rice Informatics Consortium
IRRI	International Rice Research Institute
Kb	Kilo base
Kg	Kilo gram
MAGRAMA	<i>Ministerio de Agricultura, Alimentación y Medio Ambiente</i>
MAS	Marker-assisted Selection
Mb	Mega base
MAB	Molecular Marker-assisted Breeding
MABC	Molecular Marker-assisted Backcrossing
NAA	Naphthaleneacetic Acid
NERICA	New Rice for Africa
NGS	Next Generation Sequencing
NMU	N-Methyl Nitrosurea

PEPC	Phosphoenolpyruvate Carboxylase
PGMS	Photoperiod-sensitive Genetic Male Sterility
PRF	Putative Reverse Flow
RAPD	Randomly Amplified Polymorphic DNA marker
RFLP	Restriction Fragment Length Polymorphism
RIL	Recombinant Inbred Line
RNA	Ribonucleic Acid
RNAi	Interference RNA
ROS	Reactive Oxygen Species
RuBisCO	Ribulose 1, 5-Bisphosphate Carboxylase/Oxygenase
SCAR	Sequence Characterised Amplified Region
sgRNA	Single guide RNA
SNPs	Single Nucleotide Polymorphism
SSRs	Simple Sequence Repeats or microsatellites
TE	Transposable Element
TGMS	Temperature-sensitive Genetic Male Sterility
TILLING	Targeting Induced Local Lesions IN Genomes
USA	United States of America
UV	Ultraviolet light
WARDA	West Africa Rice Development Association (Africa Rice Centre)

General introduction

1 Agriculture and human population

The world's population probably never exceeded 15 million inhabitants before the Neolithic revolution, when tribes of hunter-gatherers started their shift towards a sedentary and agrarian society. The invention of agriculture is thought to have first happened in the Fertile Crescent of West Asia around 10,000 BCE [23] and soon after, started independently in other regions [24, 25] (Fig. 1).

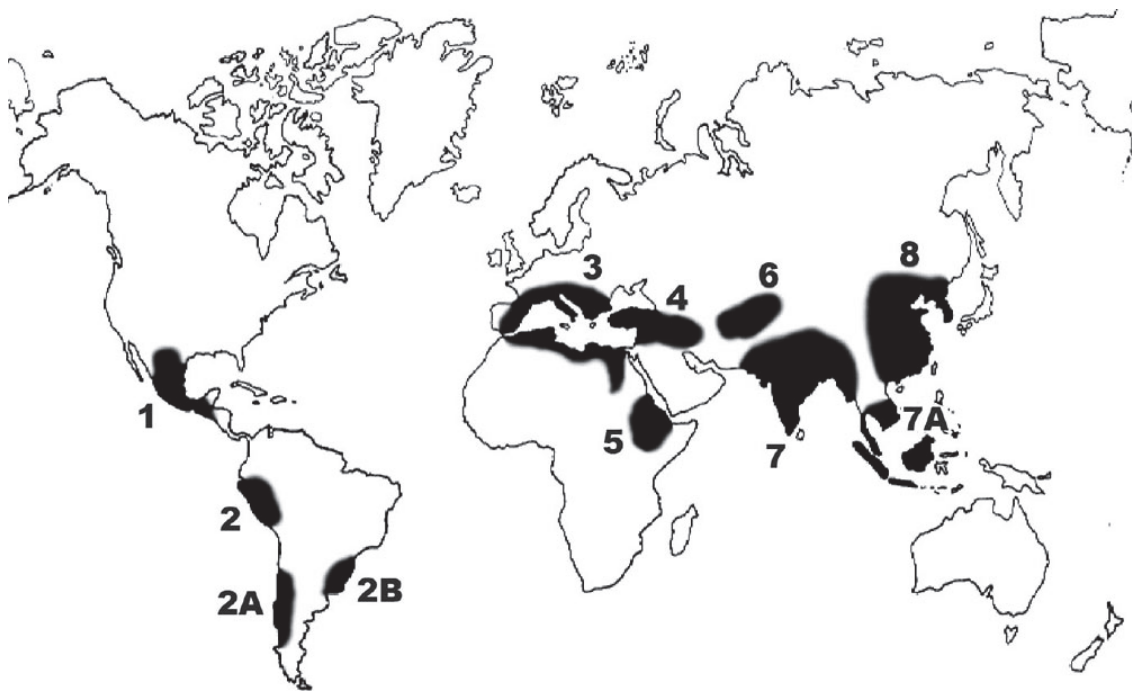


Figure 1. Proposed zones of agriculture origin: (1) Mexico-Guatemala, (2) Peru-Ecuador-Bolivia, (2A) Southern Chile, (2B) Southern Brazil, (3) Mediterranean, (4) Middle East, (5) Ethiopia, (6) Central Asia, (7) Indo-Burma, (7A) Siam-Malaya-Java, (8) China. Source [26].

From these regions, agriculture was progressively disseminated to other regions, including Europe and North America [27]. Over time, in the regions where agriculture and animal husbandry appeared, the number of human beings grew at an unprecedented rate.

Beginning about 3,000 BCE in Mesopotamia, a far more complex way of life began to appear when humans learned how to increase harvests through irrigation and other methods. Much larger populations came together in towns and cities, thanks to the substantial surpluses from farmers and stockbreeders beyond their own needs. They developed writing, specialized occupations emerged, complex religions took form, and social divisions increased. These changes marked the birth of civilization [28] and consequently world population increased reaching 200 million around 0 CE (Fig. 2).

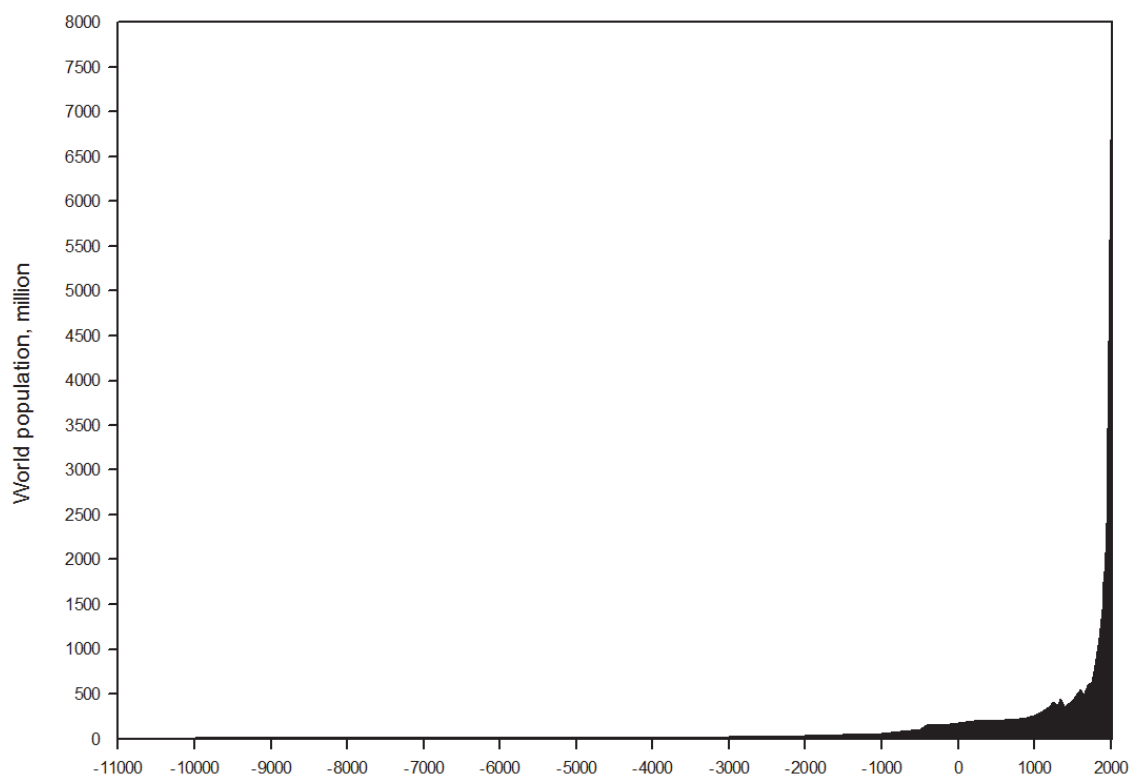


Figure 2. World population since 10.000 BCE until 2015 CE. Data source: FAOSTAT [1].

In the Middle Ages, clearing of woodlands, draining of wetlands, culture rotations, and the improvement of tools such as axes, adzes, and bill-hooks increased the crop production up to 50% [29]. Since 1600 CE new technical advances enhanced productivity, such as the wooden plough which was late improved by the addition of iron and steel parts.

By the early 19th century, world's population reached 1 billion people (Fig. 2) when agricultural techniques, implements, seed stocks and cultivars had so improved that yield per land unit was many times that seen in the Middle Ages. With the rapid rise of mechanization in the late 19th and 20th century, farming tasks could be done with a speed and on a scale never seen before. These advances had led to efficiencies enabling certain modern farms to huge output volumes of high-quality production per land unit at what may be the practical limit. Between 1700 and 1980, the total area of cultivated land worldwide increased 466% and yields increased dramatically, particularly because of selectively bred high-yielding varieties, use of fertilizers and pesticides, new irrigation systems, and more advanced machinery [30]. It is estimated that the two billionth baby was born in 1930, while the three billionth baby was born less than three decades later, in 1959 (Fig. 2).

The so called Green Revolution has improved world's crop productivity since the 1940s through massive public investments in modern scientific researches in agriculture [31]. The use of chemical fertilizers and pesticides, improved agronomy and the development of High-yielding Varieties (HYVs) through modern plant breeding techniques, soon gave an unprecedented level of national food security in developed countries [32]. The Green Revolution lifted large numbers of poor people out of poverty and helped many people avoid the poverty and hunger they would have experienced had the Green Revolution not occurred. This reduction of poverty contributed to increase the world demography, reaching four billions in 1974 [33] and 5 billion inhabitants in 1987 (Fig. 3).

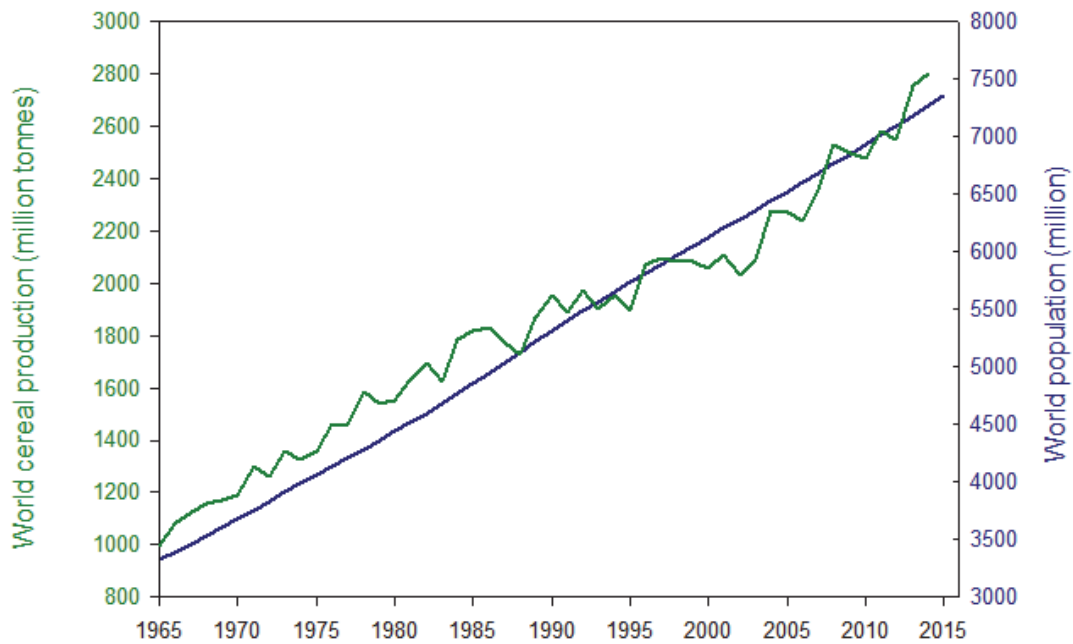


Figure 3. World population and cereal production since 1965 until 2015 CE. Data source: FAOSTAT [1].

Today world's population is 7.4 billion and expected to reach 8 billion in 2024, 9.7 in 2050 and 11.2 in 2100 according to a new UN DESA report [34]. Demographic pressures, climate change, and the increased competition for land and water are likely to increase vulnerability to food insecurity.

The Green Revolution brought negative environmental impacts that are still being adequately addressed. In too many places, yield increase is associated with management practices that have degraded the land and water systems upon which the production depends. In some of these areas, the environmental impacts accumulation in key land and water systems has now reached the point where yield could be seriously compromised. Moreover, climate change is already altering the patterns of temperature, precipitations and river flows upon which agricultural systems depend. Furthermore, arable land salinization is a growing problem in more than 100 countries [5]. The need to promote various strategies aimed at increasing potential rice yields and avoiding climate change effects enhanced plant biotechnological research and the

development of genetically modified (GM) crops [35, 36], but the question now is: can Science and Technology sustain an Evergreen Revolution leading to long-term increases in productivity without any associated ecological harm [37, 38]?

2 Plant breeding

The key change on cereal domestication, result of cultivation, was the loss of natural seed dispersal, which led to domesticated cereals with dependence on humans [39, 40]. Although selection in initial domestication seems not to be a conscientious act, the plant breeding experiments were then, most likely limited to selecting the most viable specimens from each harvest for subsequent sowing [24], which nevertheless had a profound impact on crop yield. This selection also altered the plants in new ways, since human selection was often opposite to natural selection [41]. It was realised early, that domesticated plants were not to be considered “natural” and Charles Darwin coined the term “artificial selection” in 1859 to emphasize the difference between selection in nature and human-made selection [42].

2.1 Plant breeding

Gregor Mendel devised the scientific foundation for plant breeding leading to its explosive impact over the past 150 years and to the science of genetics. Classical plant breeding uses deliberate interbreeding (crossing) of closely or distantly related individuals to produce new varieties or lines with desirable properties. This selection was initially submitted to phenotypic selectable traits. Although the traditional breeding process can take several years to cross plants and select an improved variety, Luther Burbank (1849-1926) developed over 800 different varieties of fruits and vegetables using classical plant breeding methods. Following World War II, a number of techniques were developed that allowed plant breeders to artificially induce genetic diversity by mutation breeding, induced polyploidy, and the addition or removal of chromosomes using a technique called chromosome engineering.

During Green revolution, the High yielding Varieties (HYVs) were obtained as a result of classical breeding and pedigree selection within cultivars. These new varieties were highly human

dependent because needed high amounts of fertilizers, pesticides and improved watering methods and agrarian machinery to achieve their yield potential.

Plant breeding is gradually evolving from art to science over the last 10,000 years, starting as an ancient art to the present molecular design-based science. Modern plant breeding uses techniques of molecular biology to generate, select or in the case of genetic modification, insert desirable traits into plants. Sequencing data for many plants is now available in databases. Over 86 plant species including many important crops have been sequenced to date [43]. The next challenge is to determine the junction of every gene and eventually how genes interact to form the basis of complex traits.

2.2 Molecular marker-assisted plant breeding

One of the main advances in plant breeding was the progressive replacement of traditional phenotype-based selection by molecular marker-assisted selection (MAS). MAS is an indirect selection process for a trait of interest based on genetic markers linked to this trait. As the selection is not based on the phenotypic trait, but on linked markers, large populations of plants can be screened at very early development stages. Its main application in crop breeding are the marker-assisted breeding (MAB) programs and the marker-assisted backcrossing (MABC) programs. MABC accelerates and simplifies the introgression of a trait from a donor line into the genomic background of a recipient line, while MAB is used to improve the selection of lines derived from a single cross.

Plant molecular breeding has advanced so rapidly that several types of molecular markers have been developed and used for decades. These markers are based generally on restriction and/or amplification techniques. The restriction fragment length polymorphism (RFLP) was firstly applied as DNA markers in plant genotyping [44]. RFLP technique is useful in the construction of genetic

linkage maps, but it is challenged by the complicated hybridization, radioactivity, being time consuming and having limited number of available probes [45]. With further advance of biotechnology, several types of PCR-based markers were developed and used in plant breeding programs. These PCR-based markers mainly include random amplification of polymorphic DNA (RAPD) [46], sequence characterized amplified region (SCAR) [47], cleaved amplified polymorphic sequences (CAPS) [48], simple sequence repeats (SSRs) [49, 50], amplified fragment length polymorphisms (AFLPs) [51], and direct amplification of length polymorphisms (DALP) [52]. Compared to RFLP, the PCR-based markers are amplified from individual genomic sequences under a small scale, relatively inexpensive and less time-consuming [53] being the most commonly used DNA-based markers.

In combination with the genome and expressed sequence tags (ESTs) in model plants species [54], Sanger sequencing throughput was improved to accelerate the identification of variations at the single base pair resolution [55]. The use of single nucleotide polymorphisms (SNPs) [56] as DNA markers for plant genotyping has increased the potential to score variation in specific DNA targets.

Advances in Next Generation Sequencing (NGS) have significantly lower the costs of DNA sequencing to the point that Genotyping-by-Sequencing (GBS) is now feasible for high diversity and large genome species [57]. GBS is a simple highly multiplexed system for constructing reduced representation libraries for the Illumina NGS platform [57], that provides a rapid and low-cost tool to genotype breeding populations, allowing plant breeders to implement genomic diversity study, genetic linkage analysis, molecular marker discovery, and genomic selection under a large scale of plant breeding programs.

2.2 Mutation Breeding

Exploiting natural or induced genetic diversity is a proven strategy for major food crops improvement. Whilst mutations occur spontaneously in nature, their frequency is too low to rely on alone for accelerated plant breeding. Historically, the use of mutagenesis in breeding has involved forward genetic screenings and the selection of mutant individuals with improved traits and their incorporation into breeding programmes. Over the past 70 years, more than 2,500 mutagenesis-derived varieties have been released, as listed in the Joint between FAO and the International Atomic Energy Agency (FAO/IAEA) mutant variety database [58].

Reverse genetic approaches have allowed silencing or interrupting individual candidate genes, providing the opportunity to study gene function and relate sequence information to traits. However, these methods are likely to result in the complete disruption of gene function rather than in generating allelic series of mutants with partial loss-of-function, and thus will not produce the range of mutation strengths necessary for crop improvement. Nevertheless, much of our understanding of higher organism's genetics is based upon studies utilizing induced mutations for analysing gene function.

With the recent expansion of sequence databanks, locus-to-phenotype reverse genetic strategies have become an increasingly popular alternative to phenotypic screening for functional analysis. Sequence information alone may be sufficient to consider a gene to be of interest, because sequence comparison tools that detect protein sequence similarity to previously studied genes often allow a related function to be inferred. However, hypotheses concerning gene function that are generated in this way must be confirmed empirically. Experimental determination of gene function is desirable in other situations as well, for example, when a genetic interval has been associated with a phenotype of interest. In such cases, the functions of genes in an interval can be inferred by using reverse genetic methods.

Plant breeders can generate genetic diversity within a species by exploiting a process called somaclonal variation, which occurs in plants produced from tissue culture, particularly plants derived from *callus*, although chemical or physical mutagenesis have a number of advantages over such approach. Mutagens are applicable to all plant and animal species and they can introduce random changes throughout the genome, generating a wide variety of mutations in all target genes, and a single plant can contain a large number of induced mutations in many different genes, resulting in manageable population sizes. Although this approach has been proved very successful, there are limitations imposed by the difficulty of identifying a small number of individuals with novel phenotypes within a large population, or by the genetic redundancy present in many plant species as a result of gene duplication and polyploidy, such that many mutations have no detectable effect on the plant.

Mutations can be induced by irradiation with non-ionizing (e.g. UV) or ionizing radiation; such physical mutagens often result in the larger scale deletion of DNA and changes in chromosome structure. By contrast, chemical mutagens most often only affect single nucleotide pairs. The degree of mutation is dependent on the tissue and degree of exposure (dosage \times time). Alkylating agents, who yield predominantly point mutations, have been especially valuable, since the resulting altered and truncated protein products help to precisely map gene and protein function. Furthermore, mutations at single nucleotide pairs are generally of the relevant interest to breeders because large-scale changes to chromosome structures usually have severely negative results.

Therefore, a relatively small population of individuals can provide an allelic series that includes a variety of missense changes with different effects on protein function, and nonsense or splice site changes that cause truncation of the gene product. Mutations are commonly identified by TILLING (Targeting Induced Local Lesions IN Genomes) which is based on enzymatic cleavage of PCR amplified heteroduplexed DNA followed by band visualization using fluorescent end-

labelling and denaturing polyacrylamide gel electrophoresis. The generality of the mutagenesis and the mutation discovery methods allow application of this approach to most organisms. Indeed, TILLING results have been reported for a variety of plants and animals. Chemically induced mutant populations have been performed in different plant species [59] and efficiently screened following TILLING high-throughput screening protocols [17-20] (Fig. 4). Furthermore, since mutant individuals obtained by TILLING do not involve transgenic modifications, they are attractive not only for functional genomics but also for agricultural applications.

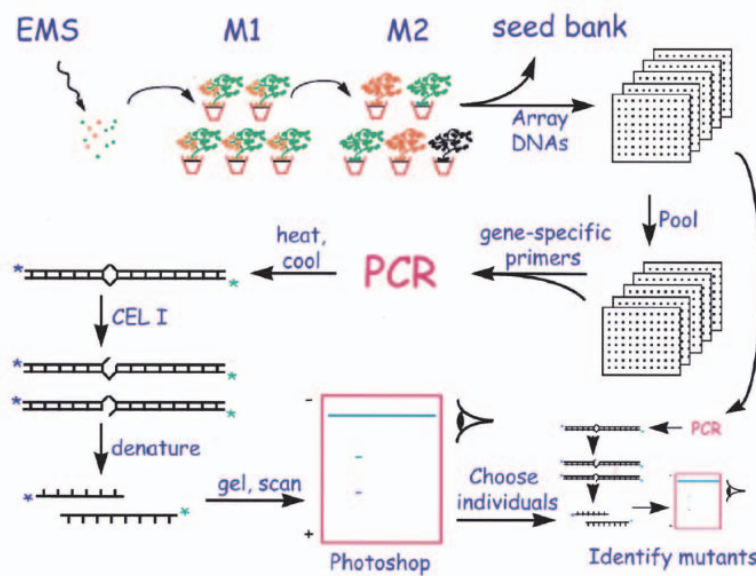


Figure 4. High-throughput TILLING. Seeds are mutagenized with ethylmethanesulfonate (EMS). M1 plants are grown and M2 seeds are sown in order to obtain the M2 generation. M2 DNAs are prepared, quantified, equalized and 8-fold pooled. PCR is performed followed by CEL I treatment, clean-up, gel electrophoresis and scanning [60].

Plant molecular mutation breeding is defined as mutation breeding in which molecular or genomic information and tools are used in the development of breeding strategies and in the implementation of the breeding process. It relies on DNA damage, repair and mutagenesis, plant molecular genetics and genomics of important agronomic traits as well as induced mutations.

Critically, mutations in important traits or genes (e.g. in nutritional quality, resource use efficiency, architecture or phenology) can be readily exploited by plant breeders without the

legislative restrictions, licensing costs, and societal opposition applied to GM approaches. This is despite the fact that transcriptomic analyses have shown that large-scale plant mutagenesis may induce greater changes in gene expression patterns than transgene insertion [61].

2.3 Anther culture and doubled haploids

Haploid plants are sporophytes that contain a gametic chromosome number (n). They can originate spontaneously in nature or as a result of various induction techniques. The potential of haploidy for plant breeding arose in 1964 with the achievement of haploid embryo formation from in vitro culture of *Datura* anthers [62-64], which was followed by successful in vitro haploid production in tobacco [65]. Many attempts have been made since then, resulting in published protocols for over 250 plant species belonging to almost all families of the plant kingdom [66].

Anther culture is an in vitro technique that generates perfectly homozygous lines through engineered meiosis over haploid material, thus permitting rapid production of homozygous doubled haploids (DH) lines from heterozygous lines or hybrids. In fact, under optimal conditions, DH lines have been routinely used in breeding for several decades, although their common use is still limited to selected species. Breeders have long recognized the advantages of DH technologies based on the knowledge that several theoretical and practical aspects of plant biology and genetics can take advantage of haploidy technology [67]. For crop improvement purposes, DH lines are developed mainly to achieve homozygosity in diploid or allopolyploid species, saving several generations in a breeding programme and producing new homozygous cultivars or parental lines for F1 hybrids [68]. While in natural offspring genetic recombination occurs and traits can be unlinked from each other, in DH plants recombination is not an issue since a recombination between two corresponding chromosomes does not lead to un-linkage of alleles or traits.

2.4 Genetic modification

Genetic modification of plants is achieved by adding a specific gene or genes to a plant, or by knocking down a gene using interference RNA (RNAi), to produce a desirable phenotype. The resulting plants from adding a gene are often referred to as transgenic or GM plants. In most cases the aim is to introduce a new trait to the plant which does not occur naturally in this species. The introduction of foreign germplasm into crops was first achieved by traditional crop breeders by artificially overcoming fertility barriers between related plant species.

Cisgenic plants are made inserting alleles found within the same or closely related species, where conventional plant breeding can occur. Some breeders and scientists argue that cisgenic modification is useful for plants that are difficult to crossbreed by conventional means (such as potatoes), and that plants in the cisgenic category should not require the same level of legal regulation as other genetically modified organisms (GMOs).

Nevertheless the most GM plants are generated by the biolistic method (particle gun) or by *Agrobacterium tumefaciens* mediated transformation [69]. The inserted genes can come from species within the same kingdom (plant to plant) or between kingdoms (for example, bacteria to plant). In many cases, the inserted DNA has to be slightly modified for its correct and efficient expression in the host organism. Transgenic plants are used to express proteins, for example the *cry* toxins from *Bacillus thuringiensis* (Bt), herbicide resistant (HR) genes and antigens for vaccinations [70].

Although plant scientists, backed by results of modern comprehensive profiling of crop composition, point out that crops modified using GM techniques are less likely to have unintended changes than those from conventionally bred crops [71-73], cultivating GM crops in an open field overcomes social problem in many developed countries. Both, GM food and GM

crops have been the subject of international trade disputes. Such a dispute arose between the USA and Europe in the early 2000s. Until the 1990s, Europe's regulation was less strict than in the United States [74]. In 1998, a *de facto* moratorium led to the suspension of approvals of new GMO in the European Union pending the adoption of revised rules to govern the approval, marketing and labelling of biotech products. Consequently, Europe has a 'de facto' ban on the approval of new GM crops and relatively small amount of genetically engineered crops are approved and cultivated in different European countries [75]. The approach chosen in the EU regarding GMOs is precautionary and imposes a pre-market authorisation for any GMO and a post-market environmental monitoring for any authorised GMO [76]. Although the newly adopted Directive (EU) 2015/412 gives Member States more flexibility to decide on the cultivation of GM crops, only one GM crop (GM maize –MON 810) is commercially cultivated in the EU, which is cultivated in 5 Member States with a total coverage, in 2013, of almost 150,000 hectares (137,000 of those in Spain). GMOs were cultivated on 175 million hectares worldwide in 2013 (mostly soy, maize, oilseed rape and cotton). For the record: in 2010, a GM starch potato, known as "Amflora" potato, was authorised for cultivation and industrial processing in the EU, although it is no longer authorised in the EU.

3 Origin and domestication of cultivated rice

Rice is a member of the *Gramineae* family and belongs to the genus *Oryza* which was probably originated about 130 million years ago [77]. Different species got distributed into different continents with the breakup of Gondwana land. By the early Holocene (7,000 BCE), Neolithic people in both north and south China may have been harvesting wild *O. nivara* rice and initiating rice cultivation that eventually led to *O. sativa* domestication [77] (Figs. 5 & 6). *O. glaberrima* was probably domesticated in Niger River delta from *O. breviligulata* wild annual ancestor.

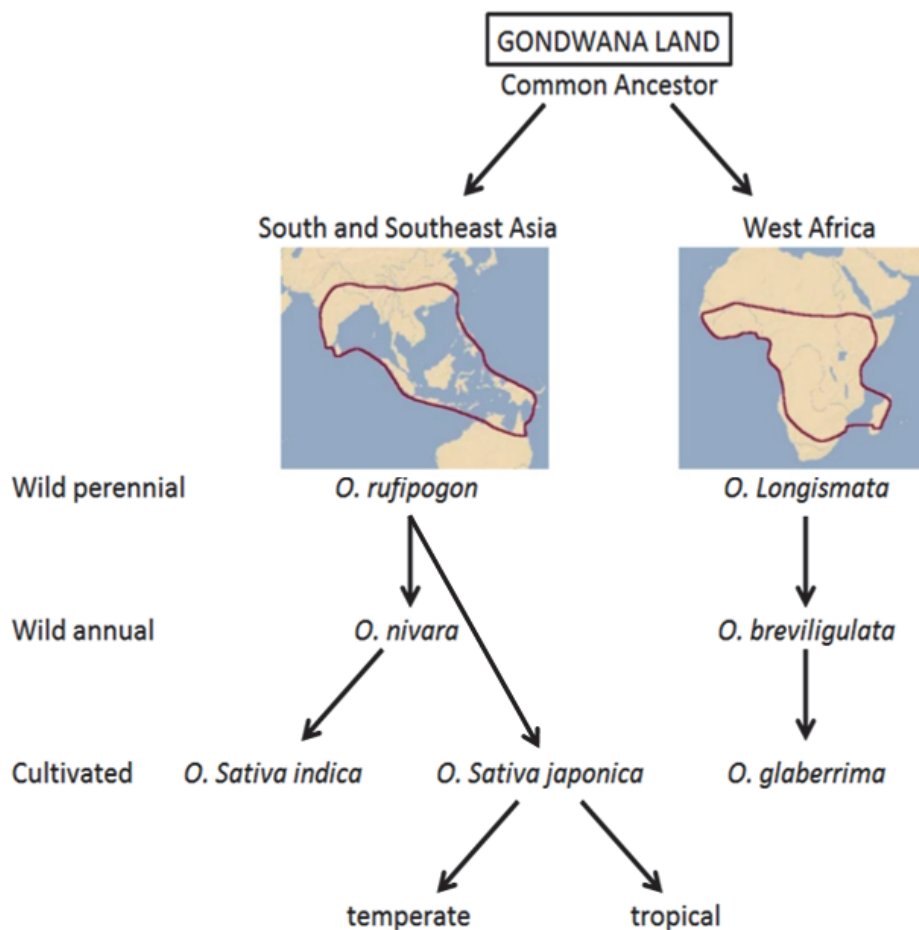


Figure 5. Evolutionary pathway of cultivated species of rice [77].



Figure 6. Panicles of wild rice *Oryza rufingopon*. Photograph credit: Pu Huang (*O. rufingopon*) [78].

The genus *Oryza* includes 20 wild species and the 2 cultivated species: *O. sativa* and *O. glaberrima*. Most of the *Oryza* species, including the rice cultigens, are diploid ($2n=24$) while nine are tetraploid ($2n=48$). Both cultivated species were originated from a common ancestor with AA genome, while the others have an AA, BB, CC, BBCC, CCDD, EE, FF, GG, HHJJ and HHKK genome. Incompatibility exists among species having different genomes. Partial sterility also shows up in hybrids when different ecogeographic races of *O. sativa* are hybridized.

Varieties of *O. sativa* are classified into six groups on the basis of genetic affinity. The rice subspecies of *O. sativa*, *indica* and *japonica*, had a polyphyletic origin. *Indica* rice (long grain) correspond to group I and *japonica* (short grain) to group VI. *Indica* rice was probably domesticated in the foothills of Himalayas in Eastern India whereas *japonica* somewhere in

South China. The so called *javanica* rice also belong to group VI and is designated as tropical *japonicas* although presenting long grain phenotype.

Wild species are widely distributed in the humid tropics and subtropics of Africa, Asia, Central and South America, and Australia [79]. Furthermore, new wild *O. sativa* conspecific relatives (weedy rice) have appeared in cultivated fields as result of spontaneous hybridization between cultivated rice and wild species (Fig. 7). These new wild rices are classified as *O. sativa f. spontanea* and have evolved overcoming one of the most notorious weeds found in rice planting areas through the world [80].

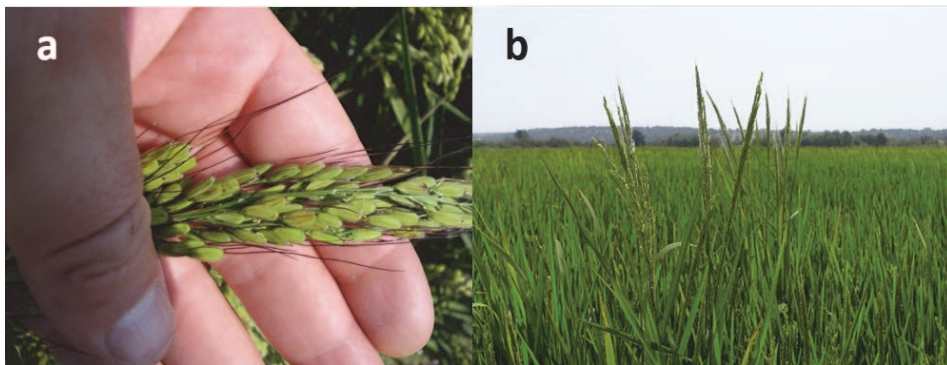


Figure 7. Weedy red rice panicle (a) and tall headed weedy red rice plants among conventional rice (b) in Ebro river delta.

The cultivated species of *Oryza* may be classified as semiaquatic plants, although extreme variants are grown not only in deep water (up to 5 meters) but also on dry land [81]. African rice (*O. glaberrima*) is confined to West Africa, whereas common or Asian rice (*O. sativa*) is now commercially grown in 112 countries covering all continents except Antarctica between 55 °N and 36 °S latitudes [82]. It is grown under diverse growing conditions (irrigated, rainfed lowland, rainfed upland and floodprone ecosystems) which has led to numerous cultivars and is believed that about 120 000 varieties of rice exist in the world.

Among the cereals, rice has the lowest water use efficiency. Therefore, rice cannot compete with dryland cereals in areas of low rainfall unless irrigation water is readily available from

reservoirs, bunds, and the like. On the other hand, the highest yields of traditional varieties have been obtained in regions of high irradiation (i.e. clear or cloudless skies), such as in Spain, California, and northern Japan [83].

O. sativa, the Asian cultivated rice, is one of the most important foods of the world's population providing more than one fifth of the calories consumed. The averaged rice consumption is about 65 Kg per person/year. In Asia, the consumption is higher than 80 Kg per person/year, in subtropical regions (Africa and Mesoamerica) is about 30 to 60 Kg per person/year, and in industrialized countries (Europe and USA) is less than 10 Kg per person/year [84]. The FAO's forecast of global rice production is 741,3 million tonnes in 2014 [6]. It is the second cereal grain in terms of production, after maize. Nevertheless, about 85% of rice production is used for human consumption [3], while maize production is substantially dedicated to other proposes like industry supply and animal consumption.

Rice production is still increasing thanks to technological advances, rather than hectare increase which is almost constant (164 million ha). Averaged world's rice yield is 4.3 tonnes per hectare (T/ha), although it varies from 0.75 T/ha in Congo's rainfed production and 9.5 T/ha in Australia using irrigated and highly mechanized farming. One to four yields a year can be obtained depending on climate conditions.

4 Rice in Europe

The Romans learned about rice during the expedition of Alexander the Great to India (c. 327—4 BCE), although they imported rice wine instead of growing the crop [83]. Nevertheless, large deposits of rice from the first century CE have been found in Roman camps in Germany [85]. The introduction of rice into Europe could have taken different routes although direct imports from various parts of Asia into Europe are also probable [83].

The extended rice cultivation in Europe was first practiced in the Cordoba Emirate wetlands (Iberian Peninsula; nowadays south of Portugal and Spain), concretely in Guadalquivir marshy lowlands (*marismas*), Guadiana estuary and “L’Albufera” lagoon in Valencia coast, just after the Islamic conquest (9th century) by the Moors (Fig. 8).

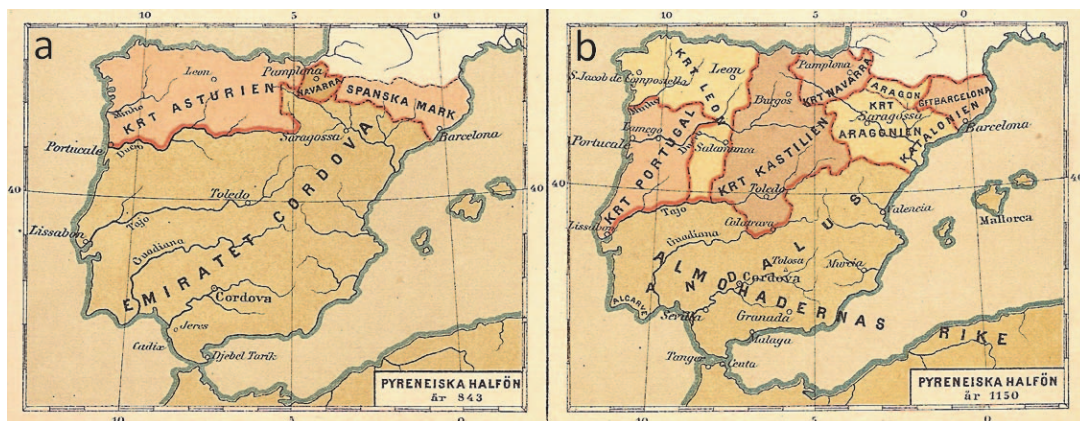


Figure 8. Historic map over the Iberian peninsula 843 CE (a) and 1150 (b) [86].

During the 12th century the Iberian Peninsula was reconquered and Spanish Christians took over the cultivation. After the 15th century, rice spread throughout Italy, then France and later to continents with no rice production, during the age of European exploration.

In 2014, paddy rice production in the European Union was 2.87 million tonnes grown in 431,062 ha (*ca.* 6.66 tonnes per hectare), mainly in Mediterranean basins [1]. The major European rice

production countries are Italy (48.26%), Spain (30.07%), Greece (9.38%), Portugal (5.64%) and France (2.9%) [1]. Rice production areas and natural protected areas having high ecological interest are highly related in Europe, such as Po Delta in Italy, La Camargue in France and Ebro Delta, *Albufera* lagoon and *Doñana* marshy lowlands in Spain. In Europe there are laws adapted to rice cultivation in protected areas to avoid contamination of surrounding ecosystems. Around two-thirds of the rice consumed by European citizens is grown in the European Union [87]. The other third is supplemented by imports of different varieties, mainly long-grain *indica* rice such as basmati from India and Pakistan. A small quantity of European produced rice (mainly *japonica*) is exported [87].

In contrast to world rice production which is mainly long grain *indica* rice, rice varieties grown in Europe mostly belong to the *japonica* group, initially associated with round to medium-long grains. Local specialty varieties are highly appreciated in local markets, especially when they are associated with an Appellation of Protected Origin emphasizing their local origin and the environment-friendly specification of the cropping practices. However, demand for long *indica*-type grain, for exotic specialty rice such as Basmati and Jasmine rice is rapidly growing with the increase in rice consumption in Europe, 6% per year. Furthermore, Spanish and Italian markets consider that pearly rice has an added value, in contrast to the international one which prefers crystalline rice and considers pearly rice as a defect confusing it with chalky rice [10] (Fig. 9).

The rice pearl is a white spot in the centre of the rice grain which that allows the absorption of flavours when cooking it with other ingredients, while chalky grain occurs when the shape, size, and packing of amyloplasts and cells in chalky grains are affected by high temperatures [88].

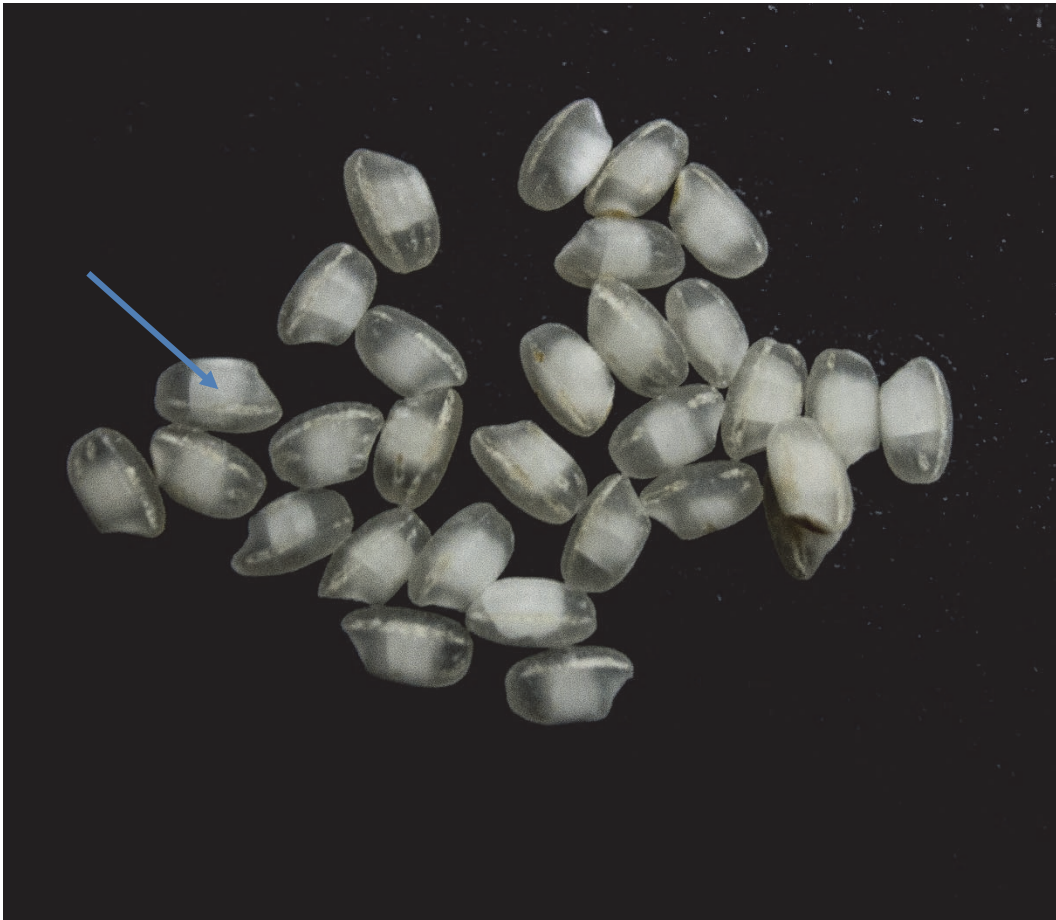


Figure 9. Characteristic pearl of Montsianell rice grains. Blue arrow indicate the pearl. Source: Camilo López-Cristoffanini.

5 Rice in Spain

In Spain, rice culture and cultivation is secular, being introduced by the moors around the 9th century in the Guadiana estuary, *l'Albufera* lagoon, *Doñana* marshy lowlands, the marshy lowlands in Majorca island (*s'albufera*) [89], and extended in 1860 to Tarragona in Ebro Delta. In 1930 rice cultivation was re-introduced in *Doñana* marshy lowlands, and from 1962 in other inner regions such as Don Benito and Miajadas in Extremadura. Nowadays, the main production areas in order of importance are: *Doñana* marshy lowlands in Seville, Ebro Delta in Tarragona, *Albufera* lagoon in the Valencia coast, Jucar Delta and the irrigated lands of Guadiana River in Extremadura.

The Rice Station of Sueca in Valencia (*Estación Arrocerca de Sueca*, EAS) opened in 1913 [90] to assist the Federation of Planters in improving rice production against recurrent famines originated by blast (*Pyricularia oryzae*) disease. Before 1927, Spanish varieties improvement consisted in mass selection of imported varieties [91]. Since then, the Spanish rice varieties have been also obtained through genealogic selection of ancient Spanish varieties and artificial breeding [92].

In 1940, after the Spanish Civil War, the EAS was integrated into the *Instituto Nacional de Investigaciones Agronómicas* (INIA; National Institute for Agricultural Research) [93]. Public investments and rice monopoly controlled by the dictatorial government helped the new artificial breeding varieties to displace traditional varieties and acclimatized foreign varieties [90]. In the 60's new semi-dwarf varieties were obtained to respond to the new production techniques based on mechanization [94].

In 2000, rice varieties obtained by breeding coupled to genealogic selection started to compete with others obtained through MAS. This technique helped plant breeders to obtain new varieties

through a more precise and faster trait selection, and consequently, new genetically improved rice varieties appeared, reducing cultivar lifetime in the Spanish market [95, 96].

In Spain, the rice production is affected negatively by biotic stresses. The worst biotic stress is caused by the fungal infection of *Pyricularia oryzae*, although other fungus species such as *Helminthosporium spp.* and *Fusarium oryzae* are also relevant. Pests such as the stem borer *Chilo suppressalis* and the pentatomid *Eusarcoris inospicuus* cause yield losses and are difficult to control. Red rice (*Oryza sativa f. spontanea*) is one of the most notorious weeds found in Spanish rice growing areas, although *Echinochloa spp.* and *Cyperaceae* are considered the most important weeds [97]. Transgenic pest and herbicide resistant (HR) lines have been also developed in public institutes using Spanish rice varieties [98-100] with no commercial success to date. However, important studies about gene flow of transgenic HR rice lines have been performed in Ebro River Delta environmental conditions [101]. *Clearfield* HR mutant lines have been recently introgressed into mid-grain rice varieties and produced in Spain by *Cooperativa de Productores de Semillas de Arroz, S.C.L.* (Copsemar, SCL). Although ecological concerns about the rapid resistance transmission to weedy rice have been reported [14-16], *Clearfield* rice is being commercialised in Spain.

Apple snail species from genus *Pomacea* are one of the worst introduced gastropod crop pest of the recent time [102-107] and now threatens to destroy Europe's rice paddy fields by eating the sown seed and the rice plantlets since it was detected in the Ebro River Delta in 2009. To date, the only successful measure to eradicate the apple snail consisted in flooding infested fields with seawater. This treatment proved 100% effective in destroying apple snail infestations [108], nevertheless residual salt concentrations affected negatively rice productivity.

In Catalonia, agricultural production methods compatible with the environmental protection of wetlands included in the Ramsar Convention on Wetlands list (www.ramsar.org), have been

applied since 1998. In order to protect birds and aquatic species, these laws prioritize substituting and reducing the chemical treatments (DARP, 2005).

In Spain there is a tradition to cultivate pearly medium or short grain. Bomba, an old variety brought by the moors, is by far the most expensive variety available in the market since its grains absorb broth to the point of doubling its volume. As other Valencian rice such as Bahía or Senia it easily retains the broth flavour with the advantage that if it is overcooked it does not cake. In spite of all those wonderful properties, the production of Bomba rice almost stopped due to its slow productivity (4 tons per hectare) and the intensive care needed. Long grain rice of *Indica* type market is growing basically for export. In fact, in 2015 the Spanish rice production estimation was 900,873 t, being 49.26% long grain rice varieties [109].

6 Rice breeding and biotechnology

International centres made a tremendous effort to educate and train rice breeders during the green revolution. One of the most difficult tasks in carrying out a successful breeding program is the choice of germplasm for it to have a desirable genetic variability. To make the right choice of parental material to be used in a breeding program, breeders must clearly know the type of product to be developed; the characteristics of the species to be bred; the combining ability of the parents in case of hybrid cultivars; the environmental conditions of the target area; the social and economic aspects of the farmers and markets; and the different breeding approaches available to achieve the proposed goals. Today, an additional element to be considered is the legal aspect in relation to the materials to be used as parents.

If one makes a global literature review on the breeding methods commonly used to develop rice varieties around the world, pedigree selection is always at the top. More than 85% of the released rice varieties published in Crop Science Society of America have been developed through pedigree selection. When there are possibilities to carry out more than one generation per year (e.g., winter nurseries) the method is combined with modified bulk or even single-seed descent to speed up the process of having pure lines for agronomic evaluation.

The first and most important aspect to successfully take advantage of the variety of biotechnology tools available to rice breeders is to have a well-structured, efficient, and effective breeding program. FAO has started a worldwide plant breeding and associated biotechnology assessment in 2002. This work has been concluded in a sample of more than 50 developing countries in all the different rice growing regions. Among other things, the results indicate that almost every country has made investments in the area of biotechnology recently. However, only a very limited number of them have reinforced their breeding activities and worse still, the great majority do not even have well-structured and fully operational breeding

programs that can incorporate biotechnology tools. To add to this, very seldom have they ensured linkages between biotechnology efforts and breeding priorities or strategies.

Biotechnology rice has been developed to address different aspects: i) concerns that focus on the profitability of rice farming such as biotic and abiotic tolerance; ii) value-adding rice through nutritional improvement; iii) using it as a vehicle to produce pharmaceutical products, and iv) as an instrument to provide environmental protection and reduce global warming.

In addition, basic studies to increase rice yield include the incorporation of genes in the C4 pathway, a more efficient converter of light energy and carbon dioxide into food assimilates [110, 111]. Moreover, basic research on hybrid rice or the production of cloned seeds has been started and promising results are being generated [112]. This will considerably reduce the cost of production of hybrid rice, an important breeding strategy in rice production [113].

6.1 High yielding rice varieties

Traditional rice cultivars have evolved to make the most of limited soil fertility, growing fast and tall to beat the weeds to the sunlight and developed large root systems in order to gain maximum nutrients from the soil. As result, they have to be widely spaced or they would be competing with each other for sunlight and nutrients, consequently, yields were quite low. If fertilisers were added to these plants they would grow too tall and they would collapse [114].

During the 60's, the International Rice Research Institute (IRRI) had its first major breakthrough when produced the first important HYV of rice called IR8 in the Philippines. This variety was developed in 1963, when one of their experiments involved crossing a short and stiff strawed rice from Taiwan with a taller variety from Indonesia that was resistant to disease. Three years later this rice was released to farmers by the name of, since it was the 8th cross they had made. It was dubbed "miracle rice" as crop yields were at least twice as high as those of traditional rice.

With good farming methods, efficient irrigation schemes, fertilizers and pesticides, yields could quadruple those of traditional rice. In Spain new semi-dwarf HYV were also obtained during the 60's [94].

In general, HYV rice varieties are much smaller and can be grown much closer together without blocking sunlight. The short stiff stem was much more rigid and could hold more grain without collapsing. The root system was much smaller as they were bred to gain all the nutrients they needed from chemical fertilisers rather than the soil. Scientific experiments led to further improvements in HYV rice. Strains were developed that could grow much more quickly, and therefore areas that grew 2 crops a year with traditional rice could grow 3 crops in one year. Later strains were also much more pest and disease resistant. These varieties are now cultivated in 70% of world's riceland. Rice production doubled between 1966 and 1990 due to large scale adoption of these improved varieties.

In the following decades IRRI developed IR36, which became the most widely planted variety in the 1980s and IR64 was the most used in the 1990s [115]. In addition to these varieties, IRRI released a large series of IRcoded varieties. However, while these newer materials were characterized by their resistance to disease and insects, they did not contribute significantly to genetic gains for grain yield. Scientists then believed that a new breakthrough in yield potential had to come through the use of new tools such as anther culture, MAS and genetic engineering, which started playing an increasing role in rice improvement.

6.2 Embryo rescue in rice

The wild *Oryza* species are a very rich source for improvements to disease and pest resistance [116, 117]. However, due to less homology between *O. sativa* genome and the wild *Oryza* related species transfer of genes between them via breeding is limited to low crossability and reduced recombination between the chromosomes.

Table 1. Chromosome number, genomic composition and potential useful traits of *Oryza* species. [118]

Wild Species	2n	Genome	Distribution	Important trait
<i>O. sativa</i>	24	AA	Worldwide	Cultigen
<i>O. nivara</i>	24	AA	Tropical and subtropical Asia	Resistance to grassy stunt, virus, blast and drought avoidance
<i>O. rufipogon</i>	24	AA	Tropical and subtropical Asia, tropical Australia	Resistance to acid sulphate soils, elongation ability, resistance to BB, source of CMS
<i>O. barthii</i>	24	A ^g A ^g	Central and West Africa	Resistance to BB
<i>O. brevilingulata</i>	24	A ^g A ^g	Africa	Resistance to GLH, BB and drought avoidance
<i>O. glaberrima</i>	24	A ^g A ^g	West Africa	Cultigen and resistance to GLH
<i>O. longistaminata</i>	24	A ^g A ^g	Africa	Resistance to BB and drought avoidance, floral characteristics for outcrossing
<i>O. meridionalis</i>	24	A ^m A ^m	Tropical Australia	Elongation ability and drought avoidance
<i>O. glumaepatula</i>	24	A ^{sp}	South and central America	Elongation ability and source of CMS
<i>O. punctata</i>	24	BB	Africa	Resistance to BPH and zigzag leafhopper
<i>O. minuta</i>	48	BBCC	Philippines and Papua New Guinea	Resistance to sheath blight, BB, BPH and GLH
<i>O. officinalis</i>	24	CC	Tropical and subtropical Asia, tropical Australia	Resistance to thrips, BPH, GLH and WBPH
<i>O. rhizomatis</i>	24	CC	Sri Lanka	Drought avoidance and rhizomatous
<i>O. eichingeri</i>	24	CC	South Asia and East Africa	Resistance to YMV, BPH, WBPH and GLH
<i>O. latifolia</i>	48	CCDD	South and central America	Resistance to BPH, high biomass production
<i>O. alta</i>	48	CCDD	South and central America	Resistance to striped stem borer and high biomass production
<i>O. grandiglumis</i>	48	CCDD	South and central America	High biomass production
<i>O. australiensis</i>	24	EE	Tropical Australia	Drought avoidance and resistance to BPH
<i>O. brachyantha</i>	24	FF	Africa	Resistance to yellow stemborer, leaf folder, whorl maggot and tolerance to laterite soil
<i>O. granulata</i>	24	GG	South and Southeast Asia	Shade tolerance and adaptation to aerobic soil
<i>O. meyeriana</i>	24	GG	Southeast Asia	Shade tolerance and adaptation to aerobic soil
<i>O. longiglumis</i>	48	HHJJ	Irian Jaya, Indonesia and Papua New Guinea	Resistance to blast and BB
<i>O. ridleyi</i>	48	HHJJ	South Asia	Resistance to stemborer, whorl maggot, blast and BB
<i>O. coarctata</i>	48	HHKK	Myanmar, Bangladesh	Resistant to sea water and submergence
<i>O. schlechteri</i>	48	HHKK	Papua New Guinea	Stoloniferous

Abbreviations: BPH, brown planthopper; GLH, green leafhopper; WBPH, white-backed planthopper; BB, bacterial blight; CMS, cytoplasmatic male sterility; YMV, yellow mottle virus

The term “embryo rescue” refers to all *In vitro* techniques whose purpose is to promote the development of an inherently weak, immature or hybrid *embryos* into a viable plant, being one of the earliest and successful *In vitro* techniques [119]. Jena and Khush [120] have demonstrated the potential role of embryo rescue as a handy tool for the production of intergenomic hybrids in the genus *Oryza* (Table 1).

Embryo rescue techniques have been applied to introgress stress tolerances in cultivated rice [116], recovering rice germplasms [121] or even obtaining new interspecific hybrid species such as NERICA [122, 123], where fertile *O. sativa* and *O. glaberrima* (Fig. 10b) progenies were obtained through backcrossing and double haploid production by Jones *et al.* [124]. The NERICA varieties provide a good example of how these techniques were used to help address some specific breeding objectives. There are not many technical publications about the development of these varieties. Information is gathered in the form of press releases, on the Africa Rice Centre (WARDA) web page [125] (Fig. 11).

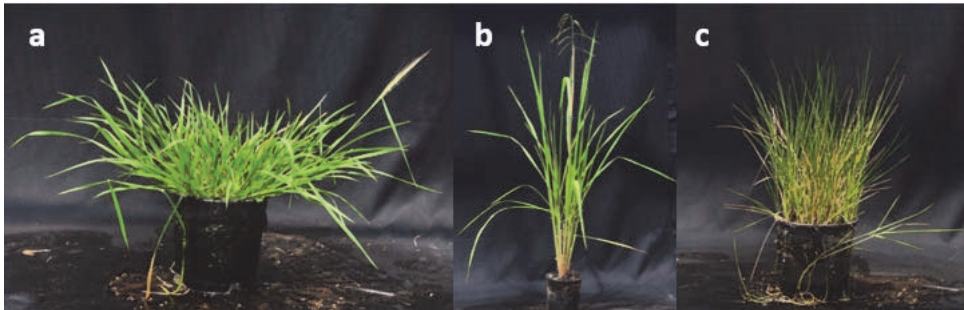


Figure 10. *Oryza nivara* (a) is the wild ancestor of *O. sativa* cultivated rice, *O. glaberrima* (b) which is the traditional African rice, and *O. coarctata* (c) which is a distant wild *Oryza* species capable to grow under salinity levels close to seawater.

A new relevant goal achieved thanks to embryo rescue technique is the “super salt-tolerant rice”. In this case, embryo rescue technique allowed obtaining the first viable F1 hybrid plant between *Oryza coarctata* (a species that can survive seawater flooding) and *O. sativa* [126] (Fig. 10c). Thus, thanks to embryo rescue this “super salt-tolerant rice” was born boosting the concept of salt tolerance to an unexpected level.

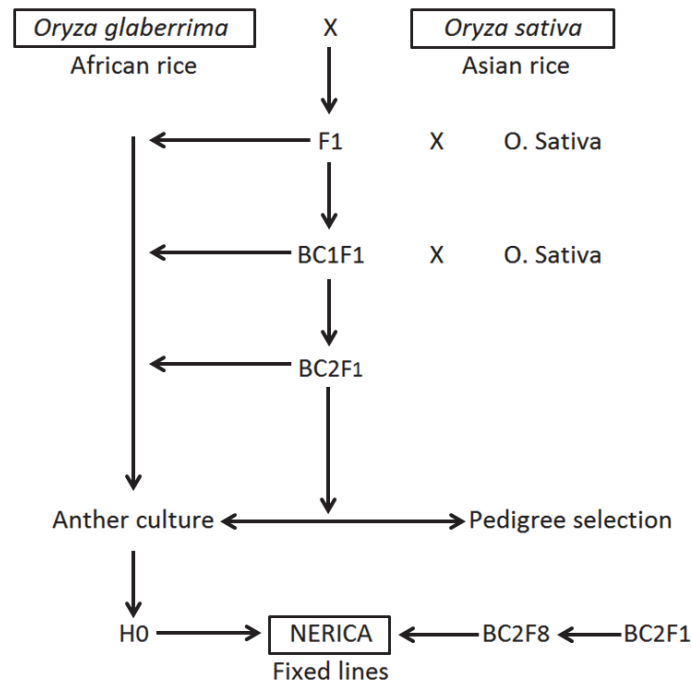


Figure 11. Hybridization scheme for the production of NERICA varieties through interspecific hybridization assisted by embryo rescue.

6.3 Marker-assisted breeding and gene mapping in rice

Several different types of molecular markers for assisting breeding and backcrossing programs are being used in rice [127, 128]. In addition, molecular linkage maps played a major role in rice MAB since knowledge of gene and marker location, linkage strength, and stability is essential.

The rice genome is one of the most studied by scientists around the world. The the first RFLP map was published in 1988 and was constructed at Cornell University by Mc Couch et al [129]. Having the rice genome fully sequenced in 2005 [9], a new and more important challenge was brought, which is to identify the biological functions of genes and their interactions with other genes and environments.

The Gramene database is a curated, open-source, integrated data resource for comparative functional genomics in crops and model plant species. It currently hosts whole annotated

genomes of over two dozen plant species and partial assemblies for almost a dozen wild rice species. It contains genetic and physical maps with genes, ESTs and QTLs locations, genetic diversity data sets, structure-function analysis of proteins, plant pathways databases (BioCyc and Plant Reactome platforms), and descriptions of phenotypic traits and mutations [130].

The International Rice Informatics Consortium (IRIC) has recently centralized information access to rice research data and provided computational tools to facilitate rice improvement via discovery of new gene-trait associations and accelerated breeding [131]. This fully available 3000 rice varieties SNPs database with phenotype and variety Information integrates the SNP genotyping data from the so called 3,000 Rice Genomes Project [132] and the phenotype and passport data for the 3,000 rice varieties from the International Rice Information System. Thus, decision-makers responsible for allocation of resources for research should not have two choices (biotechnology or plant breeding) but only one integrative way forward which is to ensure the integration of these activities.

Therefore, matching genotyping and phenotyping plays an important role. Breeding programs with excellent screening techniques and capable breeders are essential to capture the best advances of modern biotechnology and discard the rest.

6.4 Hybrid rice

Traditional rice production (i.e. non-hybrid rice) relies on rice varieties. A rice variety is a rice line that is distinguished by common characteristics of significance to agriculture and often has been assigned a commercial name. When rice is produced from a variety, a single line is planted and it fertilizes by self-pollination. When a rice variety is reproduced, it retains its distinguishing characteristics, and farmers can keep seeds for replanting next season.

In contrast, hybrid rice is the rice created by crossing two different parental lines, and that generally result in an F1 generation that is more robust than either of the parental lines. The improved qualities of the F1 generation are referred to as "hybrid vigour" or heterosis. The hybrid vigour may result in superior agronomic qualities such as higher yield, stronger resistance to diseases, more efficient use of soil nutrients, and better weed control.

Due to the difficulty of making rice hybrids, hybrid rice seeds are generally only produced by seed companies. Farmers do not keep seeds for replanting because self-fertilization will result in genetic segregation of traits. Therefore, farmers need to buy new hybrid seeds every year which may produce an economic hardship for the farmer, who has to balance the benefits of hybrid vigour with the annual cost of purchasing new seeds.

In the past, the production of hybrid rice strains was limited by rice's inherent propensity to self-pollinate. In 1974, Chinese scientists overcame this when they developed the first generation of hybrid rice using a three-line hybrid system based on cytoplasmic male sterile (CMS) lines produced by the abortive pollen system identified in the wild species *O. sativa f. spontanea* and hybrid combinations [133]. Hybrid rice would then be produced through a so-called three-line system, where one line would have the genetic-CME; the second line would be responsible for maintaining the sterility, and a third one would be used as the matching parent for the hybrid with the responsibility of restoring the fertility.

In 1996, an even more efficient second generation of hybrid rice was developed based on photoperiod-sensitive genetic male sterility (PGMS) lines. To simplify the hybrid rice production system, the concept of environmental genetic male sterility (EGMS) was introduced. The two environmental factors considered were the photoperiod (PGMS) and the temperature (TGMS) sensitivities, which are controlled by recessive nuclear genes. This technology allows the use of any genotype with good traits as male parent, to obtain *japonica* hybrids (e.g., it is difficult to

identify restorers for this group), and to develop inter-group hybrids such as *indica/japonica* [134].

The adoption of these varieties in the European countries, however, still need technologies to increase the F1 yield and lower, consequently, the cost of the hybrid seed. Promising results with these varieties have already been obtained in Spain and Italy [135] in terms of production, although quality traits are usually negatively affected.

6.5 Rice mutation breeding

The use of different sources derived from induced mutations was a popular choice to generate genetic diversity for specific traits in rice in the 1980s. Today the technique became part of the tools kit breeders have to enhance specific rice characteristics in well-adapted varieties. Maluszynski et al. [136] summarized the number officially released mutant varieties and came up with “cereals” as the group with the largest numbers followed by legumes and industrial crops. The FAO/IAEA Mutant Varieties Database indicates that there were 525 rice varieties releases recorded up to March 2007 [58]. In rice, the main improved traits were early maturity, plant height, and disease resistance. It is also worth mentioning that the famous gene *sd1* is a mutant. However, the most commonly mutated trait over all crops was “semi-dwarfism”.

6.6 Genetically modified (GM) rice

Initial studies to develop GM rice started in the early 80's as well as tissue culture experiments: playing with media components including hormones and complex amino acids and sugars; explant sources; culture conditions; and regeneration strategies. This period overlapped with the development of different genetic engineering procedures for rice.

Gene transfer in protoplasts and particle bombardment was thought to be the only efficient transformation methods in monocotyledonous plants until *A. tumefaciens*-mediated transformation was improved by supplying infection medias with acetosyringon [137, 138]. The agro-transformation resulted in higher transformation ratios and efficiency, being considered the most efficient in expressing reporter genes, for example: beta glucuronidase (*gusA*), green fluorescent protein gene (*gfp*) and selectable marker genes (herbicide and antibiotic resistance). Multigene transformation, tissue-specific or stress-specific promoters and transformation systems avoiding antibiotic resistant reporter genes has been developed, improving transgenic technology efficiency and security.

Two GM rice varieties (LLRice60 and LLRice62, both with herbicide resistance) were approved in the United States in 2000. Subsequent approval of these and other types of HR GM rice occurred across Canada, Australia, Mexico, and Colombia. However, none of these approvals resulted in commercialization. Nevertheless, research and development on genetic modification as a research tool and in developing potential GM rice varieties continues to advance in both the public and private sector around the world. Pollen-mediated Gene flow studies using GM rice in many world's rice productive areas have been published since the late 90's showing that pollen from GM plants can produce GM grains in non-GM rice plants and wild and weedy rices. These studies were carried out as it was thought that GM technology was going to be immediately applied to rice and commercialized [99, 101, 139-146].

7 Rice biotechnology applications

7.1 Pest Resistance

With the discovery and availability of pest resistance genes, GM rice was developed to improve rice's resistance to the devastating pests such as stem borers, rice weevil, leaf folder and hoppers (Table 2). Stem borer infestation of rice farms especially in the wet season poses extreme damage to as much as 30% production loss [147]. Stem borers and leaf folder are currently controlled by chemical pesticides in rice production; however, the chemical control of stem borers is now less efficient due to an increased pesticide resistance of the insect and its larvae feeding inside the stems of plants. Furthermore, increasing pesticides doses to effectively kill pests can cause severe environmental pollution [148]. A number of laboratories developed different local varieties to contain the Bt genes (*cry1Ab*, *1Ac* *1Aa*, *2A*, *1B*, or a combination of these genes) for resistance against Lepidopteran pests [148-151]. There are 140 variations of genes encoding Bt toxin that confer resistance to Lepidoptera, *Coleoptera* and *Diptera* species [152], with *Cry1Ab*, *Cry1Ac* and *Cry1Ab/Ac* being the most effective and extensively used in transgenic rice [153-157]. *Cry2A* [158] *Cry1C* [159] and other Bt genes [160-162] have also been successfully used for rice insect resistance.

Besides the *Bt* gene, some plant-derived insect-resistant genes also have been used to improve insect resistance of rice. Among them, plant lectin genes such as the *Galanthus nivalis* agglutinin [163] gene have been widely applied. Another group of plant-derived insect-resistant genes are the protease inhibitor genes like the cowpea trypsin inhibitor CpTI.

The first field testing of the Bt rice was conducted in China in 1998 [154, 156]. Nowadays, only few stem borer resistant GM rice cultivars have been approved for commercialization and planting and/or for import for food and feed use [164].

Table 2. Examples of insect-resistant transgenic rice generated since year 2000.

Gene	Target insects	Highlights	References
<i>Cry1Ab</i>	SSB, YSB.	Field trials show high protection against these insects	[154, 156, 165]
<i>Cry1Ab, Cry1Ac</i>	YSB, Leaf folder.	Field-tested insect-resistant <i>Bt indica</i> hybrid rice with high insect protection and no reduction in yield	[166-168]
<i>Cry1Ab or Cry1Ac</i>	SSB, YSB.	Transfer of insect-resistant trait into commercial varieties and/or stable expression over several generations	[169-171]
<i>Cry1Aa or Cry1Ab</i>	SSB.	<i>Bt</i> rice for Mediterranean conditions; already field tested	[100, 150, 161]
<i>Cry1Aa or Cry1Ab</i>	YSB.	Different varieties transformed for insect-resistance	[157, 172, 173]
<i>Cry1Ab</i>	SSB.	Marker-free insect resistant transgenic rice plants	[174]
<i>Cry1Ab</i>	SSB.	Wound-inducible expression of <i>Cry1Ab</i>	[175]
<i>Cry1Ab</i>	YSB, Leaf folder.	Use of different promoters for tissue-specific <i>Bt</i> gene expression	[176]
<i>Cry, Xa21 and RC7</i>	YSB, bacterial blight and sheath blight.	Gene-stacking for multiple disease tolerance	[177]
<i>Cry1Ab</i>	YSB, Leaf folder.	Field trials in India	[178]
<i>Cry1Ac, Cry2A</i>	YSB and leaf folder.	Field-trials of <i>Bt</i> rice in Pakistan	[179, 180]
<i>Cry 1C</i>	Stem borer, leaf folder.	Field-trials in China rice demonstrates that <i>Cry1C</i> rice lines are resistant to Lepidopteran	[159]
<i>Cry1Ab-1B, Cry1A/Cry1Ac</i>	YSB.	Fused cry genes transgenic rice bioassays	[148]
<i>Cry1Ab</i>	Rice stem borer.	Field-trial of <i>Bt</i> rice in North-Iran	[181]
<i>Cry1Ac, Cry2A, Cry9c</i>	YSB.	Field-trials in China using stem borer and 10 transgenic cry lines.	[182]
<i>Cry1Ia5</i>	Stem borer.	Transgenic rice with in vitro resistance to <i>Chilo agamemnon</i>	[162]
<i>Cry1Ab, Cry1Ab/Ac</i>	Thrips.	Field assays using six <i>Bt</i> lines and four Thrips species	[183]
<i>Gna</i>	Several homopteran, coleopteran and lepidopteran insects.	Snowdrop lectin gene [163]. Different rice varieties for multiple-range of insect resistance and/or no expression in seeds.	[184, 185];[186-190]
<i>Cry1Ab, Cry1Ac and/or gna</i>	Lepidopteran and sap-sucking insects.	Gene-pyramiding for protection against broad-range of insects	[172, 191]
<i>Gna, Cry1Ac</i>	BPH and SSB.	Use of linear constructs to avoid any integration of back-bone sequences in transgenic plants	[192]
<i>Itr1</i>	Rice weevil.	Constructive or endosperm-specific expression of barley trypsin inhibitor BTI-CMe for protection against rice weevil	[193]
<i>CpTi</i>	Rice stem borer.	A modified <i>CpTi</i> gene introduced in rice and assessed	[194]
<i>SbTi, gna</i>	BPH, leaf folder.	Soybean trypsin inhibitor (<i>SbTi</i>) + <i>Gna</i> transgenic <i>indica</i> rice field trials in China	[195]

Abbreviations: BPH, brown plant hopper; *Itr1*, *Hordeum vulgare* gene encoding for trypsin inhibitor BTI-CMe; SSB, stripped stem borer; YSB, yellow stem borer.

In 2009, *Pomacea maculata* Apple snail species (Fig. 12) accidentally entered the Ebro river delta in Spain. This invasion represents a serious threat to Europe's wetlands as it is spreading quickly, in summer 2015 the pest established in Ter river wetlands (ca. 300 km from its first detection). An evaluation of European Food Security Authority (EFSA) at the end of 2013 included Italy (the Po valley, which is the main rice production area in Europe) as an important hub for apple snail dispersal and epidemic. To date, the measures adopted to combat the apple snail and stop its spread have failed but, in the autumn of 2013, a new strategy was explored with permission of the European Union. This strategy was to flood 2500 ha of infested fields with seawater before the 2014 growing season. This treatment proved 100% effectiveness in destroying apple snail infestations [108], however, the residual salinity caused a loss of productivity of about 30% in some fields [196].



Figure 12. *Pomacea maculata* apple snail female individual is laying its eggs.

7.2 Disease resistance

Among the biotic stresses, blast disease caused by *Pyricularia oryzae* is the most harmful threat to high rice productivity [197] due to its wide distribution and ability to survive in a wide range of environmental conditions. This disease can cause yield losses ranging from 1 to 50 %, meaning economic losses over \$70 billion of dollar which represent the rice that can feed more than 60 million people [198]. Antifungal protein gene such as rice basic chitinase genes *RC24*, *chi-1*, *chi-2* and *chi-3*, an alfalfa β -1,3-glucanase gene [199, 200], a ribosome inactivating protein gene [201, 202], glucose oxidase gene [203], extracellular protein gene *Rirlb* [204], maize anthocyanin genes [205] and wasabi defensin gene [206] have been introduced into rice varieties, and have enhanced the rice blast resistance (Table 3). Some transgenic strategies for improving blast resistance based on the host-pathogen gene-for-gene interaction system and antifungal protein genes were developed [207, 208]. Additionally, inhibition of fungal disease development in plants by engineering controlled cell death was put forward by Strittmatter et al. [209]. In addition, foreign genes from unrelated plants and other organisms, such as cell wall degrading enzyme gene from *Trichoderma atroviride* [210] and antifungal AFP protein gene from *Aspergillus giganteus* [98], provide a new source of resistance genes (Table 3).

Bacterial blight (BB), caused by *Xanthomonas oryzae* pv. *oryzae* can cause up to 50% yield loss in severe pathogen attacks. With the discovery, identification and cloning of the *Xa21* gene in the wild rice *Oryza longistaminata* which confer broad-spectrum bacterial blight resistance, a new strategy was unfolded [211] (Table 3). A number of rice varieties were genetically-engineered to contain the gene [212, 213]. Field testing of some transgenic lines were conducted in China and the Philippines but none commercialized lines have been used so far [214]. MAB of *Xa4*, *Xa21* and *Xa27* in the restorer lines of hybrid rice have been used for enhanced disease resistance to bacterial blight [213, 215] (Table 3).

Table 3. Recent advances in transgenic rice towards resistance against viral, fungal and bacterial diseases [118].

Gene/function	Source	Performance transgenic plant	References
RDV minor core protein5S	RDV	Ribozyme-mediated resistance to RDV	[216]
RTSV/replicase gene RTBV RF2a, RF2b/transcriptional activators	RTSV/RTBV	Tolerance to RTB/disease-like symptoms	[217-220]
Gene encoding for RRSV	RRSV	Protection against RRSV infection	[221]
Viral spike protein <i>RHBV</i> nucleocapsid protein (N) gene	RHBV	Protection against RHBV	[222]
<i>Pi-ta</i> /NBS receptor class R gene	Rice	Resistant to <i>Magnaporthe grisea</i> (blast)	[223]
<i>Rif1b</i> /defence-related gene	Rice	Resistant to blast	[204]
<i>RC7</i> /chitinase	Rice	Rice resistance to <i>Rhizoctania solani</i> (sheath blight)	[224, 225]
<i>C2</i> /chalcone synthase	Maize	Expression of maize anthocyanin genes in rice for blast resistance	[205]
<i>RCH10</i> (chitinase)/ <i>MOD1</i> (maize ribosome inactivating protein)	Rice, maize	Resistance to <i>R. solani</i>	[226]
<i>chic</i> /Chitinase	<i>Streptomyces griscus</i>	Resistant to blast	[227]
<i>Ltp1</i> /lipid transfer protein	Rice	Induced by pathogen attack	[228]
<i>pinA</i> /antimicrobial puroindoline	Wheat	Resistance against blast and <i>R.solani</i>	[229]
<i>pinB</i> /antimicrobial puroindoline			
<i>Defensin</i> /Defensin	Wasabi	Resistant to blast	[206]
<i>TCS</i> /Trichosanthin, a ribosome inactivating protein	<i>Trichosanthes kirilowii</i>	Resistant to blast	[202]
<i>GOX</i> /Glucose oxidase	<i>A. niger</i>	Tolerant to multiple biotic stresses	[230]
<i>Afp</i> /Antifungal protein (AFP)	<i>A. giganteus</i>	Tolerant to blast	[98]
<i>Gns1/1,3; 1,4-β glucanase</i>	Rice	Resistant type <i>M. grisea</i> lesions	[231]
<i>RBBI2-3</i> /serine protease inhibitor	Rice	Blast resistant transgenic rice	[232]
<i>OsSBP</i> /selenium-binding protein	Rice	Tolerance against BB and fungal blast	[233]
<i>NPR1</i> / gene involved in systemic acquired resistant pathway	<i>Arabidopsis</i>	Transgenic rice tolerant to BB	[234]
<i>Xa21</i> / leucine-rich repeat receptor kinase like protein	Rice	Imparted BB tolerance to rice already made blast resistant by marker-aided selection	[163]
<i>Xa21</i>	Rice	Transgenic rice broad-pectrum BB resistant	[235, 236]
<i>Xa21</i>	Rice	Similar expression in t transgenic lines resistant to BB	[237]
<i>Xa26</i> /leucine-rich repeat receptor kinase-like protein	Rice	Another endogenous resistance gene for tolerance against BB	[238]
<i>cecB</i> /cecropin B	<i>Bombix mori</i>	Extra-cellular translocation of the transgenic protein for protection against BB	[239]
<i>ap1</i> /ferredoxin-like protein, AP1	Sweet pepper	Tolerance to BB	[187]
<i>Asthi1</i> /thionins	Oat	Transgenic rice resistant to <i>Bukholderia plantarii</i>	[240]
<i>RCH10, RAC22, β-glu, B-RIP</i>	Different species	Transgenic rice harbouring multiple antifungal genes hybridized with high resistant to BB line. Field assays revealed high resistance BB, blast, <i>Ustilaginoidea virens</i> and <i>Tilletia barclayana</i>	[241]
<i>Chi11</i> /Chitinase, <i>β-1,3-glucanase gnes</i>	Rice, Tobacco	Resistance to sheath blight using a rice chitinase and a tobacco glucanase genes	[242]
<i>Pi-d2</i> /Beta-lectin receptor kinase	Rice	Rice leaf blast and neck blast resistance	[243, 244]
<i>Xa7, Xa21, Xa22, Xa23</i>		Hybrid rice resistance to 11 races of BB	[245]
<i>GH3.1</i> / hormonal homeostasis regulator	Rice	Blast resistance in plants overexpressing <i>GH3.1</i>	[246]

Abbreviations: A, *Aspergillus*; BB, bacterial blight; RDV, Rice dwarf virus; RHBV, Rice white leaf (*hoja blanca*) virus; RTBV, Rice tungro bacilliform virus; RTSV, Rice Tungro Spherical virus.

Efforts to develop rice for resistance to sheath blight were conducted by incorporating genes coding for chitinase and glucanase enzymes that metabolize the fungal cell wall, and other pathogenic-related proteins [247, 248] (Table 3).

7.3 Herbicide resistance

Problem on the lack of irrigation in the rice paddies is aggravated by the presence of the noxious weeds that affect rice normal growth and yielding capacity. Weed control measures usually include application of herbicide combinations, crop rotation, flooding and tillage which are expensive, labour intensive, and harmful to the environment and non-target humans and animals. The early herbicides were found to be very destructive for most plants and they created undesirable environmental impacts. New chemicals such as glyphosate and glufosinate ammonium have been widely recommended for use because they are environmental-friendly as soil microorganisms are able to degrade them rapidly. For this chemicals to be used, glyphosate and glufosinate-resistant biotechnological rice were developed in 1999. Two genes commonly used to develop HR plants are bar gene isolated from *Streptomyces hygroscopicus* that detoxifies herbicide glufosinate, and EPSPS isolated from *Agrobacterium* strain CP4 that detoxifies herbicide glyphosate. Furthermore, the bar gene has been used during the early stages of rice transformation research as a reporter gene [249], but also as the priority trait for transgenic rice field trials [250, 251]. Bayer Crop Science, Inc. produced Liberty link rice LL62 by inserting the bar gene into the genetic make-up of Bengal rice, a popular rice variety grown in Southern USA. This GM rice was planned to be commercialized together with a glufosinate ammonium herbicide called *Liberty* also commercialized by Bayer. Nevertheless, no liberty link rice commercialization has occurred to date, although liberty link corn, cotton, canola, sugar beat and soybean are available in the market and Bayer's liberty link rice lines have been approved for commercialization in the USA, Canada, and Mexico since year 2000.

Significant research has been done to investigate other sources of genes that could provide herbicide tolerance in transgenic rice. For example, transgenic rice plants expressing *Bacillus subtilis* protoporphyrinogen oxidase (protox) which is a penultimate step enzyme of the branch point for the biosynthetic pathway of chlorophyll and heme, were resistant against herbicide oxyfluorfen [252-255]. Transgenic rice over-expressing a human cytochrome P450s showed variable response to herbicides [256-258], although rice expressing human genes may not be acceptable because of ethical reasons.

Rice cultivars treated with the chemical mutagen ethyl methyl sulfonate (EMS) for selection of imazethapyr-tolerant lines have been crossed to produce the *Clearfield* cultivars [144]. *Clearfield* rice in combination with imidazolinone herbicides is an effective management tool to control red rice in cultivated rice [259]. Because red rice and cultivated rice belong to the same species, transfer of imazethapyr tolerance from *Clearfield* rice to red rice has already been predicted by Moore in year 2002 [260] and reported several times [14-16, 261, 262].

7.4 Abiotic Stress Resistance

Rice is a water-loving plant that uses up to 30% of the freshwater worldwide, which is two to three times more water than other food crops [263]. In fact, it is the most salt sensitive crop and with the imminent water shortage and increased salinity brought by global warming, strategies to develop rice tolerant to drought and salinity stresses were conducted using stress-related genes and transcription factors identified in the model plant *A. Thaliana*. A large number of transgenic crops have been produced with higher tolerance to various abiotic stresses by increasing osmoprotectants such as glycine-betaine, proline, sugar alcohols (mannitol, trehalose, myo-inositol and sorbitol), and polyamines as well as by regulating K^+/Na^+ homeostasis [264] (Table 4). On the other hand, a detoxification strategy, which could effectively eliminate reactive oxygen species (ROS) produced by abiotic stress could also be used for

improving stress tolerance [265]. In addition to these, studies have shown that transcription factors play very important roles in stress tolerance mechanisms. Up to now, at least 10 types of transcription factors have been involved in abiotic stress regulations. Over-expression or loss-of-function of some genes encoding some of transcription factors can significantly increase tolerance to abiotic stresses [266].

Great advances in the comparison of genomes and transcriptomes of different organisms have contributed to the development of comparative genomics as one of the most promising fields in plant biotechnology [267-269]. In this way, finding variations in the genome or the transcriptome from the current model species related to interesting agronomic traits is of the highest importance for crop biotechnology [270].

Gene expression analysis techniques have been identified new target genes [271]. Apart from these, other signalling transduction genes also play important roles in stress responsive pathways and also can be used for transgenic improvement (Table 4). This include the expression of the *HRD* gene in rice that increased the leaf biomass and bundle sheath cells that would probably contribute to enhanced photosynthesis assimilation, water use efficiency and drought resistance [272]; and the expression of *CBF3/DREB1A* and *ABF3* in rice increased its salinity and drought tolerance [273].

Breeders have long made use of the high salinity tolerance level in landraces like Nona Bokra and Pokkali. However, negative characters in traditional varieties and the numerous and complex traits involved in salinity tolerance have presented challenges for conventional breeding to make significant progress what has led to increased interest in molecular breeding methods [274-276]. A number of mapping studies have identified QTLs associated with salinity tolerance in rice, and the *SKC1* gene was subsequently cloned and found to encode a sodium transporter that helps control K^+ homeostasis under salt stress [277]. Likewise, to characterize

the genetic components of salinity tolerance in the tolerant landrace Pokkali, a recombinant inbred line (RIL) population between the *indica* varieties IR29 and Pokkali was developed at IRRI and used in a QTL study using AFLP genotyping [274, 278]. A major QTL associated with the Na⁺–K⁺ ratio and seedling-stage salinity tolerance, named *Saltol*, was identified on chromosome 1 along with a number of minor QTLs on other chromosomes. Subsequently, RFLP and SSR markers were added to the *Saltol* region, and a test of 54 RILs in an hydroponic screen at the seedling stage revealed that this QTL explained 43% of the variation for seedling shoot Na⁺–K⁺ ratio in this population [279]. One highly salt tolerant RIL from this population, FL478 (IR 66946-3R-178-1-1), has been promoted as an improved donor for breeding programs, as it is highly tolerant to salt at the seedling stage and is photoperiod insensitive, shorter and blooms earlier than the original Pokkali landrace.

Table 4. Rice transgenics and abiotic stress tolerance

Gene/function	Source	Result	References
<i>OsDREB1</i> /DRE.binding protein 1	Rice	Drought, salt and cold tolerance	[235, 236, 280]
<i>GPAT</i> /glycerol-3P-acyltransferase	<i>Arabidopsis</i>	Chilling tolerance	[281]
<i>Mn-SOD/Superoxide dismutase</i>	Yeast	Salt tolerance	[282]
<i>HVA1</i> / group 3 LEA protein	Barley	Drought, salt and freezing tolerance	[216, 283-286]
<i>OsCDPK7/Ca-dependent protein kinase</i>	Rice	Cold, drought and salt tolerance	[287]
<i>GS2</i> /glutamine synthase	Rice	Salt and chilling tolerance	[288]
<i>MnSOD</i> /Manganese superoxide dismutase	Pea	Drought tolerance	[224, 289]
<i>naatA</i> , <i>naatB</i> /biosynthesis of mungenic acid phytosiderophores	Barley	Tolerant to iron deficiency	[290]
<i>codA</i> /glycine betaine	<i>Arthrobacter globiformis</i>	Drought and salt tolerance	[291-293]
<i>SNAC1</i> /Stress responsive NAC1	Rice	Drought and salt tolerance	[163, 294]
<i>Cat</i> /catalase	Wheat	Cold tolerant	[295]
<i>AGPAT</i> , <i>SGPAT</i> /fatty acids biosynthesis	<i>Arabidopsis</i> , spinach	Improved photosynthesis and growth at low temperatures	[296]
<i>YK1</i> /rice homolog of maize HC toxin reductase	Rice	Tolerant to multiple abiotic stresses	[297]
<i>OsNHX1</i> , <i>AgNHX1</i> /	Rice	Salt tolerance	[298]
Na ⁺ /H ⁺ antiporter <i>HVPIP2</i> ; 1/aquaporins	<i>Atriplex gmelini</i> , rice	Differential response to salt-stress	[299-301]

<i>OtsA, OtsB</i> /trehalose biosynthesis	<i>E. coli</i>	Multiple abiotic stress tolerance such as salt, drought and cold	[302, 303]
<i>Adc, Samdc</i> /polyamine biosynthesis	Ds, oat, wheat, barley	Drought and salt tolerance	[304-306]
<i>Dadc</i> /arginine des carboxylase	Ds	Drought tolerance	[306]
<i>ABF3</i> /ABRE-binding factor 3	<i>Arabidopsis</i>	Drought tolerance	[273]
<i>DREB1A</i> /DRE-binding protein 1	<i>Arabidopsis</i>	Drought and salt tolerance	[273]
<i>HvCBF4</i> /C-repeat binding factor	Barley	Drought, salt and cold tolerance	[307]
<i>OsCIPK03</i> /Calcineurin B-like protein-interacting protein kinase 03	Rice	Cold tolerance	[308]
<i>OsCIPK12</i> /Calcineurin B-like protein-interacting protein kinase 12	Rice	Drought tolerance	[308]
<i>OsTPP1</i> /trehalose-6-P phosphatase	Rice	Salinity and cold tolerance	[309]
<i>ZFP252</i> /TFIIIA-type zinc finger protein	Rice	Drought and salt tolerance	[310]
<i>AeMDHAR</i> /Monodehydroascorbate reductase	Mangrove plant	Salt tolerance	[311]
<i>OsRIP18</i> /Ribosome inactivating protein	Rice	Drought and salinity tolerance	[312]
<i>OsHsp17.0, OsHsp 23.7</i> /Heat shock proteins	Rice	Drought and salt tolerance	[313]
<i>Oshox22</i> /Homeodomain-leucine zipper I TF	Rice	Drought and salt tolerance	[314]
<i>MTH1745</i> /Disulphide isomerase-like protein	Mt	Mercury tolerance	[315]
<i>OsTZF1</i> /CCCH-tandem zinc finger protein	Rice	Delayed senescence and stress tolerance	[316]
<i>TaSIP/T. aestivum</i> salt-induced protein	Wheat	Drought and salt tolerance	[317]
<i>PDH45</i> /DESD-box helicase	Pea	Salinity tolerance	[318]
<i>OsFKBP16-3</i> /Immunophilin	Rice	Salt, drought and oxidative stress tolerance	[319]
<i>OsERF4a and 10a</i> /ethylene response factors	Rice	Drought tolerance	[320]

Abbreviations: Mt *Methanobacter thermoautotrophicum*, Ds *Datura stramonium*, TF transcription factor.

Besides that, a team of scientists at the International Rice Research Institute (IRRI) developed the so called “Super salt-tolerant rice” which is a backcross line that introgress the ability of *Oryza coarctata* to drive out salt through salt glands it has on its leaves, into a commercial *O. sativa* IR56 rice line [126]. *O. coarctata* withstand saline water (20 to 40 dSm⁻¹ E.C) submergence for quite a long period through some special unicellular salt hairs (trichomes) on the adaxial surface of the leaves, by which they efficiently maintain a low concentration of toxic salts in the plant tissue [321] and maintain the optimum mineral concentration in its tissues.

7.5 Nutritional improvement

Rice is a good source of carbohydrate, proteins, fibre, lipid and fats, minerals (potassium, phosphorous, magnesium, calcium, sodium, copper and iodine) and vitamins (thiamine, riboflavin, niacin, vitamin B6 and folic acid) [322]. In poor countries having less access to meat and fish, rice is predominantly eaten, and thus important minerals and vitamins are lacking in the diet. This can lead to a widespread occurrence of vitamin A and E, iron and zinc deficiency which afflict susceptible children, pregnant and lactating women. Food supplementation and fortification programs conducted were found to be relatively expensive, noncompliance is high, and requires infrastructure for delivery and targeting. A novel approach in biofortification uses biotechnological tools to incorporate genes for increased amounts of these essential nutrients into staple food. Biotech rice with provitamin A, *Golden Rice*, has been developed [323, 324] and is being used to transfer beta carotene loci into high-yielding local commercial cultivars through MABC breeding in the Philippines, Bangladesh and India. Biotech rice with increased ferritin content was found to replenish the haemoglobin and iron liver concentrations in rat experiments suggesting that biotechnological approaches that manipulate ferritin expression of the iron in seeds may contribute to a sustainable solution to global problems of iron deficiency [325].

Rice is devoid of essential amino acids such as threonine, tryptophan, lysine, and specially methionine [326]. Strategies to improve the lysine content of rice showed that inhibition of its degradation through the RNAi approach increased its free level, and affected the concentrations of the amino acids related to lysine metabolic pathway, such as threonine and aspartic acid [327]. Furthermore, recent results obtained from plants overexpressing a sulphur-rich 2S albumin in rice seeds revealed that reduced sulphur is limiting the protein synthesis and thus limiting the approach to increase the level of bound methionine [328]. Otherwise, a major issue with the expression of foreign proteins is their potential allergenicity as was shown for the 2S albumins from seeds of Brazil nut or sunflower [329]. To overcome these issues, direct

approaches to manipulate the biosynthetic pathway of cysteine or methionine would allow the improvement of the endogenous content of sulphur-containing amino acids in rice [330-332]. As plant proteins are the primary sources of all dietary proteins consumed by human and animals and have lower production costs compared with meat, improving their quality will make a significant contribution to future needs of resources [333].

7.6 Biopharming

Rice can be used as a vehicle to produce pharmaceuticals including vaccine, such is the case of a rice-based oral vaccine containing the vaccine antigen cholera toxin B subunit (CTB). The CTB vaccine is accumulated in the protein bodies of the starchy endosperm cells, and then after being ingested is taken up by mucosal cells of the gastrointestinal tracts for the induction of antigen-specific mucosal immune responses with neutralizing activity [334]. In addition, the rice-based CTB vaccine remained stable and maintained immunogenicity at room temperature for more than 1.5 years, and was protected from pepsin digestion in vitro. Other mucosal cell vaccines can be produced in rice to target diseases of the respiratory and gastrourinary tracts and could be administrated economically in the developing countries where need is often the greatest.

Extended use of antibiotics contributes to the development of antibiotic resistance in commensal bacteria in poultry, pigs, cattle, and humans, so the search for alternative strategies is needed. Antibacterial molecules such as lactoferrin and lysozyme were expressed in rice grains through biotechnology. Experimental feeding of broiler chickens fed with rice containing lactoferrin and lysozyme showed that they improve the feed efficiency, histological indices of intestinal health, and increased bacteriostatic activity. This strategy can also be used in maintaining intestinal health and in the prevention of diarrhoea in other young animals including human infants which can be the case of a large population in developing countries [335].

7.7 C₄ rice

Harvest index (HI, ratio of grain yield to biological yield) is an important trait associated with the dramatic increases in crop yields by introducing semi-dwarf varieties during the Green Revolution last century [336]. However, the value of HI higher than 50 % which is close to its theoretical limit has already been achieved for rice varieties. It is therefore difficult to increase HI by only manipulating plant architecture for this cereal [337].

Most terrestrial plants, including many important crops such as rice, assimilate CO₂ through the C₃ photosynthetic pathway and are therefore classified as C₃ plants. However, some plants, such as maize and sugarcane, possess the C₄ photosynthetic pathway, and are classified as C₄ plants. Rice is limited for higher photosynthetic efficiency compared with C₄ crops. This can be mainly attributed to the catalytic properties of the enzyme ribulose 1, 5-bisphosphate carboxylase/oxygenase (RuBisCO) which is not only an inefficient enzyme for carboxylation reaction with a low turnover number, but also catalyses an active oxygenation reaction resulting in photorespiration. By comparison, C₄ plants possess a CO₂ concentrating mechanism to pump CO₂ in high concentration around RuBisCO to reduce the photorespiration effectively. This CO₂-concentrating mechanism, together with modifications of leaf anatomy, enables C₄ plants to achieve high photosynthetic capacities and high water and nitrogen use efficiencies (Fig. 13). As a consequence, the transfer of C₄ traits to C₃ plants is one strategy being adopted for improving the photosynthetic performance of C₃ plants [338].

Scientists have made great efforts to produce C₄ rice by overproducing one or several key C₄ enzymes in a C₃ mesophyll cell in recent years. In C₄ cycle, the first enzyme which catalyses the fixation of CO₂ to form a C₄ acid oxaloacetate is phosphoenolpyruvate carboxylase (PEPC), thus a large endeavour has been made to produce transgenic rice overproducing PEPC of C₄ plants.

However, different research teams produced conflicting results under different experimental conditions [339-345].

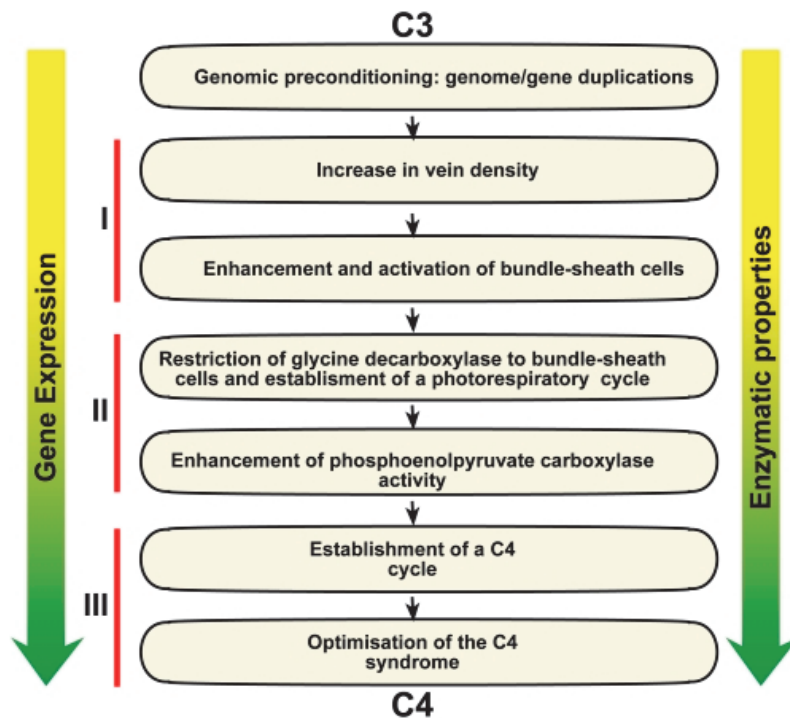


Figure 13. Stepwise evolution of C4 photosynthesis [346].

Simply overproducing C₄-specific enzymes cannot enhance photosynthetic CO₂ use efficiency in rice [347, 348]. C₄ photosynthesis is normally associated with the compartmentation of photosynthesis between mesophyll and bundle sheath cells [2]. The mechanisms regulating the differential accumulation of photosynthesis proteins in these specialized cells are fundamental to our understanding of how C₄ photosynthesis operates. Cell-specific accumulation of proteins in mesophyll or bundle sheath cells can be mediated by posttranscriptional processes and translational efficiency as well as by differences in transcription. NGS and comprehensive analysis of which genes from C₄ species are expressed in mesophyll or bundle sheath cells of C₃ plants should provide insight into how the C₄ pathway is regulated and has evolved [349].

Other strategies point to the use of naturally occurring RuBisCOs with higher CO₂ specificity than that of C₃ crops, which have been found in red algae *Griffithsia monilis* or the C₄ dicot *Amaranthus edulis* [350]. These theoretical models support the replacement of rice RuBisCO with a more efficient naturally existing one [351-353]. However, comparison of 3D structures of RuBisCOs from multiple prokaryotic and eukaryotic sources indicated that the structural differences are limited [354]. Furthermore, amino acid substitutions to different areas of RuBisCO large subunit only produced less efficient enzymes until now [355].

Successful manipulation of RuBisCO in higher plants may also need to take the RuBisCO activase into consideration, protein in charge of catalysing the removal of inhibitory sugar phosphates from the RuBisCO active site for its correct activity. Gradual improvements have been made on gathering evidence toward the function of the RuBisCO activase. By site-directed mutagenesis studies, the identity of residues required for successful interaction between RuBisCO and RuBisCO activase has been proposed [53]. Another reason to modify RuBisCO activase is that photosynthesis declines at moderately high temperatures due to the deactivation of RuBisCO, caused by the thermal inactivation of the RuBisCO activase. Recent progress has been made on generating the variants of thermostable RuBisCO activase in *Arabidopsis* by DNA shuffling [356].

It should be clear that the next great challenge for rice yield improvement is to enhance photosynthetic CO₂ use efficiency and knowledge about molecular development of C₄ morphology and the underlying gene regulatory networks and/or technical improvement on the selection process for better Rubisco are prerequisites to be successful in this endeavour. By combining approaches spanning many disciplines such as molecular genetics, genomics, developmental biology, plant phenomics, system biology and crop science, rice photosynthesis could be significantly enhanced and translated into increased crop yield in a near future.

Objectives

The general aim of this PhD Thesis was to study the application of biotechnology in Spanish rice cereal breeding. Thus, the work was focused in three specific objectives:

- 1) To understand the economic and environmental consequences of releasing GM rice cultivars in Mediterranean climatic conditions through the study of the pollen-mediated gene flow between GM rice and conventional rice in coexistence with red rice weed in real field conditions.
- 2) To re-new lifetime in market of a traditional and high quality rice cultivar through the application of an anther culture and field selection protocols improved for Spanish *japonica* varieties.
- 3) To improve a Spanish rice variety through a new and original TILLING protocol using *callus* mutagenesis and an improved mismatch detection to rapidly obtain delayed senescence mutants that are thought to affect the yield positively.

In order to accomplish the first specific objective, Senia, a common rice cultivar grown in the Ebro Delta, was transformed to obtain GM herbicide resistant Senia plants. Two consecutive field trials were performed in the Ebro Delta using a circular design where conventional Senia, GM Senia and red rice weed were used. Plants were harvested and their descendants were analysed to study and quantify different gene flow rates:

1. Gene flow rate from GM to conventional rice and the effect of the distance and the geographic orientation.
2. Gene flow rate from red rice to GM rice, the effect of its relative proportions and the generation of GM red rice weeds.
3. Gene flow rate from GM rice to red rice weed, the effect of its relative proportions and the generation of GM red rice weeds.

In order to complete the second specific objective, different protocols had to be adapted and optimized to Spanish *japonica* cultivars:

1. Pollen development and cold treatment studies to improve anther's response.
2. Anther culture *in vitro media* design and optimization for Mediterranean *japonica* commercial varieties.
3. Greenhouse and field trial designs.

Finally, to complete the third specific objective a new TILLING approach was conceived to rapidly obtain desired mutants rice. Two new inventions were successfully developed:

1. Performing a *callus* mutagenesis protocol to rapidly obtain a rice mutant population instead of a classic seed mutagenesis.
2. Using a new mismatch detection enzyme to screen the mutant population.

Outline of the Thesis

The Thesis is divided in five parts. This includes General Introduction, Objectives, Results, General Discussion and Conclusions.

The General Introduction describes the importance of plant breeding and biotechnology in relationship to the human world population, the latest plant biotechnological applications, rice biotechnology and the Spanish rice biotechnology scope.

The Results section includes three Chapters based on published papers to international journals. Chapters refer to different real applied biotechnology studies in different Spanish rice varieties.

Briefly:

- Chapter 1 studies the effect of red rice weed infestation in pollen-mediated gene flow rates when coexisting transgenic herbicide resistant and conventional rice lines under Ebro River Delta environmental conditions.
- Chapter 2 describes a rapid protocol successfully used to obtain and select anther culture-derived rice lines using a highly heterozygous seed batch of a Spanish cultivar as starting plant material.
- Chapter 3 describes an original, fast and efficient TILLING technique based on callus mutagenesis in rice for obtaining improved Spanish rice varieties.

Discussion section discusses chapter's results in relation to the most recent studies and the objectives of the thesis.

Conclusion section summarizes briefly the main resulting conclusions.

**Informe del factor d'impacte dels articles
publicats i participació del doctorand**

El Dr. Salvador Nogués Mestre i el Dr. Eric Lalanne com a Directors de la Tesi que porta per títol: “APPLIED BIOTECHNOLOGY TO IMPROVE MEDITARRANEAN RICE VARIETIES” que ha dut a terme el doctorand Xavier Serrat Gurrera, informen sobre l’índex d’impacte i la participació del doctorand en cadascun dels articles inclosos en la memòria de la Tesi. En tots els articles, el doctorand és el primer autor dels treballs.

Capítol 1. Article: “Quantification of direct and reverse pollen-mediated gene flow between GM rice and red rice weed”, publicat a la revista *AoBPLANTS*, amb un índex d’impacte de 2,273. En aquest estudi es va estudiar el flux de gens mediat pel pol·len entre varietats d’arròs convencionals, transgèniques i salvatges (arròs vermell salvatge) mitjançant dos assajos de camp amb disseny circular. Aquest va ser un dels primers estudis en el que el doctorand va col·laborar analitzant el flux de gens salvatges cap a plantes transgèniques mitjançant la tècnica d’AFLPs, analitzant les dades i redactant-ne l’article esmentat.

Capítol 2. Article: “A Mediterranean *japonica* rice (*Oryza sativa*) cultivar improvement through anther culture”, publicat a la revista *Euphytica* amb un índex d’impacte de 1,385. En aquest article es descriu un protocol d’obtenció de línies millorades a partir de cultiu d’anteres emprant un lot de llavors desestabilitzades. El protocol engloba des del cultiu d’anteres fins als assajos de camp necessàries per l’avaluació i selecció de línies amb interès comercial, les quals estan actualment en procés de registre.

Capítol 3. Article: “EMS mutagenesis on rice mature seed-derived calli as a new method to obtain rapidly TILLING mutant populations”, publicat a la revista *Plant Methods* amb un índex d’impacte 3,102. En aquesta publicació es descriu una variant nova i original del TILLING basada en la mutagènesi de calls per a l’obtenció de mutants d’arròs. El nou sistema permet assolir una alta taxa de mutagènesi amb dosis baixes de mutagen, suggerint que es poden assolir taxes molt més altes. A més, descriu el disseny de dianes per l’obtenció de mutants amb senescència retardada i l’innovador ús de l’extracte cru de fonoll per a la detecció enzimàtica dels mutants.

I per que consti a efectes oportuns

Dr Salvador Nogués Mestres

Dr. Eric Lalanne

Barcelona, 13 de Maig de 2016.

Table of performed studies

Chapters	Type of study	Field localization	Greenhouse localizations	Laboratory Localizations	Rice cultivars
1	Gene flow	IRTA-Amposta	IRTA-Cabrils	IRTA-Cabrils	Senia Red rice
2	Anther culture	CAMARA-Amposta	SCE UB-Barcelona Oryzon-Torribera	SCE UB-Barcelona	NRCV980385
3	Tilling	HISPARROZ-Alcalá de Guadaira	SCE UB-Barcelona Oryzon-Torribera	SCE UB-Barcelona Oryzon-Cornellà Oryzon-Parc Científic	Hispagran

Results

Chapter 1.

Direct and reverse pollen-mediated gene flow between GM rice and red rice weed.

Resum

El flux genètic des de plantes d'arròs transgènic (*Oryza sativa*) donadores de pol·len, cap a plantes d'arròs convencional i cap a plantes d'arròs salvatge (*Oryza sativa f. spontanea*) ha estat àmpliament estudiat en diferents condicions de camp. Alguns d'aquests estudis han demostrat que el pol·len de l'arròs transgènic és capaç de transferir els transgens a plantes d'arròs salvatge, resultant-ne llavors d'arròs salvatge transgèniques que mostren els caràcters de dehiscència i dormància típicament salvatges. A més, les plantes l'arròs salvatge poden transferir el seu pol·len a l'arròs transgènic en un fenomen que hem anomenat flux de gens revers, el qual també provoca la aparició de llavors d'arròs salvatge modificat genèticament. Hem quantificat el flux de gens revers emprant material de dos assajos de camp. Es va dur a terme un anàlisi molecular basat en AFLPs a la descendència de les plantes transgèniques, però també anàlisis fenotípics de caràcters salvatges com son l'alçada, el vigor, la coloració del pericarp i la dehiscència de les llavors. El flux genètic revers detectat va ser més gran que els flux genètic directe tant en el primer com en el segon assaig de camp. La taxa de flux genètic directe va variar en funció de la proporció relativa entre plantes salvatges i transgèniques.

L'impacte ecològic del flux genètic revers és limitat degut a que les llavors que s'obtenen en la primera generació encara no expressen els trets de dehiscència i ni de dormància que son els que fan que puguin aparèixer plantes salvatges transgèniques en els camps. La llavor híbrida romandria a l'espiga i seria collida durant la sega. De tota manera aquest fenomen ha d'ésser considerat en els camps de producció de llavor certificada i als països en desenvolupament, on els agricultors sovint guarden una part de la collita per sembrar a l'any següent. En aquests

casos, el risc d'una infestació d'arròs salvatge modificat genèticament és més alta i caldria establir un pla de supervisió adequat.



Research Article

Direct and reverse pollen-mediated gene flow between GM rice and red rice weed

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Abstract. Potential risks of genetically modified (GM) crops must be identified before their commercialization, as happens with all new technologies. One of the major concerns is the proper risk assessment of adventitious presence of transgenic material in rice fields due to cross-pollination. Several studies have been conducted in order to quantify pollen-mediated gene flow from transgenic rice (*Oryza sativa*) to both conventional rice and red rice weed (*O. sativa* f. *spontanea*) under field conditions. Some of these studies reported GM pollen-donor rice transferring GM traits to red rice. However, gene flow also occurs in the opposite direction, in a phenomenon that we have called reverse gene flow, resulting in transgenic seeds that have incorporated the traits of wild red rice. We quantified reverse gene flow using material from two field trials. A molecular analysis based on amplified fragment length polymorphisms was carried out, being complemented with a phenotypic identification of red rice traits. In both field trials, the reverse gene flow detected was greater than the direct gene flow. The rate of direct gene flow varied according to the relative proportions of the donor (GM rice) and receptor (red rice) plants and was influenced by wind direction. The ecological impact of reverse gene flow is limited in comparison with that of direct gene flow because non-shattered and non-dormant seeds would be obtained in the first generation. Hybrid seed would remain in the spike and therefore most of it would be removed during harvesting. Nevertheless, this phenomenon must be considered in fields used for elite seed production and in developing countries where farmers often keep some seed for planting the following year. In these cases, there is a higher risk of GM red rice weed infestation increasing from year to year and therefore a proper monitoring plan needs to be established.

Keywords: Field trial; gene flow; herbicide resistance; *Oryza sativa*; red rice; risk assessment; transgenic rice.

Introduction

Genetic modification technologies are widely used as a way to introduce new genetic traits into crops of interest. One of the main environmental concerns about these technologies is the non-controlled gene spread between crops at different levels. There are many studies studying

this phenomenon and reporting guidelines to minimize the risk of cross-pollination between modified plants and non-modified crop plants (Mallory-Smith and Zapiola 2008; Devos *et al.* 2009). These studies propose solutions like the use of different crop species working as a natural barrier against cross-pollination, minimum distances

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between crop fields or a delay in flowering coincidence. However, all those gene flow studies are focused on the risk of the genetically modified (GM) pollen spreading out towards the non-modified crops or to the wild species that surround the fields.

In our study, the influence of weedy red rice (*Oryza sativa* f. *spontanea*) gene flow over a GM rice line (*O. sativa*) was studied in the north-east of Spain, under Mediterranean climate conditions. The gene flow from a GM rice line to the weedy rice was also quantified in order to be able to compare both types of gene flow.

Weedy rice is one of the most notorious weeds found in rice-growing areas throughout the world. It can be defined as any spontaneously and strongly shattered rice that occurs in cultivated rice fields (Xia et al. 2011). Red rice is a conspecific weedy relative of cultivated rice that has been hybridized recurrently, increasing its genetic diversity as well as its adaptability to different rice cultural environments (Langevin et al. 1990). Although it is mainly self-pollinated, it can produce viable and fertile hybrids that exhibit the dominant traits of the parental weed (Langevin et al. 1990; Noldin et al. 1999; Gealy et al. 2002, 2003). It is characterized by superior competitive ability (Diarra et al. 1985; Burgos et al. 2006), protracted flowering and seed maturation, high shattering, varying degrees of dormancy (Cohn and Hughes 1981) and a red pericarp. When red rice is mixed with cultivated rice grains at harvest, it reduces the quality of the white rice grain (Ottis et al. 2005). This constitutes an important economic problem for rice farmers given the major impact that this has on the yield and quality of harvested rice. Crop rotation is the best method for controlling weeds, while spraying herbicides has only a limited effect on the control of red rice weed. However, when crop rotations are not possible, false seeding and puddling may also provide a degree of control (Català 1995; Messeguer et al. 2004).

According to United Nations' estimates, the world population will grow from 6 billion in 2000 to 8 billion in 2025. The relationship between the growth in the world's population and grain production has shifted over the last half-century, with it being possible to divide this period into two distinct sub-periods. From 1960 to 1985, the growth in grain production easily exceeded that of population, with per capita harvests increasing from 279 kg in 1960 to 343 kg in 1985. However, during the following 15 years, the growth in grain production fell behind that of population growth, mainly due to a slower growth in the use of irrigation and fertilization (Khush 2005). Global environmental degradation, in the form of salinization, pollution and global warming (Peng et al. 2004), has also reduced the availability of suitable arable land and water. All of these effects, combined with the high cost of energy-dependent

labour and fertilizers and phytosanitary treatments and their environmental impact, have contributed to the need to promote various strategies aimed at increasing potential rice yields, such as biotechnological research and the development of GM crops (Repellin et al. 2001; Chen et al. 2009).

A large number of transgenes, which code for a wide variety of traits, have been successfully transferred to different crop varieties using transgenic biotechnology. Biotech crops are the most rapidly adopted crop technology in the history of modern agriculture, with a 94-fold increase in their total area of cultivation: from 1.7 million hectares in 1996 to 170.3 million hectares in 2012 (James 2012). Thirty-five official field trials involving transgenic rice were carried out in the European Union between 1998 and 2006, and 264 GM rice field trials had been conducted in the USA before 2010 (GMO-Compass 2010). Although GM rice has not been officially commercialized to date, GM rice cultivation is expected to spread rapidly to China, India, Indonesia and the Philippines in the near future.

Potential risks and benefits of GM crops must be identified, tested and quantified before their commercialization, like happens with all new technologies. One of the major concerns of the different stakeholders is the proper risk assessment of adventitious presence of transgenic material in conventional fields due to cross-pollination. The importance of gaining a better knowledge of rice gene flow has become acutely significant with the rapid development of genetically engineered rice.

Rice is a self-compatible autogamous plant, but pollen-mediated outcrosses occur when different cultivars or subspecies are grown close enough together and/or when their flowering periods overlap. Given the ecological and economic importance of rice crops worldwide, the transgene flow from genetically engineered crops to other cultivars, or to their wild and weedy relatives, is currently one of the major concerns for ecologists, particularly given the risks associated with the commercial release of transgenic plants (Messeguer 2003).

In field trials performed in Europe (Messeguer et al. 2001, 2004), the pollen-mediated gene flow from GM rice plants to conventional ones showed values that were always <0.2 % for immediately neighbouring plants and <0.0125 % for those located at a distance of 10 m, but these results were strongly influenced by prevailing winds. In field trials performed by other authors, the gene flow detected was generally <1 % for GM and non-GM crops separated by a certain distance (Rong et al. 2007; Chun et al. 2011) and even when GM and conventional plants were mixed together on the same plot (Rong et al. 2005; Jia et al. 2007).

Genetically modified pollen-donor rice plants can transfer GM traits to red rice receptor plants by what we call

direct gene flow; this subsequently results in highly shattered and dormant GM red rice seeds. Studies using glyphosate- and glufosinate-resistant GM donor plants point to low direct gene flow outcrossing rates in red rice. Noldin et al. (2002) assessed adjacent rice plots in Brazil, obtaining pollen-mediated gene flow rates ranging from 0.14 to 0.26 %, depending on the red rice ecotype in question; Chen et al. (2004) reported a rate of between 0.01 and 0.05 % in Korea using mixed red rice and GM rice (1 : 3 respectively) plots; in Colombia the rate ranged from 0.03 to 0.3 % (Lentini and Espinoza 2005); and in Catalonia, a cross-pollination rate of 0.036 % was detected when using side-by-side lines (Messegueur et al. 2004). Gene flow between red rice and conventional rice varieties has also been studied using mutant (non-transgenic) imidazolone herbicide-resistant rice lines (Clearfield™ rice); the rates observed were also <1 % using different field trial designs such as adjacent plots (Estorminos et al. 2003a, b), mixed plants (Shivrain et al. 2006) or concentric circles (Shivrain et al. 2007).

Reverse gene flow, which is the gene flow from red rice to GM plants, could transfer dominant weedy traits to GM cultivars and potentially result in the emergence of GM red rice. The GM content in a given red rice population that could be associated with direct gene flow is relatively easy to monitor using herbicide tolerance as a GM trait marker. However, quantifying the reverse gene flow from red rice is more complicated and requires the identification of certain specific red rice traits. Reverse-flow hybrid seeds (F1) look exactly like pollen-receptor cultivar grains because some of their seed characteristics are of maternal inheritance (Van and Jin 2010). The weedy traits present in seeds are therefore not expressed until the following generation (F2). Vigour and height are the only phenotypic characters of reverse flow that are detectable in F1 seedlings during the initial stages of growth. Pericarp colour and shattering cannot be checked until F1 plants have completed their maturity stage. In this study, we therefore thought it necessary to use a molecular technique to verify our phenotypic results. A number of different molecular techniques can identify these specific traits, but when we are seeking the fingerprints of very closely related varieties, or when we are looking for certain characteristic differences, it is essential to obtain a very high level of polymorphism (Folkertsma et al. 1996; Janssen et al. 1996). Amplified fragment length polymorphisms (AFLPs) serve this purpose and have proven to be effective, robust, low in cost and easy to set up for any species (Becker et al. 1995; Maughan et al. 1996; Xu et al. 2000; Gort 2010); they have also been used successfully in rice DNA fingerprinting (Mackill et al. 1996; Zhu et al. 1998; Federici et al. 2001; Saini et al. 2004). The red rice fingerprint markers generated in this study made it feasible to distinguish

any red rice hybrids. This tool also helped to establish reverse gene flow rates by giving molecular strength to empirical studies based on phenotypic characters.

Red rice is usually taller than the cultivated rice varieties with which it grows and probably also offers advantages in terms of pollen dispersal (Sales et al. 2007). In this study, we investigated the levels of direct and reverse gene flow between GM and red rice under Mediterranean field conditions. In field trial 1 (Messegueur et al. 2004), we evaluated the direct gene flow (GM to red rice) when red rice and GM rice plants were grown side by side. Here, in field trial 2, we were interested in estimating the effect of the distance between the GM and red rice gene flow, although we also quantified direct gene flow from GM to conventional rice plants. We did this in order to be able to compare the results obtained in the two field trials. The reverse gene flow (from red rice to GM plants) was also quantified in both trials.

Methods

Plant material

Red rice seeds were supplied by the IRTA Experimental Station (Amposta, Tarragona). They were harvested in a paddy field in which the Senia variety (Català et al. 2007) had been cultivated for several years.

The pCAMBIA3301 plasmid (provided by the Center for the Application of Molecular Biology to International Agriculture (CAMBIA) in Canberra, Australia) was used. This plasmid bears a T-DNA containing the *bar* gene encoding phosphinothricin acetyl transferase driven by the 35S promoter, which confers tolerance to the herbicide ammonium glufosinate, and the *gusA* gene encoding β -glucuronidase driven by the 35S promoter. The plasmid was introduced into the EHA105 *Agrobacterium* strain (Hood et al. 1993), which in turn was used for the co-culture of seed-embryo-derived embryogenic calli of the Spanish *japonica* rice cultivars Senia, as described by Pons et al. (2000).

The transgenic line S 1B was used in both of the field trials described below. It was derived from the transformation event S-B and exhibited both resistance to ammonium glufosinate and expression of the *gusA* gene. Segregation studies carried out with T1 progenies obtained by self-pollination of the primary transformant S-B gave rise to a Mendelian segregation ($\sim 2/3$ resistant and $1/3$ sensitive), which indicated that the transferred DNA had been successfully integrated into the plant genome. T1 plants that were homozygous for the *bar* gene were identified by ammonium glufosinate spraying and histochemical β -glucuronidase (GUS) assays of leaf tissue from their T2 progeny. All 48 T2 plantlets tested for the S 1B line exhibited full resistance to the herbicide and a strong expression of

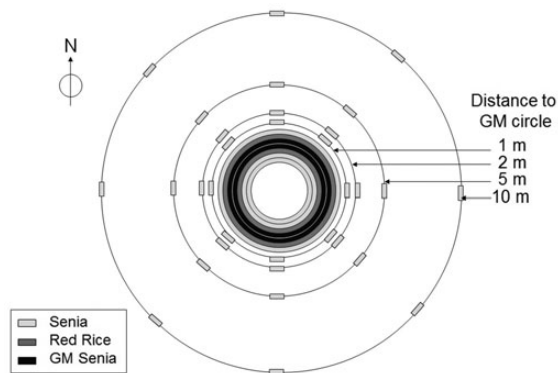


Figure 1. Field trial 1 design: scheme detailing the composition of the circles. One hundred and seven GM Senia plants forming two circles (of 57 and 50 plants) were planted next to 107 red rice plants organized in two circles (63 and 44 plants) to evaluate the resulting gene flow. The circular distribution of the plants was designed to maintain the 1 : 1 plant ratio and to neutralize the effect of the prevailing SW wind. Non-GM Senia plants were also planted at different distances. The results of direct gene flow from the GM Senia to the red rice and Senia plants were published in [Messeguer et al. \(2004\)](#).

the *gusA* gene; this showed the homozygous status of both genes. Detailed Southern blot analyses of the S 1B transgenic line, using appropriate restriction enzymes and probes consisting of *bar* and *gusA* coding sequences, revealed that S 1B contained insertions at a single locus of two T-DNA copies, both of which contained the *bar* and *gusA* genes ([Messeguer et al. 2004](#)).

Field trials

A first field trial (field trial 1) was carried out in order to assess gene flow between transgenic and conventional rice and red rice. Seedlings were transplanted to the field in concentric circles with inter-plant distances of 25 cm. The inner circle (3 m diameter) was planted with non-transgenic plants and surrounded by a circular arrangement of red rice plants. This, in turn, was surrounded by two successive circular arrangements of transgenic plants. There was then a circular arrangement of red rice plants and then a final circular arrangement of non-transgenic plants (Fig. 1). This field trial was also used to quantify the reverse gene flow from red rice to GM plants. In this case, 27 GM ammonium glufosinate-resistant Senia plants from the field trial were harvested at the ripening stage and 500 viable seeds from each plant were sown on trays in a peat-vermiculite substrate. At the 3- to 4-leaf stage, the seedlings showing the fastest growth rate were isolated for subsequent analysis by AFLP and cultivated to obtain progeny for subsequent phenological analysis.

A second field trial (field trial 2) was also performed (Fig. 2). The design of field trial 1 was modified in order to better detect the frequency of reverse gene flow and

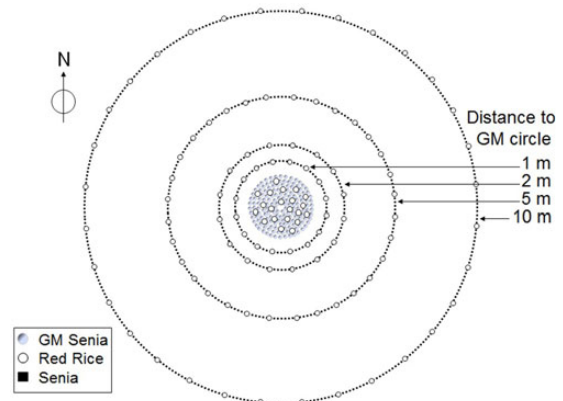


Figure 2. Field trial 2 design: scheme detailing the composition of the circles. One hundred and forty-nine transgenic S 1B plants, arranged in seven concentric inner circles, formed a 3.5-m-diameter transgenic nucleus. Non-transgenic Senia plants were planted in the external concentric circles at distances of 1, 2, 5 and 10 m from transgenic plants. Twenty-one red rice plants were grown in the transgenic inner nucleus (density $2.3 \text{ plants m}^{-2}$), 16 red rice plants were grown in each of the circles that were 1 and 2 m from the centre, and 32 red rice plants were grown in each of the circles located at distances of 5 and 10 m from the centre.

also to study the effect of distance on direct gene flow from GM rice to red rice (Fig. 2). Transgenic S 1B T2 homozygous seeds, certified non-transgenic Senia seeds and red rice seeds were all sown in a peat-vermiculite substrate under greenhouse conditions and then transplanted to a paddy field when they reached the 4- to 5-leaf stage. One hundred and forty-nine herbicide-resistant transgenic Senia plants were placed in seven concentric inner circles that formed a 3.5-m-diameter transgenic nucleus. Non-transgenic isogenic Senia plants were planted in concentric circles at distances of 1, 2, 5 and 10 m from the inner circle. In total, the numbers of Senia plants planted in the circles at distances of 1, 2, 5 and 10 m were 53, 78, 136 and 262, respectively. Twenty-one red rice plants were grown in the transgenic inner nucleus (at a density of $2.3 \text{ plants m}^{-2}$). Sixteen red rice plants were also homogeneously spaced in each of circles 1 and 2 (which were located 1 and 2 m from the centre, respectively) and 32 red rice plants were similarly spaced in each of the other circles (located 5 and 10 m from the centre). The trial was conducted according to standard seed production practices. After flowering, the red rice panicles were covered with a mesh to prevent seed dissemination. Panicles from all of the plants were harvested manually and individually, and their respective geographic locations were recorded. In both trials, wind speed and direction were measured using a Delta-T Type AN1 anemometer and a Delta-T Type Wd1 potentiometer, which had been placed in the middle of the field and just a few centimetres above the spikes. Wind speed and direction data were



Figure 3. Appearance of seedlings after herbicide treatment. Seedlings at the 3- to 4-leaf stage were treated with a commercial herbicide (Finale from AgrEvo Co.) at a dosage equivalent to 800 g of active ingredient per hectare. The trays of seedlings coming from conventional (non-transgenic) plants appear pale yellow while transgenic trays (blue arrows) are completely healthy and green. Few direct gene flow herbicide-resistant individuals can be seen among conventional seedlings (green plantlets).

registered using a Delta-T Datalogger (Delta-T Devices Ltd, Bruwell, Cambridge, UK). Both field trials were approved by the Spanish Biosafety Commission (ref. B/ES/00/07 and B/ES/01/07).

Analysis of direct gene flow

Harvested seed samples from each non-transgenic *Senia* and from the red rice plants were sown in a greenhouse in $48 \times 28 \times 7$ -cm trays containing a peat-vermiculite substrate. The average germination rate was used to weight seeds in order to average values for 500 seedlings per tray. Transgenic *Senia* S 1B seeds harvested in field trial 2 were also sown as a positive control. Seedlings at the 3- to 4-leaf stage were treated with a commercial herbicide (Finale from AgrEvo Co.) at a dosage equivalent to 800 g of active ingredient per hectare (Fig. 3). After 3–4 weeks, all the surviving seedlings were transferred to individual pots for histochemical GUS assays, further development and the final harvest.

Analysis of reverse gene flow

Seedlings from S 1B plants were used to detect reverse gene flow. Progenies from 27 GM ammonium glufosinate-resistant *Senia* plants from field trial 1 were sown in $48 \times 28 \times 7$ -cm trays containing a peat-vermiculite substrate. The average germination rate was used to weight seeds in order to average values for 500 seedlings per tray. A glufosinate treatment (Finale from AgrEvo Co.), with a dosage equivalent to 800 g of active ingredient per hectare, was carried out at the 4- to 6-leaf stage to verify that all the seedlings tested came from homozygous glufosinate-resistant transgenic plants



Figure 4. Appearance of reverse-flow seedlings after herbicide treatment. A tray of transgenic herbicide-resistant seedlings was compared with non-transgenic trays. The circled PRF seedling is taller than the others.

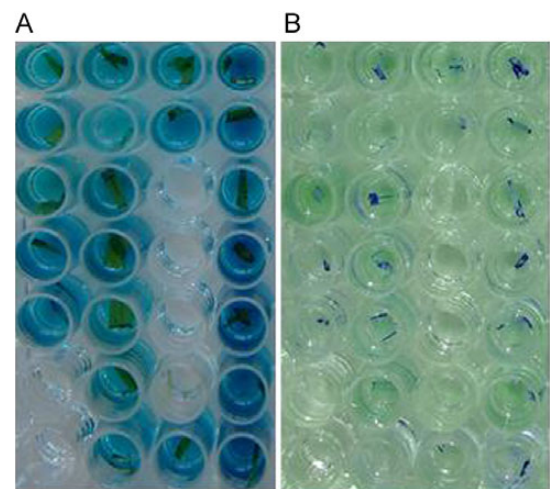


Figure 5. GUS staining of some reverse-flow plants from field trial 1. Samples incubated for 24 h at 37 °C with GUS staining solution (A) and the same samples after chlorophyll extraction by soaking the tissues in 70 % EtOH for 24 h (B).

(Fig. 4). It was observed from the field trial 1 assay that a few seedlings (43 in total) grew faster and more vigorously than the others (Fig. 4). These seedlings (which we called putative reverse-flow or PRF seedlings) were planted in pots for GUS expression analysis (Fig. 5) and AFLP fingerprinting analysis. Dehiscence and grain colour were recorded at the ripening stage.

The selection of PRF from field trial 2 seedlings was more exhaustive. In this assay, 250 seedlings were grown in each tray in order to discard substrate competition. After applying the herbicide treatment, at the 4-leaf stage, the tallest plants were selected from the trays containing herbicide-resistant descendent seedlings. To be sure that almost all the PRF seedlings were analysed, the tallest and most vigorous seedlings from those remaining were also selected a few days later. In this case, two levels of

vigour selection criteria were therefore used. All these PRF seedlings were analysed using AFLPs and planted in pots until the ripening stage in order to obtain the resulting grains and samples for phenotypic analysis confirmation.

AFLP analysis

Seedlings from transgenic Senia plants and red rice plants were used to determine their molecular fingerprint pattern by identifying their specific polymorphic peaks by AFLP. A DNA pool from 10 different Senia plants and a further DNA pool from 10 red rice plants were first purified and then analysed. DNA was double digested with EcoRI and MseI, following the protocol of Vos et al. (1995). Labelled fragments were then run on an Abi-prism 310 Automated DNA Sequencer (Perkin Elmer–Applied-Biosystems) and analysed using GENESCAN Analysis Software 2.0. The three differential peaks presented in the red rice pattern were identified using MseIAAG/EcoRICAC, MseIAGC/EcoRICAA and MseIACA/EcoRICAC primers. The samples used in red rice and Senia DNA pools were then separately analysed, plant by plant, in order to detect useful polymorphic peaks. Three clearly reproducible and non-overlapping polymorphic peaks were selected as a red rice fingerprint. All of the red rice samples analysed presented all of these red rice polymorphic peaks, whereas none of the Senia samples exhibited any of them. The PRF samples were then analysed, including Senia pools and red rice pools as controls.

Histochemical glucuronidase assay

Leaves from the seedlings that survived the herbicide treatment were subsequently assayed for expression of the *gusA* gene; this was carried out following the histochemical staining procedure described by Jefferson et al. (1987) but using a modified extraction buffer described by Van Altvorst et al. (1995) and reducing the ferrocyanide solution 10-fold. After incubation for 24 h at 37 °C, chlorophyll was extracted by soaking the tissues in 70 % EtOH for 24 h (Fig. 5).

Results

Field trial 2 (Fig. 2) was initially designed to detect the effect of distance on direct gene flow (from GM to red rice) and also to quantify the reverse gene flow (from red rice to GM rice) affecting immediately neighbouring plants. The gene flow from GM to conventional rice was also evaluated so that we could compare our results with those obtained in field trial 1 (Messegueur et al. 2004), in which the direct and reverse flows were also quantified.

The agronomic behaviour of transgenic and non-transgenic Senia plants and red rice plants was as expected. The time to 50 % heading for both transgenic

and non-transgenic Senia plants was 7 weeks after the plants were transferred to the soil, and flowering was fully synchronous between the non-transgenic and transgenic plants. The 50 % grain ripening stage occurred approximately 3 months after planting. However, the red rice flowered 3–4 days before the Senia plants (Table 1). Random analysis of 100 spikes, conducted on a daily basis, showed that the overlap between the flowering periods of transgenic and red rice was limited to a 13-day period during which cross-pollination could have taken place. As expected, the red rice plants were more vigorous and produced more spikes than the cultivated Senia variety. During early stages of development (28 days of culture), seedlings from the red rice plants were already taller and more vigorous than those from the GM and conventional plants.

Effect of wind and distance

During field trial 2, wind sensors were set up at the rice panicle level at flowering time and they recorded data on a daily basis throughout the trial. Figure 6 shows the average daily wind run for each of the eight points of the compass. Wind run is calculated by multiplying wind speed by the frequency with which the wind blows in a given direction. This measurement shows the amount of wind passing through the station during a certain period. The total amount of wind passing the meteorological station from the south-west reached a daily distance of 25.2 km. This represented the maximum distance that an imaginary weightless particle could have travelled in a day when propelled by the wind.

To quantify the gene flow from GM to conventional rice, we analysed 290 000 Senia seedlings harvested from plants that had been located at different distances from the GM pollen source (Fig. 2); of these, 61 incorporated the transgenic trait (0.021 %). We observed a clearly asymmetric distribution of the results obtained for the circles located at distances of 1, 2, 5 and 10 m. When the circles located at different distances were analysed by applying the Watson one-sample U^2 test, the null hypothesis

Table 1. Field trial 2. Flowering date and plant height at 28 days and at the ripening stage.

Variety	Flowering date	Height (cm) at 28 days	Height (cm) at the ripening stage
GM Senia	27 July	23.18 ± 0.59	90.2 ± 1.3
Conventional Senia	28 July	21.48 ± 0.14	88.7 ± 0.4
Red rice	24 July	25.52 ± 0.53	107.2 ± 0.7

(data from a given population are uniformly distributed around the circle) was rejected in each case ($U^2 = 4.0207$, $P < 0.0005$), which suggested the presence of a vectorial factor. It was therefore possible to attribute the asymmetric distribution to the direction of the prevailing

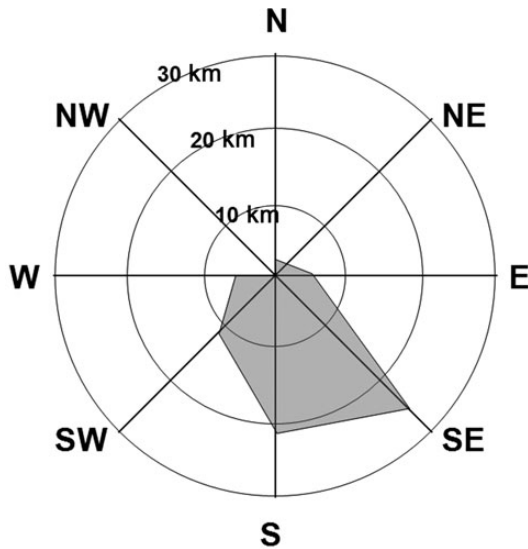


Figure 6. Average daily winds for each of the eight compass points. Wind run was calculated by multiplying the wind speed by the frequency with which the wind blew in a given direction. This measurement provides a good indication of the ‘amount’ of wind passing the station during a given period. The total ‘amount’ of wind passing the meteorological station from the south-west covered a daily distance of 25.2 km; this was the maximum distance that an imaginary weightless particle could have travelled per day when propelled by the wind.

wind at flowering time. The locations of the transgenic seedlings with respect to the direction of the prevailing wind are shown in Fig. 7. The differences in percentages of transgenic seedlings when comparing the north-west (NW) quadrant with the other three quadrants were quite large and could be explained by the influence of the prevailing wind blowing from the south-east. These results agreed with those obtained in field trial 1, previously published in Messegueur et al. (2004).

In field trial 2, the red rice plants were not only located in the central nucleus but also in different circles and at different distances (Fig. 2). We were therefore able to extrapolate the influence of distance and wind direction on the gene flow that would have occurred between GM fields and red rice plants located along the borders or in neighbouring fields. Five hundred seedlings per red rice plant were analysed (8000 red rice seedlings from the circles located at distances of 1 and 2 m from the transgenic nucleus, and ~16 000 red rice seedlings from the circles located at distances of 5 and 10 m). The rate of gene flow in each circle was very low. In Fig. 7, each point represents the average %GM flow detected per analysed plant progeny and its standard error. The values obtained also presented a clear asymmetry, with the greatest accumulation being observed in the NW quadrant. When the Watson one-sample U^2 test was applied to all the values, the null hypothesis was rejected ($U^2 = 3.449$, $P < 0.002$), also suggesting the effect of the prevailing wind at flowering time. The gene flow rates from the GM rice to both the conventional Senia rice and the red

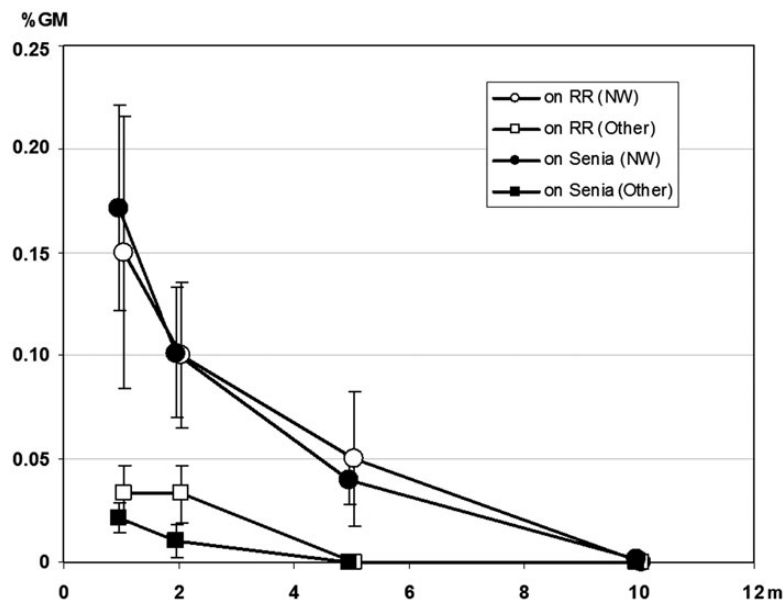


Figure 7. Transgene flow detected in Senia and red rice plants. Each point represents the average %GM flow detected per analysed plant progeny with its standard error. The seedlings analysed were taken from plants growing at different distances. In total, 61 herbicide-tolerant Senia seedlings, out of a total of 290 000 treated plants, and 22 herbicide-tolerant red rice seedlings, out of 57 500 treated plants, were identified. The NW direction (the direction of the prevailing wind) corresponds to plants located in the quadrant delimited by west and north.

rice that were assessed in field trial 2 were very similar (Fig. 7). This could be explained by the fact that the red rice used in these field trials was an ecotype collected from paddy fields in which the Senia variety had been cultivated for several years. During this period, there had probably already been some cross-pollination and, in consequence, this red rice strain may have already acquired some of the agronomic characteristics of the Senia variety.

In field trial 2, the gene flow values were very low and at a distance of 10 m we did not identify any resistant individuals among the 16 000 red rice seedlings analysed. This result demonstrates that the gene flow to red rice was dramatically reduced by distance. It also suggests that it would have been very unlikely for transgenic traits to have been transferred to red rice plants located on the field borders or in neighbouring fields.

Red rice: direct gene flow

The direct gene flow from the GM rice plants to the red rice was evaluated in the central nucleus of field trial 2 where the red rice plants were totally surrounded by GM rice plants. There, the proportion of GM rice to red rice was 7 : 1. This corresponded to 2.3 red rice plants m^{-2} , which would be considered a high infestation rate under agronomic conditions. We were only able to detect a low degree of asymmetry in the distribution of transgenic seedlings and this was mainly found in the NW quadrant. In this case, the Watson one-sample U^2 test gave a significant result ($U^2 = 1.84, P < 0.05$), which suggested that the prevailing wind also influenced direct gene flow.

The herbicide treatment and subsequent GUS assay confirmation showed a direct gene flow rate of 0.137 ± 0.038 % in field trial 2 (13 seedlings from 9500 analysed) (Table 2), which was higher than 0.036 ± 0.001 % found in field trial 1 (Messegueur et al. 2004). This may be explained by the different design used in the field trials because in the second trial there was a higher density of GM plants. The proportion of GM rice to red rice in field trial 1 was therefore 1 : 1, whereas in field trial 2 it was 7 : 1.

Red rice: reverse gene flow

Viable seeds (13 500 in total) from 27 herbicide-resistant GM Senia plants (line S 1B progenies) from field trial 1 were sown and treated with herbicide to confirm that all of them were transgenic (Table 2). One week later, 43 PRF seedlings were selected from among them. All of these seedlings grew faster than the others and expressed the *gusA* gene (Fig. 5), but only 30 hybrid transgenic red rice plants were confirmed by AFLP analysis (Fig. 8). The percentage of reverse flow per plant was 0.222 ± 0.028 . These plants were then grown until maturity to study their progeny.

Table 2. Results for direct and reverse gene flow between GM Senia and red rice. Percentages are noted on a plant-by-plant basis with the respective standard errors. *This standard error was then recalculated from the original data. The previously published SE value (0.036 ± 0.006) was calculated by grouping plants according to wind direction according to the compass (Messegueur et al., 2004).

	Field trial 1	Field trial 2
Total GM Senia plants	107	149
Total red rice plants	107	21
Ratio of GM Senia : red rice	1 : 1	7 : 1
Direct flow		
Red rice plant progenies analysed	107	19
Total GM seedlings detected	46	13
%GM flow per plant	$0.036 \pm 0.001^*$	0.137 ± 0.038
Reverse flow		
GM Senia plant progenies analysed	27	41
Total 'wild' seedlings detected	30	46
% reverse flow per plant	0.222 ± 0.028	0.448 ± 0.056
T-test (H_0 : Reverse = Direct)	$P < 0.001$	$P = 0.039$

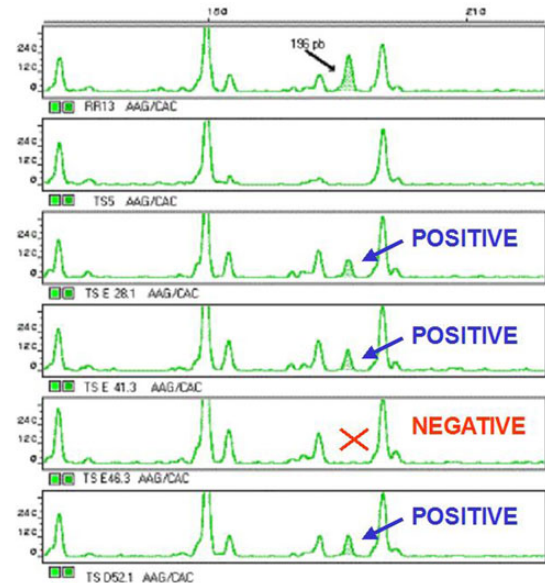


Figure 8. The AFLP pattern results representing a small area in which a 196-bp polymorphic peak was detected using MseI AAG/EcoRI CAC primer combinations. From top to bottom: red rice sample showing the positive hybrid pattern, Senia sample showing the negative hybrid pattern, and four samples out of the 43 PRF (30 positive and 13 negative) investigated samples.

All the PRF seedlings with red rice traits confirmed by AFLP analysis had red rice offspring, which exhibited red pericarps and signs of dehiscence. These results confirmed

the effectiveness of AFLP analysis for the detection of seedlings produced by reverse gene flow. However, bearing in mind that the sowing density was quite high (0.38 viable seeds cm^{-2}) some hybrids could have escaped PRF selection due to substrate competition between plantlets. Here, seed dormancy could not have had any effect on our results because only germinated seeds were taken into account. Calculations of the percentage of gene flow were therefore not based on the number of seeds sown but rather on the number of seeds that germinated.

Viable seeds ($10\,250$ in total) from 41 GM herbicide-resistant Senia plants collected in field trial 2 were sown and treated with herbicide (Table 2). In total, 82 PRF plants were analysed. In a first selection, the 37 tallest seedlings were analysed by AFLP and it was found that they were all transgenic red rice hybrid plants. In a second selection, the 45 tallest seedlings from the remaining seedlings were analysed. In this case, only nine of the seedlings were found to be transgenic red rice hybrid plants. The percentage reverse flow per plant was 0.448 ± 0.056 . Pericarp colour and dehiscence analysis showed that all 46 plants were transgenic red rice plants.

Although the sowing density of the GM progenies in field trial 2 was only half of that used in field trial 1, it is still possible that some hybrid plants may not have grown as quickly as expected and that, in consequence, they would not have been selected for further analysis. In this sense, and taking this factor into account, we can confirm that the reverse flow rate was at least 0.448 ± 0.056 % when the weed infestation density was 2.3 plants m^{-2} and the test was conducted under Mediterranean environmental conditions.

In both our field trials, the reverse gene flow was greater than the direct gene flow. The percentages were registered on a per-plant basis. The *t* test (Table 2) showed a significant difference between means, with the values being greater for the reverse gene flow in both cases.

Discussion

Numerous studies have been conducted to quantify pollen-mediated gene flow from transgenic rice under field conditions (Lu and Yang 2009). This review shows that the frequency of rice-to-rice gene flow was generally low (<1 %). Gene flow frequency may, however, fluctuate substantially, depending on several factors including the distance between the pollen donor and the recipient, wind direction and speed, and sexual compatibility. In field trial 2, we quantified the gene flow between transformed and non-transformed examples of the same variety. Even though there was full sexual compatibility between the GM and conventional rice, the rate of gene flow detected was very low (0.021 %) and clearly

influenced by wind and distance. Chun *et al.* (2011) had previously reported a crop-to-crop gene flow of 0.039 % at a distance of 0.5 m and one of 0.0007 % at 7 m. Rong *et al.* (2007) obtained similar results, with crop-to-crop gene flow ranging from 0.28 % at a distance of 0.2 m to <0.01 % at 6.2 m. In field trial 2, according to the direction of the prevailing wind, we observed gene flow values ranging from 0.15 % at 1 m to 0.001 % at 10 m. With other wind directions, the registered gene flow values were much lower (ranging from 0.07 % at 0.2 m to 0 % at 10 m). These values were fully comparable with those obtained in field trial 1 (Messegueur *et al.* 2004) in which the detected gene flow rates were also very low, decreased with distance and were strongly influenced by the direction of the prevailing wind during the flowering period.

As with many other weed varieties, red rice has complex patterns of dormancy and asynchronous germination and, in consequence, red rice flowering could occur at almost any time during the crop season. Nevertheless, the majority of red rice seeds usually germinate when the climatic conditions are most favourable. As a result, red rice tends to flower at almost the same time as its cultivated counterpart in many rice-growing regions. Spontaneous hybridization between cultivated rice and red rice often occurs in the same fields, as reported by Xia *et al.* (2011). In field trial 1, we found a cross-pollination rate of 0.036 ± 0.001 % between GM and red rice when the herbicide treatment was administered and subsequent GUS assay confirmations were performed. This cross-pollination rate was strongly influenced by wind direction (Messegueur *et al.* 2004). In field trial 2, the direct gene flow detected via the herbicide treatment and subsequent GUS assay confirmation was 0.137 ± 0.038 % (when 13 seedlings out of 9500 red rice seedlings were identified), which was a little higher than in field trial 1.

This difference could be explained by the different field trial designs. In field trial 1, red rice and GM plants were sown in concentric circles in such a way that they were only in direct contact along one side. In contrast, in field trial 2, the red rice plants were sown in the centre of the transgenic nucleus and, in consequence, they were completely surrounded by GM plants (Fig. 2). However, perhaps the most important factor influencing the gene flow rate could have been the different proportion of transgenic to red rice plants. In field trial 1, the ratio was $1:1$, whereas in the central nucleus of field trial 2 this ratio was $7:1$. Gene flow is the result of competition between the pollen produced by the plant and that coming from outside. This means that gene flow is influenced not only by distance but also by the respective proportions of the donor and receptor pollen plants. The results obtained in the field trials that are presented here clearly show that direct gene flow rates vary according

to the relative proportions attributable to the donor (GM rice) and receptor (red rice) plants.

In field trial 2, the exact position of each red rice plant was also recorded. This allowed us to detect the influence of the prevailing wind, which, in turn, explained the asymmetric distribution of the hybrid seeds detected.

Few published studies have investigated the influence of distance from the pollen source on outcrossing rates involving red rice. Most of the hybrids detected in the field trial carried out by [Shivrain *et al.* \(2007\)](#), who used Clearfield™ (CL) rice, were located within 1 m of the CL rice pollen source. However, a few hybrids were found at distances of up to 6 m from the source; this was the greatest distance at which hybrids were detected in those experiments. [Chun *et al.* \(2011\)](#) reported a decreasing gene flow rate from herbicide-tolerant GM Dongjin rice to weedy rice, with values ranging from 0.024 % at a distance of 0.5 m to 0.0025 % at 7 m. However, weedy rice received higher gene flow than any of the other pollen-receptor cultivars studied except for the Dongjin isogenic conventional line. This was despite there being only a small overlap between the flowering periods of red rice and Dongjin. [Chun *et al.*](#) suggested that wind speed and direction helped the flow of GM pollen to weedy rice. In our study, however, the cross-pollination rate between GM and red rice decreased with distance and was clearly influenced by the direction of the prevailing wind (Fig. 6).

With respect to the direction of the prevailing wind and other wind directions, direct gene flow rates from GM Senia plants to red rice were very similar to those observed from GM Senia plants to the conventional Senia cultivar. Several authors, including [Xia *et al.* \(2011\)](#) and [Jiang *et al.* \(2012\)](#), have analysed several weedy rice populations from China and Italy and detected a certain degree of allelic introgression from rice cultivars to coexisting weedy rice. In fact, the gene flow from rice cultivars to weedy rice is well documented and it has been demonstrated that different weedy rice populations exhibit a degree of genetic differentiation. This differentiation is linked to hybridization with the coexisting rice cultivar ([Lu and Yang 2009](#); [Xia *et al.* 2011](#); [Jiang *et al.* 2012](#)). In these studies, the pollen donors were different rice cultivars and the receptor was weedy rice. In our study, the red rice ecotype used was collected from a field in which the Senia variety had been grown for a long time. It was therefore highly probable that this ecotype had introgressed some characteristics from the coexisting Senia rice, thereby making it more compatible with this variety.

[Xia *et al.* \(2011\)](#) studied conspecific crop–weed introgression in China, and found that outcrossing rates can significantly affect the heterozygosity of populations, which may shape the evolutionary potential of weedy rice. Introgression from the conspecific crop rice can influence the

genetic differentiation and possibly evolution of its coexisting weedy rice populations. In the Delta del Ebro Mediterranean region no natural coexisting weedy rice species were found until direct seeding was adopted during the 1970s and red rice started to be a problem at the end of the 1980s ([Català 1995](#)). In addition, the red rice strain used in this study has been obtained from a field where the Senia variety had been cultivated for several years.

In our study 102 clearly distinguishable AFLP fingerprint peaks shared by all Senia, red rice and analysed PRF plants were obtained after revising AFLP data. This result contrasts with only three polymorphic peaks exclusive for the red rice. It suggests that the red rice strain used in this study is genetically highly similar to its coexisting counterpart as we suspected.

There are two ways to obtain GM red rice weed. In one scenario, GM plants can pollinate red rice weeds producing shattered and dormant GM weedy seeds. This is what we have called direct gene flow to red rice. However, in the other scenario, red rice can pollinate GM rice through reverse gene flow.

In this study we have quantified this reverse flow. Recently formed reverse-flow hybrid seeds look exactly like GM Senia seeds due to maternal inheritance of seed characteristics. The most important phenotypic character that was detectable in reverse-flow seedlings was vigour. As shown in Table 1, the red rice ecotype used in this study grew faster than GM and conventional rice, and reverse-flow seedlings would be expected to acquire this characteristic. We therefore initially selected PRF seedlings based on this criterion. The PRF plants were analysed using AFLPs. This high-resolution and low-cost technique has previously proven useful for the rapid screening of closely related rice strains and the generation of a pattern with replicable markers ([Zhu *et al.* 1998](#)). The PRF plants were transplanted and grown until seed maturity for further shattering and pericarp colour analysis. In our study, the AFLP results matched the phenotypic results in all cases.

The reverse gene flow detected in field trial 1 was six times greater than the direct gene flow detected in the same trial. Even so, this result had no real agronomic relevance in this case, because the proportion of GM to red rice plants in this trial was 1 : 1 and no commercial field would ever contain such a high proportion of weedy rice because it would not be profitable.

The central nucleus in field trial 2 was designed to simulate a real situation in a commercial field with a high infestation rate of red rice (2.3 plants m⁻²). Although the red rice plants had a clear numerical disadvantage with respect to the GM rice (1 : 7), reverse gene flow from the red rice to the GM rice was more than three times greater than the direct gene flow from the GM rice to the

red rice ($0.4488 \pm 0.056\%$ and $0.137 \pm 0.038\%$, respectively). As reported by other authors (Diarra et al. 1985; Langevin et al. 1990; Noldin et al. 1999; Estorninos et al. 2005), red rice plants are usually taller and more vigorous than agronomic varieties. In our study, differences in height could be detected in the early stages of development and by the flowering stage (Table 1); some plants were more than 15 cm taller than others. This difference in size could be an advantage for red rice as it would enhance pollen dissemination, but it would be a disadvantage for direct gene flow in which pollen grains from GM plants would have to reach stigmas from taller red rice plants.

The ecological consequences of reverse gene flow are limited in comparison with those of direct gene flow because non-shattered and non-dormant seeds would be obtained in the first generation due to the maternal inheritance of these characters (Van and Jin 2010). These consequences are significantly dependent on agricultural practices and on the presence or absence of natural *Oryza* biodiversity in the cultivation area. In developed countries, where farmers usually cultivate certified seeds, the reverse gene flow from red rice could have only limited ecological consequences, because the hybrid seed would remain in the spike and most of it would be removed during harvesting. There would be only a very small possibility of one seed falling to the soil and becoming a GM weed. Nevertheless, this phenomenon must be taken into consideration in fields used for elite seed production. However, in developing countries, and most of the area where there is natural biodiversity of *Oryza*, most farmers do not cultivate certified seeds. Farmers often keep some seeds for planting the following year. In this case, the probability of the proportion of GM red rice weed increasing from year to year is higher and, in consequence, a proper monitoring plan should be established.

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Contributions by the Authors

E.M. conceived the experiments and the field trial designs and J.M. directed the work. J.M. and G.P. obtained the herbicide-resistant Senia lines. M.M.C. performed the field trials and provided the red rice line. E.M. and R.E. performed the herbicide treatment. R.E. and X.S. performed the AFLP analysis. G.P. and X.S. carried out the GUS stainings. E.M. carried out the statistics. X.S. drafted the manuscript. The final manuscript main contributors were X.S., R.E., J.M., E.M. and G.P. All authors read and approved the final manuscript.

Conflicts of Interest Statement

None declared.

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Chapter 2.

A Mediterranean *japonica* rice (*Oryza sativa*) cultivar improvement through anther culture.

Resum

Els productors de llavor certificada seleccionen i propaguen any rere any i de forma sistemàtica les varietats registrades per així mantenir-ne la uniformitat i els caràcters originals. Tot i així pot aparèixer variabilitat degut a mutacions naturals, encreuament espontanis entre varietats i la contaminació accidental amb llavors de diferent varietat. NRVC 980385 es una varietat d'arròs japònica (*Oryza sativa ssp. japonica*) registrada l'any 2002. L'any 2005 els tests de certificació de llavor van detectar una parcel·la que diferia clarament de l'original en termes d'uniformitat i alçada suggerint la presència d'una certa heterozigosi. Aquest material va ser vist com una oportunitat per ser emprat en l'obtenció noves línies dihaploids estabilitzades que podrien competir en el mercat de llavors estatal d'arròs de gra mitjà.

En aquest estudi es descriu un protocol *In vitro* de cultiu d'anteres seguit d'assajos de camp emprat per a l'obtenció de quatre línies dihaploids de NRVC 980385 noves, millorades i estabilitzades llestes per ser registrades amb finalitats comercials. En total varen caldre quatre anys des de la recol·lecció de les anteres fins als assajos de les línies a gran escala. Aquest protocol escurça el temps d'obtenció línies dihaploids assajades en camp i te avantatges considerables tant en el manteniment les caràcters originals de les varietats registrades com en la generació de noves línies derivades.

A Mediterranean japonica rice (*Oryza sativa*) cultivar improvement through anther culture

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Abstract Certified seed producers systematically select and propagate registered varieties year after year in order to maintain their uniformity and the original registered cultivar traits. However, natural mutations, spontaneous breeding between varieties and alien grain contamination can introduce undesirable variability. NRVC 980385 is a temperate japonica rice cultivar (*Oryza sativa* ssp. japonica) first registered in Spain in 2002. In 2005 certification tests detected a plot differing from the original traits in terms of uniformity and height suggesting the presence of a certain heterozygosis. This material was therefore seen as an opportunity to obtain newly stabilized doubled haploid (DH) lines which could compete in the Spanish short grain seed market. In this study, an in vitro anther culture protocol is defined which also covers the field tests selection to obtain four new, improved and stabilized DH derived lines ready to be registered for commercial proposes. This

took just 4 years from the initial anther collection until new lines were grown in large scale field trials. Consequently, this protocol reduces the time for obtaining field assessed DH lines thereby having considerable advantages over other techniques by both maintaining the original registered cultivars and/or generating new derived varieties.

Keywords In vitro · Anther culture · Rice · Dihaploid · Doubled haploid

Introduction

In Spain, artificial breeding varieties obtained through genealogic selection of ancient Spanish varieties and artificial breeding (Herruzo 1986) started to displace traditional varieties and acclimatized foreign varieties about 1940. In the 1960s new semi-dwarf varieties were obtained to respond to the new production techniques based on mechanization (Campos et al. 1966). In 2000, rice varieties obtained by breeding coupled to genealogic selection started to compete with others obtained through molecular marker assisted-selection and doubled haploid (DH) selection. This techniques help plant breeders to obtain new varieties through a more precise and faster characters selection, and consequently, lots of new genetically improved rice varieties had appeared, reducing cultivar lifetime in the Spanish market (Català et al. 2007, 2009).

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The anther culture technique was first developed in rice by Niizeki and Oono (1968). This technique allows one to obtain completely stabilized DH plants which bypass the inbreeding process (Brar and Khush 2006; Germana 2011). It is the fastest method for DH production as it only takes between 8 and 9 months (Agache et al. 1989). This technique manipulates the male sex cells in immature anthers, to induce haploid *callus* formation, which are subsequently converted to double haploid embryos (Niizeki and Oono 1968). Genetic recombination occurs during haploid sex cell production so that each microspore (immature pollen) which is produced is genetically unique. Consequently, each DH line obtained in this way will produce a new stabilized and unique line. This breeding tool has been used not only to establish parental pure lines saving the long inbreeding process, but also to speed up descendant's selection after an artificial cross, bypassing the classical pedigree selection process (Martinez et al. 1996; Courtois 1993; Moon et al. 2003).

Transgenic pest and herbicide resistant lines have been also developed in public institutes using Spanish rice varieties (Coca et al. 2004; Messeguer 2003; Marfa et al. 2002) with no commercial results to date, although foreign Clearfield herbicide resistant mutant lines have been recently introgressed into mid-grain rice and produced in Spain by Cooperativa de Productores de Semillas de Arroz, S.C.L. (Copsemar, SCL). Its commercialization is expected in the near future, although the ecological concerns about the rapid resistance transmission to weedy rice reported (Shivrain et al. 2008; Kaloumenos et al. 2013; Goulart et al. 2012).

When a rice cultivar has been cultivated for many years, agronomic traits may begin to differ from the original traits as a result of natural mutations and spontaneous breeding between cultivars due to seed stock contamination in storage works, mechanical harvesters, seed dryers and transport, see Rice Seed Health (IRRI 1988). Certified Seed production programmes are currently working to counter this phenomenon through plot supervision and specific tests in order to guarantee the maintenance of its identity, genetic purity and physical, physiological and sanitary qualities (CIAT 1981).

The general suggested procedure for sowing panicle-rows or plant-rows (Briggs and Knowles 1967; Jennings et al. 1979) is commonly used to ensure the

uniformity of breeder seed by way of the elimination of off-types that could result from residual heterozygosity. Breeder seed also called G-0 or Pre-Base 5 (PB5) plants are grown and usually 10 spikes collected from each breeder seed plant are sown in rows the following year, this is G-1 or PB4 seed. A single 10-row group showing the highest visual uniformity and fidelity to the original variety traits is selected by comparing data between rows and within rows. Only one row is harvested among the best 10-row groups, and its self-pollinated seedlings (called G-2 or PB3) are grown next year in a separate plot. At this point, several traits such as productivity and uniformity are supervised under pre-defined tests to select the Stage I foundation seed (also called PB2). This seed is then grown to obtain Stage II foundation seed (also called Base seed), and finally Stage II foundation seedlings are grown in order to obtain R1 and R2 certified seed to be sold commercially.

Different countries use slightly different procedures and tests to obtain similar results. This depends mainly on government regulation and the amount of certified seed needed to satisfy the market: see Silva et al. (1988), the Philippines; Rao (1988), India; Munoz and Rosero (1988) and the book from Centro Internacional de Agricultura Tropical (CIAT 1981), Colombia; Masajo et al. (1988), Colombia and Africa; Botchey (1988); Asea et al. (2010), Africa; Boletín Oficial del Estado (BOE 2010), Spain; INIA report, Venezuela (Torres et al. 2006) and in general, Rice Seed Health from the International Rice Research Institute (IRRI 1988).

NRVC 980385 is a temperate japonica rice (*Oryza sativa*) cultivar derived from a traditional cultivar called Bahia which in turn was derived from the Balilla cultivar (Herruzo 1986). It was registered in 2002, and certified seeds have been produced and distributed by the rice farmers cooperative called La Càmara Arrossera del Montisà, SCCL (from now on La Càmara). Since then it has been grown in the Ebro River Delta at high and constant percentages when compared with other japonica varieties (Català et al. 2007). It is acclimatized to Ebro River Delta and Albufera de València environmental conditions. It has good germinability in direct seeding and even under cold conditions (Català et al. 2010).

NRVC 980385 was the shortest rice cultivar grown in the Ebro River Delta from 2002 until 2005, but new shorter japonica varieties have appeared in the rice

seed market since (Català et al. 2007). In the Mediterranean climatic region, the weather is usually windy and rainy at the end of the growing season; therefore NRVC 980385 is more easily affected by lodging phenomena than are shorter competitor cultivars. Reduction of plant height has traditionally been the main target in order to improve lodging resistance (Keller et al. 1999) and semi-dwarf lines were introduced to prevent lodging and increase yield during the “green revolution” (Keller et al. 1999; Khush 2001; Sasaki et al. 2002).

The objectives of this study are (1) to produce NRVC 980385-derived DH lines from a seed batch showing heterozygosity from an undetermined origin and (2) to select in controlled environment (i.e. greenhouse) and field conditions the best lines showing enhanced productivity, uniformity and short plant height, while at the same time maintaining traditional NRVC 980385 rice qualities and characteristics as demanded by the local seed market. In order to reach these aims we developed an *in vitro* anther culture protocol that allowed us to produce in a short time four new improved and stabilized DH derived lines ready to be registered for commercial proposes.

Materials and methods

Plant material and growth conditions

NRVC 980385 is a commercial temperate japonica rice cultivar grown in the Ebro River Delta region in the south of Catalonia (Spain). Plant material was obtained from a highly heterozygous NRVC 980385 seed batch which was first detected during certification test processes in 2005 by the La Càmera seed producer cooperative. One hundred seeds were sown in stages between January and March 2007 in greenhouse conditions at the Experimental Fields Service at the University of Barcelona (Fig. 1a). Plants were grown in 4 litre plastic containers filled with rice substrate: Floratorf peat moss (Floragard Vertriebs, Oldenburg)—vermiculite (2:1 v/v) substrate supplemented with Osmocote (The Scotts Company LLC, USA) controlled-release fertilizer mix [Osmocote Exact (15 + 9 + 9 + 3 MgO + micronutrients) for 6 months and Osmocote high K (11 + 5 + 15 + 1.2 MgO) for 9 months (1:1), 1 g/litre of substrate]. One gram CaCO₃ per peat litre was added to adjust the substrate pH (around 6).

Tiller cold treatment, spike sampling and disinfection

Tillers were selected in the booting stage, which is characterized by a panicle formation growing inside the flag leaf sheath (boot leaf). Distance between the atria of the last two leaves was previously correlated to the correct spikelet developmental stage for the NRVC 980385 cultivar, between 4 and 8 cm (Fig. 1b). Harvest time was from 8:00 to 9:30 as recommended by Chen et al. (1991). A three-step disinfection process was carried out as follows. Harvested tillers were soaked in 70 % ethanol for 1 min and rinsed four times with sterilized water. Surface sterilized tillers were then cold treated: 7–12 days at 7 °C in polystyrene bags and in darkness to further enhance *callus* induction (Trejo-Tapia et al. 2002a; Trejo-Tapia et al. 2002b; Cai and Chen 1984; Lentini et al. 1997). Cold treated tillers were surface disinfected again with 70 % ethanol before dissection. Panicles were then obtained under sterile conditions from the tillers by removing the leaf sheaths (Fig. 2c). The third disinfection step was carried out before anther plating as follows. The first 41 bare panicles were soaked for 3 min in 70 % ethanol and rinsed five times in sterile distilled water. While the last 196 panicles were sterilized by soaking in 10 % sodium hypochlorite solution for 3 min which was supplemented with Tween 20 (30 drops L⁻¹) and HCl 35 % (50 drops L⁻¹), and followed by 1 min in 70 % ethanol and rinsed 6 times in distilled water as described by Lentini et al. (1997). These were then plated.

Anther culture

Anther derived *callus* induction was obtained using media Chu N6 (Chu et al. 1975) modified as follows: N6 standard salts and vitamins fortified with 1 g L⁻¹ casein hydrolysate, 250 mg L⁻¹ L-proline, 2 mg L⁻¹ 2,4 dichlorophenoxyacetic acid, 1 mg L⁻¹ kinetin, 500 mg L⁻¹ 2-(*N*-morpholino) ethane sulphonic acid (MES), 30 g L⁻¹ sucrose and 3 g L⁻¹ Gelrite. Sterilin 90 mm petri dishes (Sterilin LTD, Cambridge) were filled with 25 ml media after autoclaving. Microspore stage determination was carried out in different stage panicles and spikelets to enhance anther culture efficiency (Fig. 1d). Anthers samples from the first plated panicles were fixed daily in acetic acid–ethanol (1:3) with 2 % FeCl₃ for 24 h, and stained with 2 %

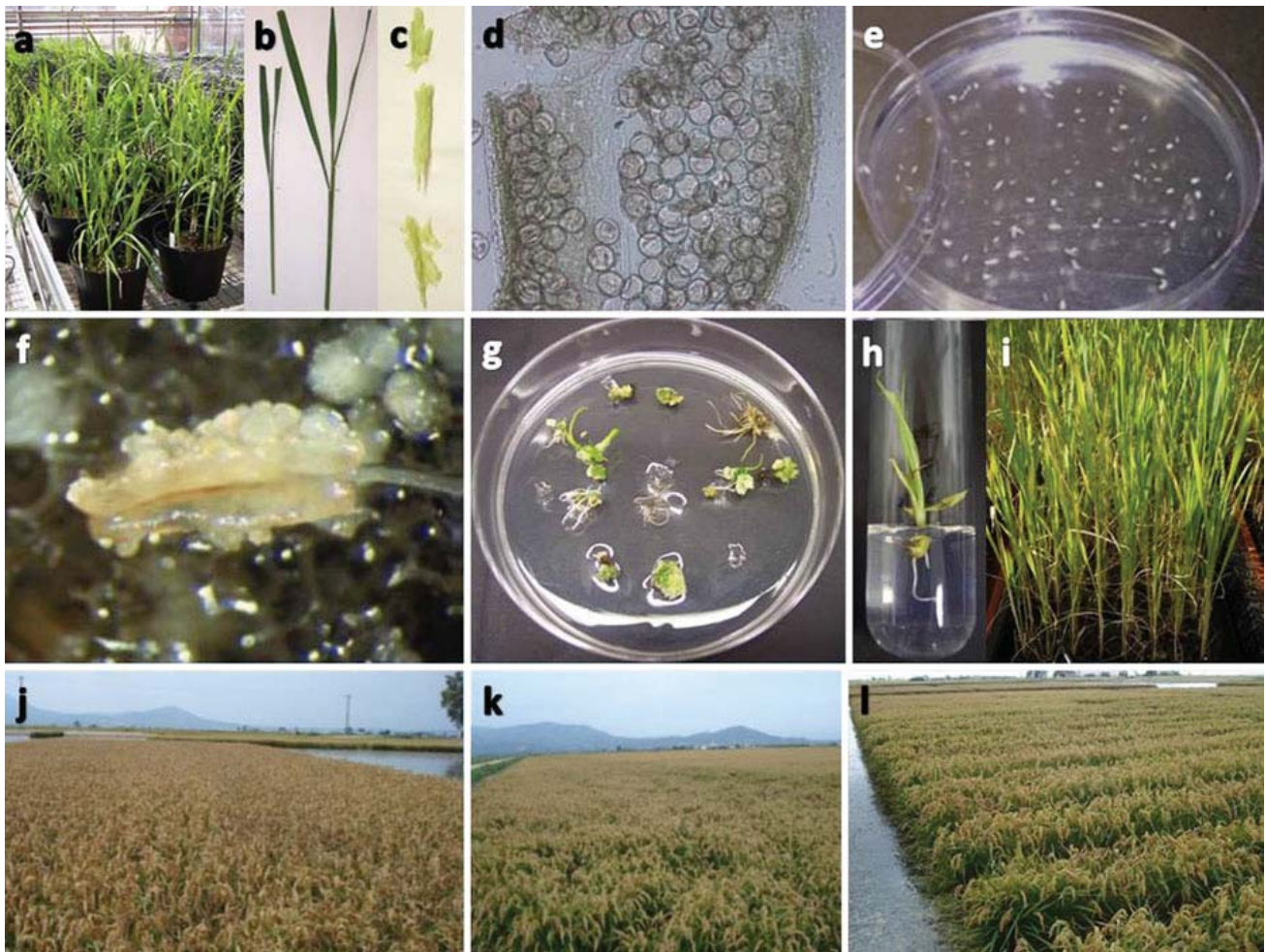


Fig. 1 Obtaining in vitro rice DH lines and subsequent field trials. **a** Donor plant material, **b** shoot selection, **c** spikelet selection, **d** microspore stage determination, **e** anther plating, **f** callus grown from anther culture, **g** plantlet regeneration from

anther derived calli, **h** plant propagation, **i** acclimatization to greenhouse conditions, **j** experiment 2 (year 2009) on DH8 line, **k** experiment 3 (year 2010) on DH8 line, **l** experiment 4 (year 2010) on DH8 line

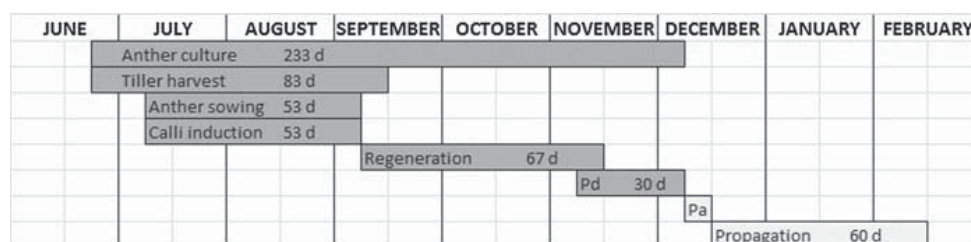


Fig. 2 Schematic representation of NRVC 980385 rice cultivar anther culture schedule to obtain DH lines and its propagation from June 2007 until February 2008. Total days (d) per step are indicated. *Pd* plantlet development *Pa* ploidy analysis took place in 5 days

acetocarmine for microspore stage determination following Mercy and Zapata protocol (1986), however, this procedure was stopped after 1 week of plating anthers as results matched the expected microspore developmental stage ratios. Yellow to slightly green spikelets (Fig. 1c) were found to be

related to the optimum rate of late uninucleate microspore developmental stage, matching results obtained by Afza et al. (2000), Shahjahan et al. (1992). Panicles were placed on sterile paper and spikelets were dissected under sterile conditions (Fig. 1c). As described by Lentini (Lentini et al.

1997), spikelet tips were first held with forceps and then the basal part was cut in the lower third part so that the anther filaments were cut at the same time. Anthers from selected spikelets that came from the same panicle were plated in each petri dish (Fig. 1e). Petri dishes were sealed using Parafilm (Pechiney Plastic Packaging Company, Chicago) and incubated at 24 °C for 6 to 8 weeks until the first anthers to respond produced the first *microcalli* (Fig. 1f). Anthers were cultured in darkness as light is not necessary to induce *calli* and in fact, darkness helps *calli* growth (Lentini et al. 1997). Anther plating was stopped as soon as the first *microcalli* had been obtained. *Microcalli* were transferred to fresh *callus* induction media and grown until 2 mm in diameter before transferring to *callus* regeneration media petri dishes.

Green plantlet regeneration was carried out using Chu N6 (Chu et al. 1975) standard salts and vitamins fortified with 1 g L⁻¹ casein hydrolysate, 250 mg L⁻¹ L-proline, 1 mg L⁻¹ naphthaleneacetic acid, 2 mg L⁻¹ kinetin, 500 mg L⁻¹ MES, 30 g L⁻¹ sucrose and 3 g L⁻¹ Gelrite. IWAKI 94 mm petri dishes (Asahi Techno Glass Corporation, Amagasaki) were filled with 25 ml media. Regeneration was done at 25 °C and 50–70 μmol m⁻² s⁻¹ fluorescent light under a 16/8 h day/night photoperiod until plantlet formation occurred (Fig. 1g). Frequently, ploidy duplication occurs spontaneously in rice during the in vitro regeneration process; therefore no antimetabolic compound was used.

Fully tiny formed R1 plantlets (0.5–3 cm length) were first transferred to hormone free MS (Murashige and Skoog 1968) media tubes; the custom made 17 cm long in vitro glass tubes were filled with 12 mL hormone free MS media: standard salts and vitamins fortified with 500 mg L⁻¹ MES, 30 g L⁻¹ sucrose and 2 g L⁻¹ Gelrite for 2–3 weeks. Next, these R1 plantlets (10–15 cm) were then propagated using a propagation media (Fig. 1h) which is the same hormone free MS media except fortified with 0.5 mg L⁻¹ 6-benzylaminopurine. Culture was carried out at 25 °C and 50–70 μmol m⁻² s⁻¹ fluorescent light under a 16/8 h day/night photoperiod and was extended so as to have enough R1 plants to carry on the ploidy determination and additional assays (Figs. 1 and 2).

All media components were supplied by Duchefa (Duchefa Biochemie BV, The Netherlands). Medias

were prepared using distilled water and the pH was adjusted to 5.7 by adding KOH (Sigma-Aldrich Co) solutions. All components including hormones were added before standard autoclave sterilization (120 °C for 20 min).

Ploidy determination

About 1 cm² of newly formed leaf tips from each line were placed on 55 mm diameter plastic petri dishes; the tissue was then sliced and chopped into small pieces by addition of 1.6 ml of lysis buffer and use of a razor blade and incubated for 14 min. Lysis buffer composition was 15 mM Tris-HCl pH: 7.5, 2 mM disodium EDTA, 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl, 0.2 % Triton X-100 and pH 7.5 adjusted with 1 M NaOH. The homogenate was filtered through a 33 μm nylon mesh. Then, 15 μL propidium iodide was added to 600 μL of filtered solution and after incubation for 30–45 min at room temperature, the fluorescence intensity of nuclei was measured using a Coulter EPIC XL flow cytometer (Beckman Coulter, Miami, FL) at the Cytometry Unit (Scientific and Technological Centers, University of Barcelona). Summit Software v4.3 (Cytomation, Fort Collins, Colorado) was employed for data analysis. Two diploid NRVC 980385 samples were used as the reference ploidy controls where the controls produced the same single peak. Accessions were classified as DH if they produced a clearly defined single peak corresponding to the peak of the diploid controls. Accessions producing a half fluorescence intensity peak were classified as haploids (Fig. S1). The main haploid R1 clones were discarded such that only one R1 haploid individual per line was kept.

Propagation, ex-vitro acclimatization and seed production

R1 DH lines were in vitro propagated in tubes for 2 months using propagation media (Figs. 1h and 2) until a minimum of 100 clones from each line were obtained. Propagated R1 clones were ex vivo acclimatized as follows: media was carefully removed from the roots using tap water and plants were transplanted into 96-well multipots and each pot was filled with 35 cm³ of rice substrate (Fig. 1i). Recently transplanted R1 plants were placed in an acclimatization tunnel inside the greenhouse. Relative humidity

was reduced over 100–70 % for 2 weeks. After the acclimatization process plants were transplanted to 4 L plastic pots (3 plants per pot) filled with rice substrate and grown in greenhouse conditions as described in Plant materials and growth conditions, however, the temperature was maintained at 19 °C. R1 plants were fertilized during the panicle formation period using high ammonium (50 % Total N) soluble fertilizer (NPK 19 + 6 + 6) supplemented with 4 % w/w micronutrients and 4 % w/w iron chelate diluted in osmotized water with a final electroconductivity adjusted to 1,200 μ S. DH plants were harvested individually in May 2008 and R2 seeds from the most vigorous and shortest cycle lines were used for Experiment 1 in the summer (see below). A haploid individual from each haploid line was also acclimatized to check flow cytometric ploidy assay results. R1 plants were ratooned and fertilized again during the panicle formation period to obtain more R2 seed for further field trials (Table 1; Figs. 1 and 2).

Experimental design of field trials

Field trials were carried out in La Càmara fields. All plots were at least 15 m apart from one another in order to avoid cross pollination. Distances between field trial and other rice fields were always more than 20 m. Standard fertilization and field management principles were applied. At least eight plants were randomly selected from each line and experiment in order to record data measurements. General traits such as the number of spikes per plant, different height measurements, grain/spike phenological stage, uniformity, pests/diseases and plant shape were recorded weekly. Other specific traits such as fungal lesions on leaves, flag leaf width, length and angle, fungal

infection in spikes, days to heading, spike position and exertion, and late flowering ratio were also recorded weekly. Plants were harvested at maturity to obtain the yield results. Three different experiments were performed as mentioned below.

Experiment 1 was designed to test basic agromorphological traits mentioned above and to select the most interesting lines using R2 seeds obtained from the initial R1 regenerated population. In the summer of 2008, the first 10 lines (DH1 to DH10) were tested. R2 seeds were sown in greenhouse conditions just 3 weeks after harvesting the original propagated and acclimatized R1 DH lines (DH1 to DH10). R2 plants were planted in the field late in the season, on June 2nd 2008. In summer of 2009, ten more lines (DH11 to DH20) were tested, plots were more homogeneous in number of plants and planting was done throughout the whole growing season (Table 1). In both years, three to four leaf stage plantlets were transported to La Càmara Experiment 1 field, being hand-planted with a spacing of 25 \times 20 cm in small plots (Fig. S2). NRVC 980385 plots were also planted in the same way and at the same time in order to compare results. This field was designed as follows: 18 plots (2 m \times 5 m) were reserved for assessed lines and NRVC 980385 controls. The bottom margin of the Experiment 1 field corresponds to the 5 m width access road; the distance from plots to the road was only 15 m. A minimum 15 m distance between lines and 20 m to adjacent field was designed to avoid gene flow-mediated hybridization.

Experiment 2 field trials were carried out to test R3 seeds from Experiment 1 selected lines on a medium scale (Table 1). Parcels were planted by hand with a spacing of 25 \times 20 cm using almost all the available R3 seed; with parcels ranging from 1242 to 4980 m² in size (Table 2; Fig. 1j). The NRVC 980385 controls were also hand-planted in parcels adjacent to each DH line using the same format and field conditions as experimental plots so as to be able to compare results. Experiment 2 was performed twice as follows (Table 1):

DH3, DH8 and DH9 seeds obtained from experiment 1 (R3) and certified NRVC 980385 seeds were sown in 2009 in greenhouse conditions and hand-planted in three rice parcels to assess traits as described above. DH3, DH8 and DH9 lines were grown in 4,480, 2,333 and 4,060 m² parcels respectively. Field size was mainly determined by seed availability.

Table 1 Field size of different experiments and DH lines assayed during 2008, 2009 and 2010 (see Sect. 2)

Experiment	Field size	DH lines assayed	Years
1	Small	DH1 to DH10	2008
		DH11 to DH20	2009
2	Medium	DH3, DH8, DH9	2009 and 2010
		DH12, DH16, DH18	2010
3	Large	DH3, DH8, DH9	2010

Table 2 Yield data from experiments 2 and 3

Line	Experiment 2 2009		Experiment 2 2010		Experiment 3 2010	
	Surface (m ²)	Yield (Kg/Ha)	Surface (m ²)	Yield (Kg/Ha)	Surface (m ²)	Yield (Kg/Ha)
DH3	4982.7	6966.16	1835.4	7273.62	47807.4	7299.77
DH8	2333.0	6763.82	1830.4	7659.53	44517.9	8106.16
DH9	4060.7	7368.12	1919.4	6892.78	44649.5	7091.98
DH12	n/a	n/a	1325.5	9038.10	n/a	n/a
DH16	n/a	n/a	1242.0	7181.96	n/a	n/a
DH18	n/a	n/a	1642.5	6295.28	n/a	n/a
CONTROL	10964.5	7753.20	5745.5	7019.41	n/a	6863.00 ^a

^a Average NRVC 980385 seed production yield in 2010. NRVC 980385 cultivar was used as control

In 2010, Experiment 2 was repeated using R4 seed from DH3, DH8 and DH9 lines, and likewise, R3 seeds from DH12, DH16 and DH18 obtained in Experiment 1 were also assessed. Six additional NRVC 980385 fields were used as controls, one control per line, each one growing next to each study line. In this experiment, a similar number of plants and plot dimensions were used thanks to seed availability. DH3, DH8 and DH9 were grown in 1835.4, 1830.4 and 1919.4 m² parcels respectively, and DH12, DH16 and DH18 were grown in 1325.5, 1242 and 1642.5 m² parcels respectively.

Experiment 3 was a large scale field trial (Table 1; Fig. 1k). R4 seeds from DH3, DH8 and DH9 lines were machine-sown in different areas, soils and fertilizer conditions in order to test the yield performance of each line under real conditions using standard seed production practices. Farmers' observations were also taken into account although traits recorded in Experiment 1 and 2 were also recorded weekly in Experiment 3.

Two different fields were used per line. The fields were rented out by La Càmara to six different cooperative associate farmers. The sum of Ha per line in associate farmers' fields ranged from 4.5 to 4.8 Ha. Yield was tested and compared to the average NRVC 980385 yield obtained that year from seed production fields grown by La Càmara farmer associates.

DH3 was grown in 2.85 and 1.93 Ha fields called Field No. 1 and Field No. 2 respectively. Field No. 2 was over fertilized. DH8 was grown in 3.57 and 0.88 Ha fields called Field No. 3 and Field No. 4 respectively. Field No. 3 was placed beside a NRVC 980385 registered seed production field and in this case control data was taken. Field No. 4 was highly

clayey and fertilized using natural guano. DH9 grown in 2.05 and 2.41 Ha fields were called Field No. 5 and Field No. 6 respectively.

Results

Anther culture and DH lines

The DH lines obtaining from anther culture took a total of 233 days (Figs. 2 and S3). In our experimental conditions booting stage tillers were harvested late in the season, from June to September 2007 and in total 42,660 anthers originating from 237 spikes were sown over 53 days.

Sixty-three out of 237 petri dishes were discarded due to contamination. Ethanol panicle disinfection resulted in a 40 % plate contamination rate, while Clorox disinfection reduced contamination to 22.25 %, but no *calli* were obtained from this second sterilization process.

Anther plating and *callus* induction was stopped on the 53rd day as soon as the first 4 anthers to respond produced the first globular and clear *microcallus* masses which were growing from different parts of the anthers (Fig. 1f). *Microcalli* grew during the regeneration process and produced green spots and small roots which were followed by shoot development (Fig. 1g). The first plantlets had fully formed 30 days after the regeneration process had begun (Fig. 2). The regeneration process yielded 42 green plantlets, 6 albinos and 1 green and white chimera. Regenerated green plantlets represented 85.71 % of the total plantlet regeneration. Flow cytometry assaying identified 29 DH and 13 haploid lines (Fig. S1), thus DH lines

represented 69 % of total green plantlet lines. One chimeric white and green plantlet was also analysed and was found to be a triploid plant.

All DH green plantlet lines were efficiently propagated over a 2 month period giving a minimum of 100 plants per line. Plantlets were acclimatized and grown until maturity in greenhouse conditions. Acclimatized haploid individuals were dwarf and sterile as expected (Niizeki and Oono 1968), confirming flow cytometry results. DH line seedlings were harvested individually in May 2008 (Fig. 1) and plants were ratooned in order to obtain more seed for further experiments.

Experiment 1

The tiller per plant ratios were strongly increased in DH3, DH8 and DH9 lines (43, 70 and 58 % increases, respectively). DH3 was the shortest line, 15 cm shorter in comparison to NRVC 980385. This line did not look like NRVC 980385 as flag leaves were 41 % longer. Line DH8 was about 10 cm shorter and flag leaves were only 16 % longer. Furthermore, DH9 characteristics were most similar to those of NRVC 980385 but plants were only 8 cm shorter. Height results are shown in Fig. 3a. Yield results were not taken into account since plots were too small and were of different sizes.

In 2009, the same Experiment 1 was carried out throughout the entire season, and 10 more lines, from DH11 to DH20 were tested. The number of plants per plot was more balanced. Height results were worse in comparison to results from the previous year (Fig. 3b). Finally, three lines were selected (DH12, DH16 and DH18) in accordance with yield and grain quality, although all other plant agronomic data was also revised to take the decision.

Experiment 2

The first Experiment 2 performed in 2009 confirmed height results of DH3, DH8 and DH9 lines (Fig. 4a). In this Experiment 2, tiller number increase was not significant in comparison to NRVC 980385, but lines were shorter than NRVC 980385 with DH3 being the shortest line (Fig. 4a, b). Yield results were very similar between lines and NRVC 980385 controls (Fig. 5a).

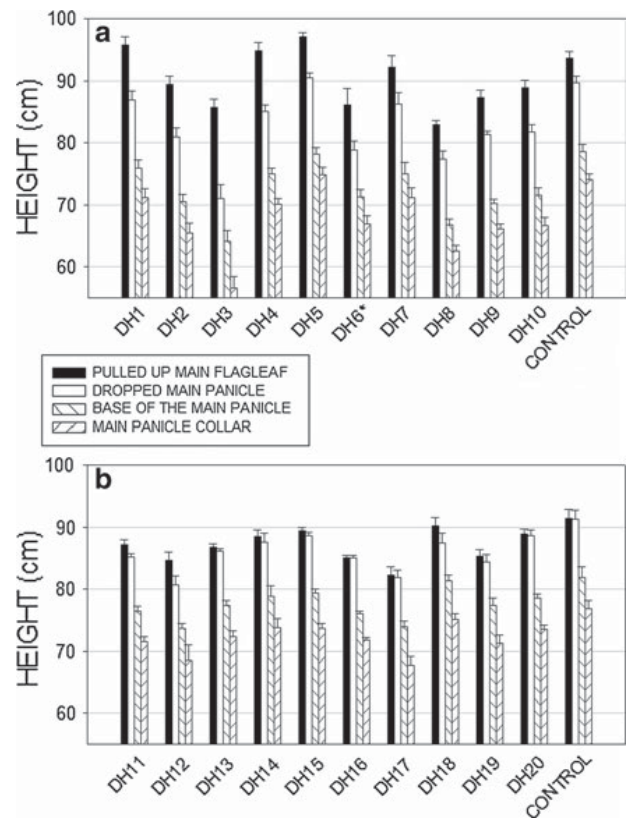


Fig. 3 Height average for experiment 1 tested lines and NRVC 980385 controls in both 2008 (a) and 2009 (b) trials. Each bar is the average of at least 8 replicates \pm standard error

In order to confirm these results, Experiment 2 was repeated in 2010 using seeds harvested from the DH3, DH8 and DH9 lines from Experiment 2 performed the previous year. In addition, seedlings from three more lines (DH12, DH16 and DH20) which were also selected from the second Experiment 1 (2009) were tested. Height results were confirmed again for DH3, DH8 and DH9 (Fig. 4b), while height reduction was poor in second set lines. DH12, DH16 and DH20 lines were taller than controls, DH3, DH8 and DH9 assessed in the same experiment and in the same year (Fig. 4b, c), although height was almost acceptable for DH12 and DH16 (Fig. 4c). Yield results showed that DH12 had nearly 30 % more productivity, while yields in all other lines were similar to NRVC 980385 controls (Fig. 5).

Experiment 3

DH3 was once again the shortest line and also the distance between the panicle collar and the first leaf was clearly shorter than in that of NRVC 980385. In

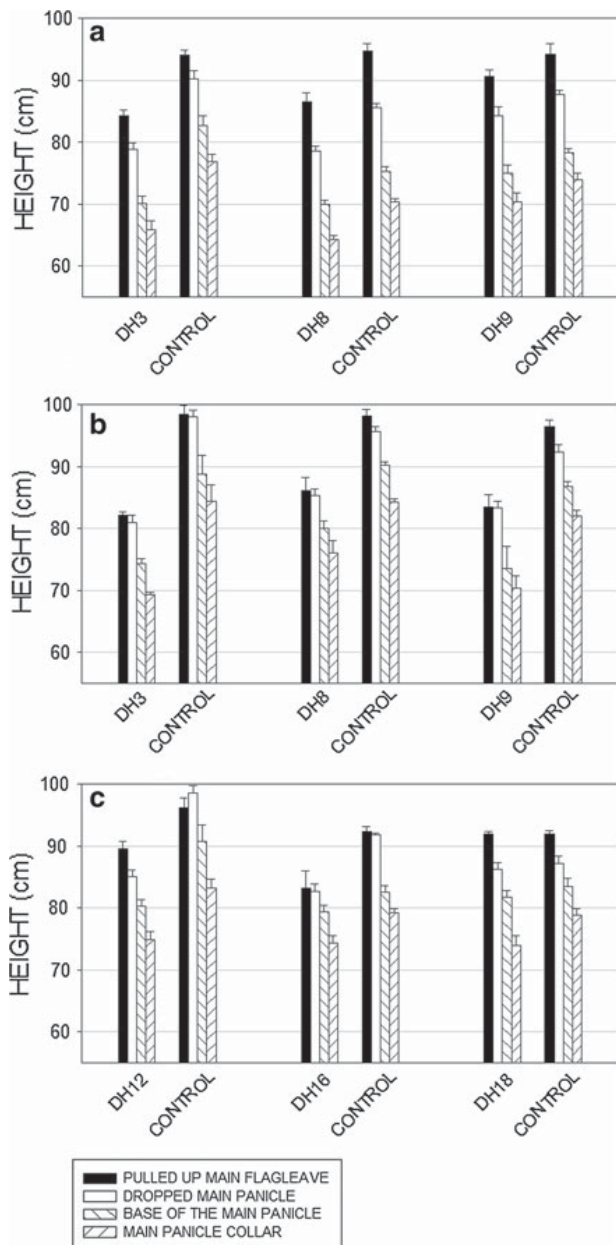


Fig. 4 Height average for experiment 2 tested lines and NRVC 980385 controls in (a) 2008 trial and (b and c) 2009 trials. Each bar is the average of at least 8 replicates \pm standard error

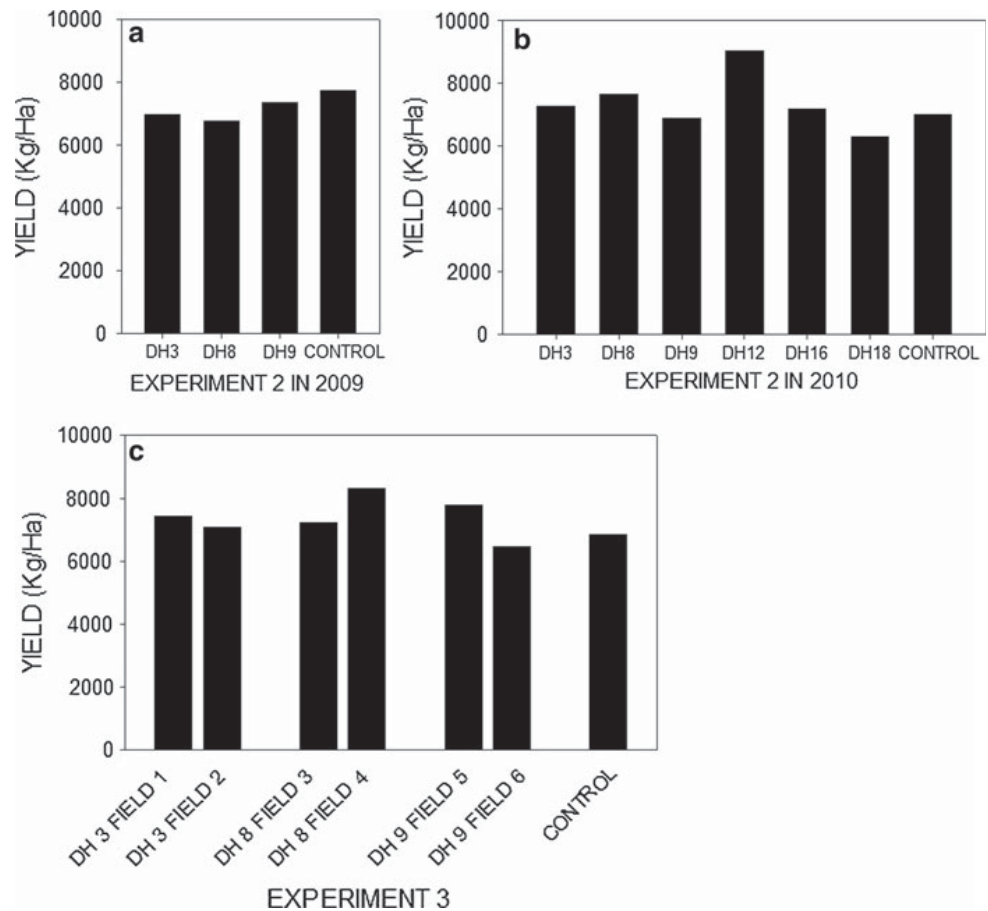
Field No. 2, the farmer over fertilized the field. Therefore, plants were taller and greener than in Field No. 1 but yield was reduced and a higher level of fungal attack and lodging was detected. DH8 was grown in Field No. 3 surrounded by an NRVC 980385 registered seed production field managed by the same farmer, from which control data was taken. Here, an average height reduction of 13 cm was scored. DH9 was clearly shorter than NRVC 980385 but plant shape was similar. Yield results are shown in Table 2 and Fig. 5.

Discussion

An anther culture method based on the Lentini et al. protocol (1997) has been modified (see above) in our lab in order to efficiently obtain DH lines from NRVC 980385 cultivar plants grown in greenhouse conditions using spikes from plants sown in season which resulted in a high rate of *callus* production. However, in this study plants were sown from January to March, 3 months in advance of the normal growing season. Although the greenhouse was heated and NRVC 980385 is photoperiod insensitive, total irradiance was lower than in season and furthermore, 50 of the 100 donor plants had to be ratooned in order to obtain a second set of panicles. Ratooning plants and collecting spikes out of season could affect *callus* induction (Chen et al. 1991; Lentini et al. 1997; Raina and Zapata 1997; Guzman and Arias 2000; Heberlebers 1985), however Guzman and Arias (2000) reported higher green plantlet regeneration using ratooned Taipei-309 indica rice cultivar.

Although harvest time was from 8:00 to 9:30 am following Chen's recommendations (Chen et al. 1991) and the collected spikes were cold treated to enhance *callus* induction (Trejo-Tapia et al. 2002a, b; Cai and Chen 1984), success in obtaining DH lines through anther culture is highly related to the amount of cultured microspores in the middle to late uninucleate stage present in plated anthers (Chen 1977; Gupta and Borthakur 1987; Bishnoi et al. 2000). This microspore developmental stage has been correlated to the distance between the atria of the last two leaves, but may vary depending on plant genotype and the environmental conditions in which it is placed (Lentini et al. 1997). Several authors have studied this correlation to improve anther culture and green plantlet regeneration rates, each one using different cultivars; Chen and Chen (1979) recommended collecting tillers with a distance of 5 cm between the atria of the last two leaves in Tainan 5 indica cultivar; Abbasi et al. (2011) reported 4–8 cm while Herath and Bandara (2011) reported 3–7 cm, both using indica-japonica hybrids. Our investigations in NRVC 980385 cultivar prior to this present work concluded that a 4–8 cm distance between the atria of the last two leaves and the flag leaf had the highest ratio of yellow to slightly green spikelets which also contained the highest ratio of middle to late uninucleate stage microspores according to Afta et al. (Afza et al. 2000). In the

Fig. 5 Yield data from primary selected lines. Experiment 2 data from 2009 assay (a) and 2010 assay (b) is compared to NRVC 980385 control grown by the lines and cultured the same way. Experiment 3 data (c) is compared to NRVC 980385 average production in seed production fields in the same year



present study, the developmental stage in microspores was determined only in the first panicles since they matched our previous results. Efforts were therefore directed towards sowing as many anthers as possible in order to ensure enough DH lines were yielded. Nevertheless, the relationship between atria distances and microspore developmental stages could have changed during the plating process as donor plants were sown out of season and some of them had been ratooned.

Contamination is quite common in anther culture as spikelets cannot be completely sterilised without affecting anther viability (Lai et al. 1980). In our study, the high average contamination rate (26.58 %) in the anther derived *callus* induction process could be due to endogenous contamination and soft ethanol disinfection. This contamination rate also affected *callus* induction efficiency. The ethanol sterilisation process applied to the first 41 spikes resulted in a 40 % plate contamination, thereby a Lentini's Clorox sterilization process was followed (Lentini et al. 1997) and contamination incidence was reduced (22.5 %), however, no *calli* were obtained.

Although some authors succeeded in obtaining rice DH plantlets directly sowing anthers in one-step culture media without transferring to regeneration media so as to save time and resources (Karim and Zapata 1990; Marassi et al. 1993), two-step culture media protocols are widely used and described by authors (Lentini et al. 1997). As optimal media composition is highly affected by the genetic backup of the plants (Talebi et al. 2007) we adapted the protocol of Spanish Hispagan rice variety, genetically similar to NRVC 980385, assessing N6 and MS salts and vitamins with different hormonal doses in order to induce anther derived *calli* formation and subsequent regeneration. N6 based medium yielded the best results in *calli* formation both in Hispagan and NRVC 980385, which agrees with results from other authors where the application of N6 medium is quite suitable for japonica rice, but usually not for indica rice cultivars (Reddy et al. 1985).

However, the anther derived *callus* induction media for NRVC 980385 required few modifications on the previously established Hispagan protocol. The concentration of 2,4-D was reduced from 2.5 to 2 mg L⁻¹,

while kinetin (1 mg L^{-1}) was maintained. This 2,4-D doses is in the range of that used by other authors (Shimada et al. 1999; Islam et al. 2004). Also, (Herath et al. 2009) coincided assessing 2 mg L^{-1} 2,4-D and 1 mg L^{-1} kinetin in Hu lo tao japonica rice variety.

The use of 4 % maltose as a carbon source was tested and rapidly discarded after different assessments with no *callus* formation although better results were obtained in indica rice by (Bagheri et al. 2009) using Iranian rice cultivars, (Niroula and Bimb 2009) using Nepalese varieties and (Javed et al. 2007) Indian rice cultivars. Proline was found to increase *callus* induction as reported by Cho and Zapata (1988), being 250 mg L^{-1} the optimal dose for both Hispagan and NRVC 980385 cultivars.

Albinism is a common problem in anther culture and generation of DHs. Loss of chlorophyll pigments and incomplete differentiation of chloroplast membranes is partially influenced by environmental conditions, media composition and culture conditions, but genetic factors are the major determinants (Kumari et al. 2009; Yamagishi 2002). In our study, the proportion of albino plants among anther derived regenerated rice plants was only 14 % which is quite low in comparison to 36 % in the Nipponbare rice model cultivar (Yamagishi et al. 1998). Although, Wang reported that frequencies of albino shoot forming microspore *calli* range from 5 to 90 % in different temperate japonica cultivars (Wang et al. 1981).

When in vitro anther cultures are performed, diploid plants are mainly homozygous DHs, but heterozygous diploids produced by the somatic tissue can occur, although in rice this is very unlikely (Chen et al. 1982). Some authors discard heterozygous diploids using isozyme analyses, RAPD markers, SCARs or SSRs to assess homozygosity (Germana 2011). In addition, conventional cytological techniques can be employed to determine the ploidy level of regenerated plants, but ploidy level can be more easily assessed by way of flow cytometry analysis (Bohanec 2003).

Our results indicated that plants were mainly DH (69 %), although one polyploid and some haploid plants were also detected. The DH percentage obtained among green regenerated plantlets is similar (56.4 %) to that obtained by Mercy and Zapata (1986). Consequently, flow cytometry helped us to discard non-DH plantlets thereby saving time and resources in

in vitro propagation works. In our case, field experiments resulted in high visual homogeneity in all offspring and no apparent character segregation was observed in any line.

A first selection was carried out when acclimatized plantlets were grown in greenhouse conditions until seed set. Only the first 20 DH lines producing seeds were tested in Experiment 1. All DH lines were stable and homogenous. Consequently, twenty healthy, stable and homogeneous DH lines having the shortest cycle were tested in Experiment 1 small field trials. Due to seed availability, the number of individuals per plot was from 50 to 120 plants in Experiment 1 performed in 2008. The number of tillers per plant appeared to be strongly increased in DH3, DH8 and DH9 lines (43, 70 and 58 % increases respectively) in Experiment 1, however, no significant increases in this trait were obtained from subsequent experiments. It was suspected that this apparent increased tiller formation was due to the fact that NRVC 980385 controls had more plants per plot and it decreased the border effect in comparison to DH line plots.

Selection criteria was initially mainly focused on reduced height, enhanced tillering and enhanced yield, although all other weekly measures data records such as different height measurements, grain/spike phenological stage, uniformity, pests/diseases, plant shape, fungal lesions on leaves, flag leaf width, length and angle, fungal infection in spikes, days to heading, spike position and exertion and late flowering ratio were taken into account. Six lines were selected from Experiment 1, three from the 2008 Experiment 1 field trial, which were considered to be the most interesting in terms of height reduction and yield having no evident bad results in other characters. Three more lines were selected from the 2009 Experiment 1 which demonstrated insufficient height reduction but which had interesting yield results. These six lines were assessed on medium scale field trials (Experiment 2) and from these, the three lines obtained in 2008 experiment were assessed in real conditions large scale trials (Experiment 3) as they had obvious height reduction, high homogeneity and acceptable productivity. Nevertheless, the DH12 line had an exceptional and unexpected yield result.

Rice production and yield assays have to be repeated for at least three consecutive years and using replicates and control replicates, as field trial results are usually variable (Fig. 5). In our study, field

dimensions were different in each experiment due to seed availability and they were placed in different locations due to field availability. Nevertheless, this three seed lines have been successfully submitted to Spanish registration process and other lines are still today under study.

Selected lines

The DH3 line differed from NRVC 980385 in a variety of traits: higher uniformity, shorter plants, shorter flag leaf to spike distance (half the distance), wider and bigger spikes, however, with similar rice production. DH8 differed from NRVC 980385 in terms of early flowering and maturation (up to 2 weeks) and shorter height, although it had a similar yield. DH9 is highly uniform, shorter than NRVC 980385 but taller than the DH3 and DH8 lines. DH12 turned out to be the most interesting line as it was the most productive.

Conclusions

In conclusion, an in vitro protocol based on the anther culture technique has been designed and optimized to obtain DH lines for the Spanish Mediterranean temperate japonica variety NRVC 980385. This protocol allows one to move rapidly from the anther collection stage to large scale field trials, thereby achieving the production of field assessed DH lines in a much reduced time period. This can now be adapted to other temperate japonica rice cultivars in order to obtain improved lines, and therefore avoid long breeding and inbreeding processes. It can also be adapted to obtain selected DH lines from F1 or F2 hybrids.

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Chapter 3.

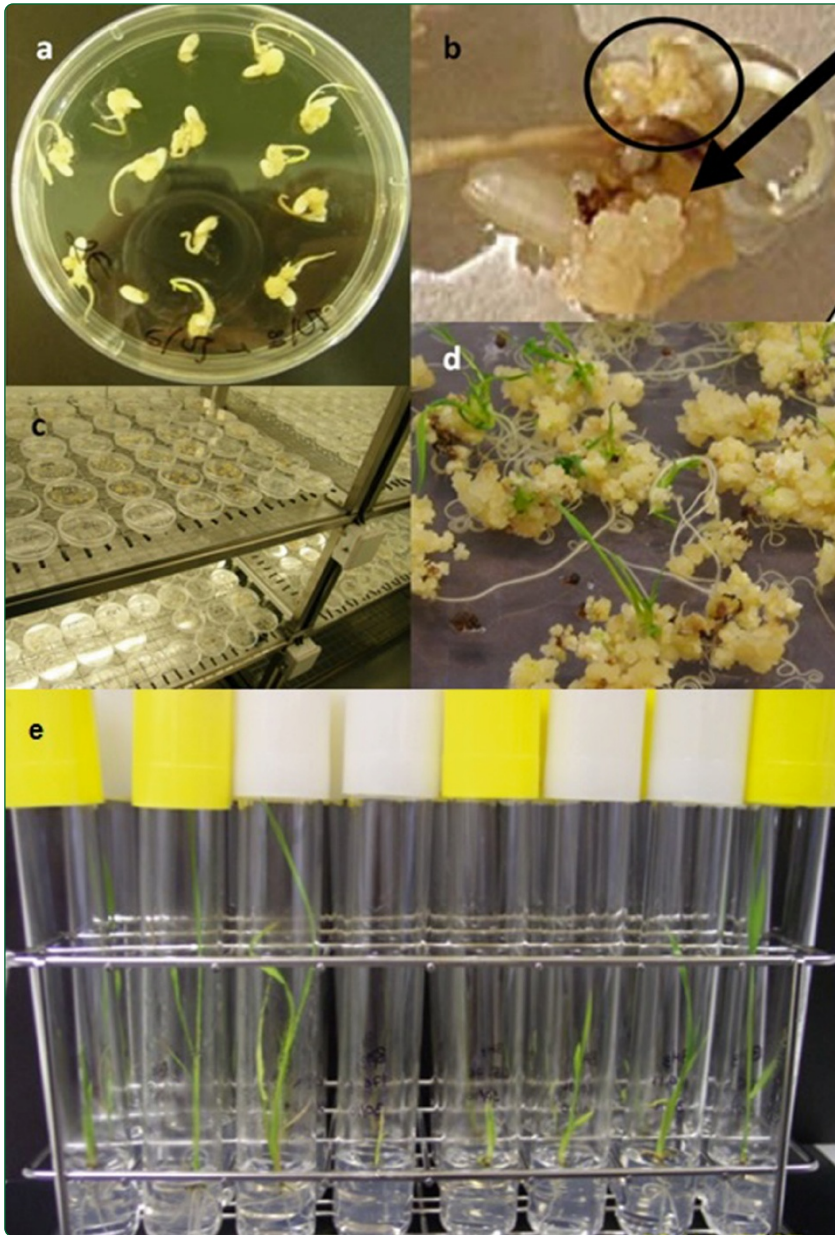
EMS mutagenesis in mature seed-derived rice *calli* as a new method for rapidly obtaining TILLING mutant populations.

Resum

S'anomena TILLING (*Targeting Induced Local Lesions IN Genomes*) al mètode emprat en genètica reversa que combina la mutagènesi química amb garbellats *high-throughput* de genomes sencers per detectar individus amb mutacions puntuals en gens d'interès. Una de les grans dificultats per desenvolupar aquesta tècnica és obtenir poblacions de mutants amb una taxa de mutacions suficientment alta. A més els protocols de mutagènesi de plantes requereixen de la producció de dos generacions successives de poblacions mutants (M1, M2) abans que es pugui començar el garbellat de mutants ja que altrament les mutacions no estarien fixades.

En aquest article es descriu una nova variant del TILLING en arròs basada en la mutagènesi amb etil metasulfonat (EMS) de calls derivats de llavor madura seguit del garbellat directe de les plantes regenerades *in vitro*. Al garbellar 2400 individus d'una població de 6912 mutants, s'obté una alta taxa de mutagènesi (p. ex. una mutació cada 451 Kb) i es detecten un total de vuit mutacions silencioses i onze mutacions de canvi de sentit en tres fragments de gens relacionats amb senescència retardada.

Aquesta nova tècnica representa un avantatge significatiu en quant a estalvi de temps (p. ex. mesos de 8 mesos), espai d'hivernacles i volum de feina durant la generació de la població mutant. A més redueix els volums de mutagènesi i la quantitat total de mutagen emprat, assegurant taxes de mutagènesi altes i útils per a estudis de genètica reversa.



EMS mutagenesis in mature seed-derived rice *calli* as a new method for rapidly obtaining TILLING mutant populations

Serrat *et al.*



METHODOLOGY

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EMS mutagenesis in mature seed-derived rice *calli* as a new method for rapidly obtaining TILLING mutant populations

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Abstract

Background: TILLING (Targeting Induced Local Lesions IN Genomes) is a reverse genetic method that combines chemical mutagenesis with high-throughput genome-wide screening for point mutation detection in genes of interest. However, this mutation discovery approach faces a particular problem which is how to obtain a mutant population with a sufficiently high mutation density. Furthermore, plant mutagenesis protocols require two successive generations (M1, M2) for mutation fixation to occur before the analysis of the genotype can begin.

Results: Here, we describe a new TILLING approach for rice based on ethyl methanesulfonate (EMS) mutagenesis of mature seed-derived *calli* and direct screening of *in vitro* regenerated plants. A high mutagenesis rate was obtained (i.e. one mutation in every 451 Kb) when plants were screened for two senescence-related genes. Screening was carried out in 2400 individuals from a mutant population of 6912. Seven sense change mutations out of 15 point mutations were identified.

Conclusions: This new strategy represents a significant advantage in terms of time-savings (i.e. more than eight months), greenhouse space and work during the generation of mutant plant populations. Furthermore, this effective chemical mutagenesis protocol ensures high mutagenesis rates thereby saving in waste removal costs and the total amount of mutagen needed thanks to the mutagenesis volume reduction.

Keywords: *Oryza sativa*, *Scutellum*, Mature seed, *Calli*, Mutagenesis, EMS, TILLING, Reverse genetics

Background

Rice (*Oryza sativa*) is one of the most important food crops in the world. It is also a model cereal plant [1] for molecular biology and genetics due to its small genome size relative to other cereals, the availability of the entire genome sequence [2], its ease of transformation and regeneration, and the availability of a variety of mutants. Since sequencing of the rice genome was completed in December 2004 [2], functional genomics has been used to determine the function of all of the approximately 50,000 annotated genes [3,4]. This objective has already been reached through the development of a variety of gene knockout strategies [5-7].

There are 3 ways in which to induce mutations, by either using: 1) biological agents such as transposons

and T-DNA, 2) physical agents such as fast neutron, UV and x-ray radiation, or 3) chemical agents such as N-methyl-N-nitrosourea (MNU), 1,2:3,4-diepoxybutane (DEB) or ethyl methanesulfonate (EMS). Among these compounds, EMS has become one of the most effective, reliable, powerful and frequently used chemical mutagens in plants [8]. EMS mainly induces C-to-T substitutions resulting in C/G to T/A transitions [9,10] and at a low frequency, EMS generates G/C to C/G or G/C to T/A transversions through 7-ethylguanine hydrolysis or A/T to G/C transitions through 3-ethyladenine pairing errors [9-12].

Irradiation and chemical mutagenesis have long been used to produce mutant plants for breeding purposes [13,14]. Molecular screening of mutations was developed much later after the efficiency of chemical mutagens producing small deletions and point mutations had been improved [8,9,15,16] and DNA sequencing allowed for the identification of such point mutations. Nevertheless, direct sequencing in large populations is a slow and

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expensive process. Therefore, several mutation detection techniques based on physical properties were developed [17-24] before enzymatic mismatch detection methods provided the key to efficient mutant population screening. The endonucleases obtained from *Aspergillus* [25], *Vigna radiata* [26] or *Penicillium* [27] were the first enzymes used to detect DNA mismatches. Oleykowski et al. [28] improved this technique by using the CEL I endonuclease obtained from *Apium graveolens* combined with an electrophoresis step, thereby paving the way for high throughput screening technology. Since then, the enzymatic detection methods in combination with high throughput genotyping have been improved for the efficient detection of genetic polymorphisms [29-32]. Consequently, there has been growing interest in using irradiation and chemical mutagenesis in model organisms for use in functional genomics research [33,34].

Chemically induced mutant populations have been generated in different plant species [11] and efficiently screened following Targeting Induced Local Lesions IN Genomes (TILLING) high-throughput screening protocols [35-37]. These combine random chemical mutagenesis with polymerase chain reaction (PCR) amplification of target genes, heteroduplex formation and identification of a range of allele changes [38] by using enzymatic mismatch cleavage and electrophoresis. Achieving a genome-wide saturated mutant population in plant species with large genomes is challenging. Small genome species such as rice are more suitable for TILLING [30,39,40]. As a result, many rice mutant populations have been efficiently screened using this technique [36,41-43].

In TILLING chemical mutagenesis protocols, germinating seeds are incubated in a mutagenic solution. The first generation (*M1*) that is produced directly from the mutagenic treatment cannot be screened because the majority of generated mutations are somatic and are not transmitted to the progeny [30]. To solve this problem, the *M1* mutant population has to be grown and then self-fertilized. The mutations in *M1* sexual structures can produce whole mutant *M2* descendants, thereby avoiding any ambiguities caused by mosaicism. The resulting *M2* progeny can be screened for mutations.

Tissue culture methods and mutagenesis techniques currently available could significantly shorten the breeding process and overcome some substantial agronomic and environmental problems. In most cases, the embryo-derived rice *callus* regeneration is only generated from a few cells. Thus, the regenerated *M1* plantlets from mutant *calli* could be screened directly without waiting for a self-pollinated *M2* population. Few attempts at mutagenesis for breeding purposes in rice using immature embryos, *calli* derived from mature seeds or single zygotic cells in recently fertilized spikelets have been reported [44-47]. Recently, mutagenesis of suspension-cultured rice cells for

phenotypic detection of mutants has been reported [48]. However, to date no studies of chemical mutagenesis in mature seed-derived rice *calli* in order to obtain mutant populations for TILLING have been reported.

The aim of this work is to carry out a new TILLING strategy based on the production of a plant mutant population from EMS mutagenised embryo-derived *calli* followed by a mutational screening on the regenerated plants. This mutational screening focusses on two genes related to senescence since this developmental process in annual cereal crop plants overlaps with the reproductive phase and may reduce crop yield when it is induced prematurely under adverse environmental conditions.

Results

Mutagenised population

In order to till rice we followed a new approach that differs from the traditional TILLING procedure (described in the introduction section, see above, Figure 1) in two aspects: i) mutagenesis was applied to embryo-derived *calli*, and ii) mutational screening was carried out on regenerated plantlets after acclimatization.

Rice seeds were cultured in *callus* induction *media* (OryCIM) for three weeks before *Scutellum*-derived *callus* masses were picked and mutagenised avoiding those *calli* obtained from the radicle (Figure 2a and b). No apparent differences were detected between partially disaggregated mutagenised and non-mutagenised *calli* except that the first showed a certain degree of browning. Both *calli* grew normally when cultured in OryCIM *media* for four weeks. Plantlets from both mutagenised and control *calli* started regenerating just three weeks after the growing *callus* masses were transferred to regeneration (MSM) *media* (Figure 2d). Mutant plantlets did not show any apparent phenotypic differences with respect to control plantlets. The control material was discarded after observing that the regeneration rate was satisfactory and similar in both treatments.

The *callus* regeneration process yielded 6912 individual plantlets obtained from 395 different mutagenised *callus* masses. From these, 2400 plantlets were sampled and their DNA was extracted, pooled fourfold and organized into a 96-well format for TILLING screening.

Molecular screening

Two target genes of agronomic interest were selected: *OsACSI* (Os03g0727600) and *OsSGR* (Os09g0532000). Among the six ACS isozymes identified in rice, *OsACSI* is the most closely related (86% identity) to ACS6 of *Zea mays* (Swiss-prot: Q3ZTU2), a protein encoded by the *ZmACS6* gene whose expression is largely responsible for directing natural, dark-induced and drought-induced senescence in maize [49,50]. *OsSGR* exists as a single

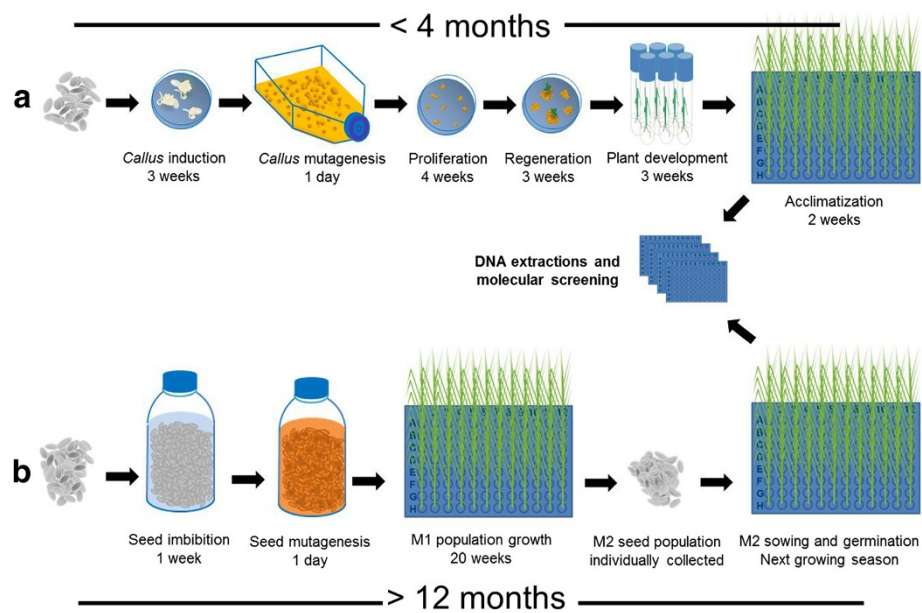


Figure 1 Diagram comparing callus mutagenesis and seed mutagenesis protocols in rice TILLING. **(a)** In the proposed rice TILLING protocol through callus mutagenesis, calli are induced, mutagenised, and the regenerated plants provide DNA for molecular screening of mutations. **(b)** In the basic TILLING method, seeds are mutagenised, the resulting M1 plants are self-fertilized and the M2 generation of individuals is used to prepare DNA samples for mutational screening while their seeds are inventoried. The steps represented cover from callus induction **(a)** or seed imbibition **(b)** to DNA extractions for molecular screening of mutations. Duration of each step is indicated.

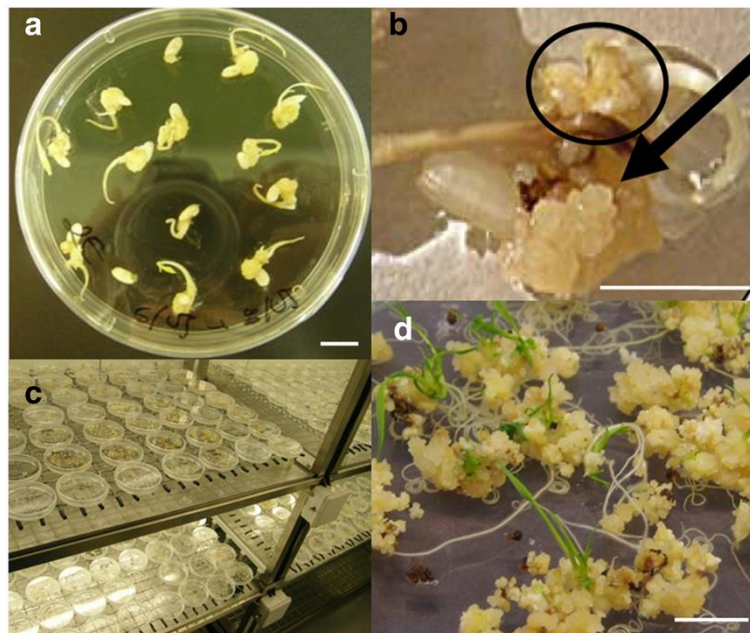


Figure 2 Plantlets regeneration of *Oryza sativa* var. Hispagram from EMS mutagenised calli. **(a)** *Oryza sativa* cv. Hispagram dehusked seeds forming callus masses after 18 days culture in darkness using N6 medium supplemented with 0.5 mg L⁻¹ casaminoacids, 1 g L⁻¹ L-proline, 2 mg L⁻¹ 2,4 dichlorophenoxyacetic acid and 0.5 g L⁻¹ 2-(N-morpholino) ethane sulphonic acid. **(b)** development of a scutellum-derived callus mass (black arrow) and a callus mass growing from radicle (black circle), **(c)** and **(d)** plantlet regeneration from mature seed-derived callus (0.2% EMS mutagenised) in MS medium supplemented with 1 g L⁻¹ casein hydrolisate, 3 mg L⁻¹ kinetin, 0.5 mg L⁻¹ 6-benzylaminopurin, 0.5 mg L⁻¹ 1-naphtalenic acid and 0.5 g L⁻¹ 2-(N-morpholino) ethane sulphonic acid under 18/6 h light/dark cycles (details in Methods). Scale bar 1 cm.

copy in the rice genome and is mapped onto the long arm of chromosome 9 [51].

OsACS1 is a four exon rice gene that codes for a 487 amino acid protein [Swiss-prot: Q10DK7]. *OsACS1* was screened in two fragments called *OsACS1* 1–3 and *OsACS1* 4. *OsACS1* 1–3 is a 1014 base pair (bp) fragment harboring the first three exons, while *OsACS1* 4 is a 1480 bp fragment spanning exon 4 (Figure 3).

Figure 4 shows the detection of three *OsACS1* mutants (*acs1* 152 s3, *acs1* 228 s1 and *acs1* 576 s1) based on different heteroduplex banding patterns (Figure 4a) and the identification of nucleotides changes by sequencing (Figure 4b). *acs1* 152 s3 resolve into four bands of approximately 250, 450, 550 and 750 bp and has two missense mutations, T → G and G → A transitions at gene nucleotide positions 58 and 174, in the target fragment *OsACS1* 1–3 (Table 1). The G → A transition generates a modification (GT → AT) in the splicing donor site of the first intron. The T → G change resulted in the amino acid substitution C → G at position 20 in exon 1 (C20G, Table 1). *acs1* 228 s2, *acs1* 398 s4 and *acs1* 576 s1 are missense mutations in the target fragment *OsACS1* 4 which result in the amino acid substitutions S314N, A246P and L354P at gene positions 1266, 1220 and 1351 bp respectively (Table 1). In addition, one mutation in intron 3 (*acs1* 43 s3, T → A at gene position 707 bp), one silent mutation in exon 1 (*acs1* 418 s2, C → T at gene position 84 bp) and one silent mutation in exon 3 (*acs1* 558 s2, G → A at gene position 535 bp) were detected in the *OsACS1* 1–3 fragment. Furthermore, two silent mutations in exon 4 coded *acs1* 83 s2 (C → T at gene position 1177 bp) and *acs1* 364 s2 (G → A at gene position 1669 bp), and one +73 bp C → T downstream mutation

were also detected in the *OsACS1* 4 fragment (Table 1). A total of 11 nucleotide changes in the *OsACS1* gene were detected after screening 2400 individuals. This is a one in every 457 Kb, 2.19×10^{-6} mutation frequency.

The analysis of the deduced mutant amino acid sequences using the GOR (Garnier-Osguthorpe-Robson) Secondary Structure Prediction application [52] is summarized in Table 1. According to the Eukaryotic Linear Motif resource for Functional sites in Proteins (ELM) [53], the conserved functional motif (50–432 amino acids) of the wild type rice protein ACS1 is divided into two sub-domains 50–148 amino acids and 275–404 amino acids.

The C20G (*acs1* 152 s3 mutant) change is located in the first functional domain and generates a weak α -helix domain modification (+1) (Table 1). The mutations L354P (576 s1 mutant) and S314N (228 s1 mutant) are located in the second functional sub-domain resulting in a loss and a weak modification of an α -helix motif respectively (Table 1). The change A246P (*acs1* 398 s4 mutant) does not affect any functional domain; it produces a complete α -helix domain lost mutation. Finally, the *acs1* 152 s3 mutant which has a GT → AT change at the intron 1 splicing acceptor site may affect splicing.

OsSGR contains three exons that code for a 274 amino acid protein [Swiss-prot:Q652K1]. A single 926 bp fragment harbouring the first two exons was screened in this study (Figure 3b). Following the same strategy described above, one mutation in exon 1 (*sgr* 24 s1 G → A at gene position 67 bp), one mutation in exon 2 (*sgr* 389 s2 G → A at gene position 484), one silent mutation in exon 1 (*sgr* 855 s2 C → T at gene position 78 bp) and one mutation in intron 1 (*sgr* 854 s1 G → A gene position

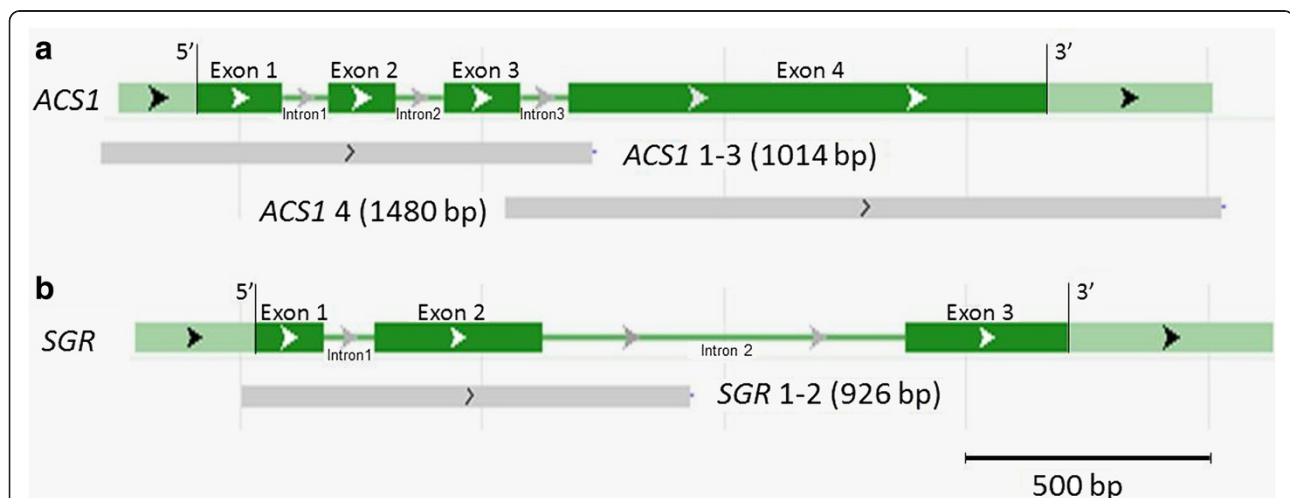


Figure 3 Targeted genes diagrams and PCR amplicons. Diagrammatic representation of the *OsACS1* (Os03g0727600) (a) and *OsSGR* (Os09g0532000) (b) genes obtained by blast searches at NCBI. Exons are represented by dark green boxes, introns by dark red lines and 5'-UTR and 3'-UTR by pale green boxes. Exons are designated Exon 1-Exon 4 (*OsACS1*) or Exon 1-Exon 3 (*OsSGR*). Intron numbering follows exon numbering. Targeted screened gene segments (grey boxes) are designated *ACS1* 1–3, *ACS1* 4 and *SGR* 1–2.

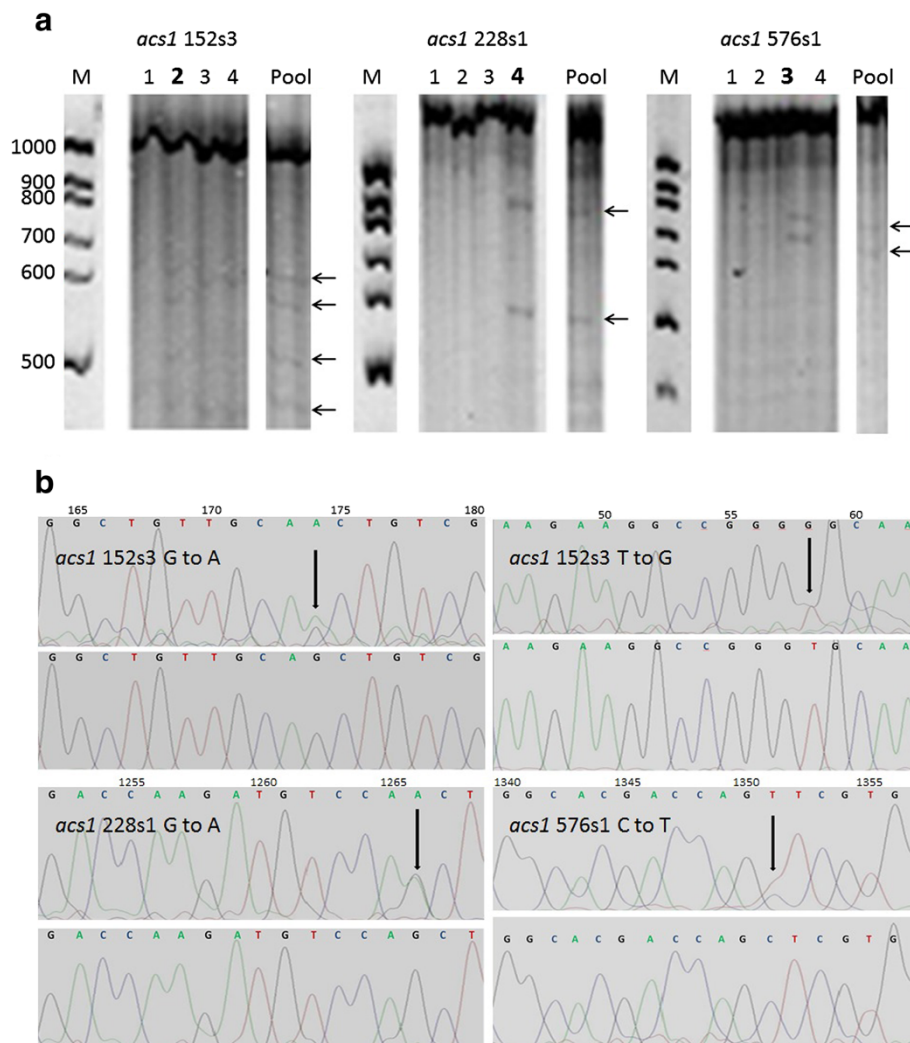


Figure 4 Identification of *OsACS1* mutants. (a) Heteroduplex mobility assays identifying three of the *OsACS1* mutants (*acs1* 152 s3, *acs1* 228 s1 and *acs1* 576 s1). Denaturing polyacrylamide gel electrophoresis showing different heteroduplex DNA band patterns formed after PCR amplification and mismatch cleavage by Fennel Crude Extract (FCE) incubation. The presence of two bands of about 600 and 850 bp in the case of *acs1* 228 s1 sample and 700 and 750 bp in the case of *acs1* 576 s1 sample, indicate heteroduplex digestions in the 1480 bp amplicon (*ACS1* 4). The presence of two mutations in the same 1014 bp amplicon (*ACS1* 1–3) of the same individual (*acs1* 152 s3) generates four bands of about 250, 450, 550 and 750. M: molecular weight marker (100 bp marker, Thermo Fisher Scientific Inc.). Pool: positive mismatch pool formed by mixing four individual DNA samples. Arrows indicate mismatch digested bands. Lanes 2–5: individuals DNA samples of the positive mismatch pool. Mutant samples are indicated by bold number lane. (b) Identification of nucleotide changes by sequencing. Heterozygous *acs1* mutants (upper panel) and wt (lower panel) sequences are represented. Black arrows indicate the base substitution. Direct nucleotide sequencing of the *acs1* 152 s3 mutant revealed the heterozygous G to A and T to G transitions at gene nucleotides position 174 and 58 respectively which resulted in substitution of GT/AG at intron 1 5'UTR and the amino acid C to G change at position 20 (C20G). Direct nucleotide sequencing of *acs1* 228 s1 and *acs1* 576 s1 mutants revealed the heterozygous G to A and C to G transitions at gene nucleotides position 1266 and 1351 which resulted in substitution of amino acids S to N and L to P at amino acid positions 314 (S314N) and 354 (L354P) respectively.

238 bp) were detected after screening 2400 individuals. This is a one in every 436 Kb, 2.30×10^{-6} mutation frequency. The GOR application predicted that amino acid substitutions A23T and V127M induced modification in α -helix domains in exon 1 (*sgr* 24 s1) and exon 2 (*sgr* 389 s2) respectively (Table 1).

Individual mosaicism was discarded after re-analysing 8 new independent leaf samples from all of the 14 detected

mutant individuals, while the incidence of multiple clone regeneration was studied by revising mismatch cleavage results of all plants that had originated from the same *callus* which had regenerated the 14 detected mutant plants. In total, 112 leaf samples were subjected to mismatch cleavage detection and sequencing, resulting in no individual mosaicism (Additional file 1: Table S1). All the mismatch cleavage results from samples of plants

Table 1 Mutations discovered in the *Oryza sativa* cv. Hispagan population obtained from 0.2% EMS-mutagenised callus

Mutant gene	Mutant code	Target fragment	Nucleotide change	Gene bp position	Effect	Predicted protein structure modification
<i>acs1</i>	152 s3	<i>ACS1</i> 1-3	T->G	58	C20G	Exon 1 α -helix domain modification (weak effect)
<i>acs1</i>	418 s2	<i>ACS1</i> 1-3	C->T	84	S28S	Exon 1 silent mutation
<i>acs1</i>	152 s3	<i>ACS1</i> 1-3	G->A	174	GT->AT	Intron 1 5'UTR splicing donor site GT modification that may affect splicing
<i>acs1</i>	558 s2	<i>ACS1</i> 1-3	G->A	535	Q114Q	Exon 3 silent mutation
<i>acs1</i>	43 s3	<i>ACS1</i> 1-3	T->A	707		Intron 3 mutation
<i>acs1</i>	83 s2	<i>ACS1</i> 4	C->T	1177	F231F	Exon 4 silent mutation
<i>acs1</i>	398 s4	<i>ACS1</i> 4	G->C	1220	A246P	Exon 4 α -helix domain lost
<i>acs1</i>	228 s1	<i>ACS1</i> 4	G->A	1266	S314N	Exon 4 α -helix domain modification (+1)
<i>acs1</i>	576 s1	<i>ACS1</i> 4	C->T	1351	L354P	Exon 4 α -helix domain modification affecting functional domain
<i>acs1</i>	364 s2	<i>ACS1</i> 4	G->A	1669	L395L	Exon 4 silent mutation
<i>acs1</i>	408 s3	<i>ACS1</i> 4	C->T	+73		Downstream mutation
<i>sgr</i>	24 s1	<i>SGR9</i> 1-2	G->A	67	A23T	Exon 1 α -helix domain modification (weak effect)
<i>sgr</i>	855 s2	<i>SGR9</i> 1-2	C->T	78	L26L	Exon 1 silent mutation
<i>sgr</i>	854 s1	<i>SGR9</i> 1-2	G->A	238		Intron 1 mutation
<i>sgr</i>	389 s2	<i>SGR9</i> 1-2	G->A	484	V127M	Exon 2 α -helix domain modification (+2)

Confirmed *acs1* and *sgr* mutants detected by TILLING screening and its predicted effect using GOR (Garnier-Osguthorpe-Robson) Secondary Structure Prediction application.

that had originated from the same *callus* which had generated the 14 mutant plants resulted negative (Additional file 1: Table S1).

In total 15 mutations were obtained in both target genes after screening 2400 individuals. This is a one in every 451 Kb, a 2.22×10^{-6} mutation frequency which is useful for reverse genetic studies and breeding proposes [54].

Homozygous mutant lines

Mutants were self-pollinated in order to obtain homozygous mutants. Selection was completed in two steps. First, in order to detect and discard heterozygous mutants, individual DNA was subjected to PCR amplification, denaturation and re-annealing to perform heteroduplexes, and FCE incubation and electrophoretic analysis to detect FCE-cut products. Then, with the aim of detecting and selecting homozygous mutants, individual samples of potential homozygous mutants selected in step 1 were mixed with wild type DNA (1:1 w/w). From this point onwards, screening was identical including PCR amplification, FCE digestion and electrophoresis. An example of this screening using 16 descendants of a self-fertilized *sgr* 389 s2 mutant is presented in Figure 5. In the case of heterozygous descendants, mismatch cleaved bands of 380 and 506 bp were observed, while no bands were detected in homozygous descendants (either wild type or mutants) (Figure 5a). In the second step, mismatch cleaved bands only appear

in samples where wild type DNA is mixed with DNA from homozygous mutants (Figure 5b).

Four wild type, 7 heterozygous and 5 homozygous mutant lines were obtained from the *sgr* 389 s2 descendants, X^2 statistic for "goodness of fit" with the expected Mendelian segregations was $X^2 = 0.375$ confirming the null hypothesis (2 degrees of freedom, $p = 0.05$) (Additional file 1: Table S1). Preliminary results indicated that they were completely fertile and showed a delayed senescence phenotype. In contrast, strong effect mutations of *OsACS1* such as *acs1* 228 s1, 398 s4 and 576 s1 (Additional file 2: Table S2) were partially sterile, no mutant homozygotes were obtained and about 25% of seeds were unable to germinate. Segregations did not fit Mendelian segregation, but when scoring non-germinated seeds as lethal homozygous mutants, X^2 test for goodness-of-fit with expected segregation resulted in $X^2 = 1.500$, $X^2 = 1.444$ and $X^2 = 0.200$ respectively, being less than 5.991 when considering 2 degrees of freedom and $p = 0.05$ (Additional file 2: Table S2).

Discussion

A new rice *callus* mutagenesis protocol using EMS was established. Mutant plants were efficiently obtained by mutagenizing *scutellum*-derived *callus* masses containing primary, embryogenic and non-embryogenic *calli* [55] (Figure 2). Root *callus* was the only *callus* mass to be discarded as it is unable to regenerate plants and it is

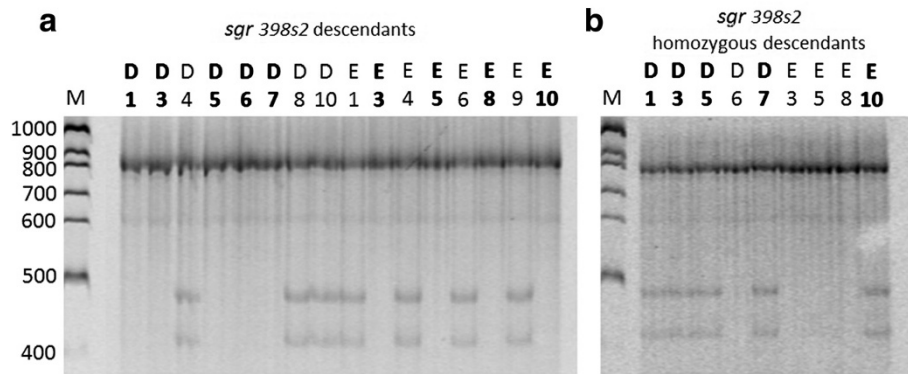


Figure 5 Identification of homozygous mutants among *OsSGR 389 s2* mutant descendants. (a) Electrophoretic analysis to discard heterozygous mutant plants obtained by self-fertilization of *sgr 389 s2*. Individual DNA samples were subjected to PCR amplification, denaturation, re-annealing, Fennel Crude Extract (FCE) heteroduplex mismatch cleavage and denaturing polyacrylamide gel analysis. The 926 bp band corresponding to the amplified fragment is present in all samples, while cleaved bands (380 and 506 bp) were detected in heterozygous mutant descendants (D4, D8, D10, E1, E4, E6 and E9). No cleavage bands were observed either in wild type or mutant homozygous descendants (D1, D3, D5, D6, D7, E3, E5, E8 and E10) highlighted in bold. (b) Electrophoretic analysis to discard homozygous wild type descendants. Homozygous descendants (D1, D3, D5, D6, D7, E3, E5, E8 and E10) individual DNA was mixed with wild type DNA (1:1 w/w) and subjected to PCR amplification, denaturation, re-annealing, FCE mismatch cleavage incubation and polyacrylamide gel analysis. Cleaved bands (380 and 506 bp) were detected in homozygous mutant descendants (D1, D3, D5, D7 and E10 in bold). Homozygous wild type individuals DNA could not form mismatches, and consequently no mismatch cleaved bands were visualized (D6, E3, E5 and E8). M: Molecular weight marker (100 bp marker, Thermo Fisher Scientific Inc.).

easy to identify as it grows separately from the radicle. The expensive and time consuming embryogenic *callus* selection process which is commonly used in transformation protocols proved to be unnecessary for successful *callus* mutagenesis, since embryogenic *calli* are contained in *scutellum*-derived *callus* masses [55] (Figure 2b).

In order to ensure sufficient *callus* availability 1,200 Hispagan seeds were sown, although only less than 20% of total *calli* was used for mutagenesis. It would have been possible to mutagenise the same amounts of *calli* using less than 200 *callus* forming seeds, although the capability of obtaining and regenerating embryogenic *callus* depends on the cultivar and culture media used [55]. Nevertheless, it is highly recommendable to use mutagenesis flask replicates in order to ensure that fungal and/or bacterial contamination free *in vitro* material is obtained.

In general, in seed-propagated plants, the chemical mutagenesis protocols use a seeds under germination process, so that the mutagen has to be absorbed by the germinating embryo and reach the meristematic region where the germ cells are contained. Other alternative plant material has been mutagenised, such as pollen, microspores, single zygotic cells in recently fertilized eggs and suspension cultured cells. Although pollen mutagenesis has been performed in maize, no pollen mutagenesis attempts have been reported to date in rice as pollen lifetime is too short and manual pollination is much more complicated than in maize [56-58]. Iftikhar and Mumtaz [59] mutagenised microspores using EMS for a

practical mutation breeding programme in the genetic improvement of oilseed brassicas. On the other hand, Suzuki et al. [46] found high mutagenesis rates when treating single zygotic cells in recently fertilized rice spikelets by using N-methyl-N-nitrosourea. However, a low cell survival was obtained and the resulting seeds (*M1*) had to be grown, fertilized and harvested until the *M2* population was ready for screening. Recently, suspension-cultured cell mutagenesis using EMS has been reported [48] where regenerated mutant plants were self-pollinated in order to obtain 302 *M2* lines for phenotypic analysis in field conditions which subsequently achieved high mutagenesis rates.

In this context, *callus* mutagenesis is more effective when compared to the traditional mutagenesis technique in seeds since this technique allows the mutagen agent to easily reach the target uncoated embryogenic cells rather than complex fully-formed embryos. Considering that somatic embryogenesis is the main regeneration method in the culturing of rice *in vitro*, and that somatic embryos arise from single cells, each mutated single cell can develop into a somatic embryo and regenerate a mutant plant. [48]. Furthermore, all mutants were unique; no clones were detected in the mutant population after screening the whole population and upon revision of all the results involving any plant that had shared the same *callus* of origin.

The intensity of mutagenesis applied is an important component in a TILLING project, and it is necessary to find a compromise between mutagen toxicity, genome mutation saturation and possible accumulation of undesirable

phenotypes. In our study, 0.2% EMS for 2 hours was effective to generate a whole rice mutant population with a sufficiently high mutation density. This mutagen dose applied in order to mutagenize rice seeds is in the range of values (0.2-2%), however, the duration of treatment is lower, 2 hours versus 6 hours reported by Chakravarti et al. [60] (0.2% EMS), and 12 hours reported by Wu et al. [43] (0.4% to 1% EMS) and Talebi et al. [61] (0.25 to 2% EMS). Regarding rice cell culture, Chen et al. [48] reported that a treatment of 0.4% EMS for 18–22 hours is optimal in order to induce mutagenesis. Consequently, the effectiveness of our low doses and low volume of mutagen presents an advantage since it implies a considerable financial saving and reduces the amount of residues generated. The mutagenized *callus* regeneration rate was similar to that of non-mutagenized control *calli*, which means that this treatment neither causes apparent lethality, which is one of the main problems associated with chemical mutagenesis [61], nor affects the plantlet regeneration rate.

Characterization of the two mutant populations obtained through screening with *ACS* and *SGR* target genes revealed mutation densities of 1/457 kb and 1/436 Kb, respectively, which are satisfactory and suitable for high throughput TILLING [42]. These mutations densities are in the range of those found by Wu et al. (1 per 1 MB for cv. IR64) [43], Till et al. (1 per 300 Kb for cv. Nipponbare) [42] and Suzuki et al. (1 per 135 Kb for cv. Taichung 65) [46] using rice seed mutagenesis protocols.

In twelve out of 15 mutants the most common EMS induced mutation was present, which is the C/G to T/A (C → T or G → A) substitution [9,12], while one case of G/C to C/G (C → G) transition was detected as expected in *Arabidopsis* EMS mutagenesis experiments [10]. Although T → A transversions are the second largest expected in *Drosophila* EMS mutantagenesis experiments [62], T → A and T → G detected transversions were unexpected in EMS rice mutagenesis. The origin of these mutations is unknown. We used 1200 seeds obtaining 395 *callus* masses which subsequently regenerated 6912 plantlets, and from these, 2400 individual mutants were screened. Polymorphisms in the Hispagan cv. seeds batch used to generate the mutant population could not explain these mutations since at least more than one other identical mutant should have been found, however all mutants were unique and furthermore, no other identical mutants were observed within the mutant population, even in those sharing the same *callus* of origin.

In this work, mutations were detected using a heteroduplex digestion assay with crude fennel juice extract (FCE) instead of crude celery juice extract (CJE) [63] or CEL I nuclease from celery, the most common enzyme used in TILLING projects [42,64]. The disadvantage of using juice extracts is that they contain multiple mismatch-

cleaving enzymes which collaborate in the digestion of heteroduplex DNA substrates. However, Till et al. [65] concluded that juice extracts and highly purified preparations in optimal conditions yielded similar mismatch detection results. Crude FCE exhibits lower mismatch-cleavage activity than that of CJE [66] however, it cleaves A/C and T/G mismatches preferentially, matching them with the most likely substitutions induced by EMS treatment, while the commercial CEL1 purified enzyme cleaves C/C mismatches preferentially [28]. In addition, it is inexpensive when compared with the substantially higher cost of the CEL I purified enzyme. Nevertheless, FCE nucleases mismatch cleavage was under study, and its 3'-5' exonuclease activity and other aspects implied the need for specific incubation conditions [67].

Functional analysis of the *acs* and *sgr* rice mutant lines would be needed in order to shed some light on the segregation of these genes and the roles that they play in the improvement of rice culture (i.e. delayed senescence and increase in rice yield), through performing field trials. To date, we are not able to predict the effect of *OsACSI* mutations on senescence since the amino acidic changes detected do not affect critical amino acids for catalysis, interaction and correct orientation of pyridoxal 5'-phosphate and substrate recognition [68] or any of the seven strongly conserved regions described by Wong et al. [69]. With respect to *sgr* mutants, the A23T and V127M changes do not correspond to amino acids critical for the correct functioning *SGR* proteins of rice [51,70], pepper and tomato [71]. As far as we currently know, all rice *sgr* recessive mutants obtained to date [51,70] belong to non-functional type C-*sgr* mutants in which chlorophylls are retained in senescent leaves as their photosynthesis efficiency decreases [72]. Given that *SGR* is a highly conserved protein in plants and does not show a large degree of similarity to any other proteins, we are not able to predict the effect of the two mutations which were obtained. Homozygous lines obtained from both mutants are completely fertile, and they remain greener longer than in Hispagan plants. Therefore, these results suggest that they could be functional stay-green rice mutants. If this is the case, these mutants could be the first functional *sgr* mutants in rice to have been found and they could potentially be useful for rice improvement. On the other hand, if these mutants produce a non-functional *SGR* phenotype, they would be useful for the study of the chlorophyll degradation pathway. Further work is now underway to understand the effect of these mutations on rice.

Conclusions

In conclusion, our results showed that combined EMS mutagenesis in *callus* with FCE heteroduplex digestion assay is a powerful tool for the identification and genetic

characterization of rice mutants. In our study, we were able to identify 15 nucleotide changes. The estimated mutation density is in the range of that previously reported for rice.

Our mutagenesis protocol avoids the problem of the inhibitory effect of the mutagen on seed germination and can be adapted to any *callus* induction/regeneration media with two modifications which are: the removal of agar and the addition of antioxidants during mutagenesis and rinsing. In addition, *callus* mutagenesis makes it possible to rapidly obtain a mutant population with a time saving of more than eight months when compared to classical seed mutagenesis. Furthermore, it saves greenhouse resources and work, the amount of mutagen needed to produce a mutant population and, consequently, the amount of residues generated.

This methodological approach could be easily adapted to any rice variety or even other plant species (e.g. cereals) and also cell suspensions.

Methods

Plant material

Hispagran temperate japonica rice (*Oryza sativa*) cultivar is a high yielding variety grown in Extremadura and Seville (Spain). Certified Hispagran seeds were supplied by the *Instituto Hispánico del Arroz, S.A.* (Hisparroz). The possibility of polymorphisms in targeted gene fragments of Hispagran was studied in advance by sequencing the targeted fragments in 80 wild type Hispagran individuals. The nucleotidic sequences were compared with the GenBank database of Nipponbare japonica rice cultivar (GenBank: AP008209.2).

Callus induction

To ensure enough *calli* were obtained, 1200 Hispagran certified seeds were dehusked and surface disinfected after soaking and stirring for 1 minute in 70% ethanol followed by 30 minutes in bleach sterilization solution; 40% commercial bleach supplemented with 8 drops of Tween 20 (Sigma-Aldrich, Madrid, Spain) per litre. After five rinses (5 minutes per rinse) using sterilized water, seeds were sown in solid *Callus* Induction Media (OryCIM),

based on rice *Callus* Induction Media [73] that was optimized to Mediterranean japonica rice varieties; Chu N6 [74] standard salts and vitamins were supplemented with 0.5 g L⁻¹ casaminoacids, 1 g L⁻¹ L-proline, 2 mg L⁻¹ 2,4 dichlorophenoxyacetic acid (2,4 D), 0.5 g L⁻¹ 2-(N-morpholino) ethane sulphonic acid (MES), and 30 g L⁻¹ sucrose. The pH 5.7 was adjusted using 1 M KOH solutions and 2.5 g L⁻¹ Gelrite™ was added before autoclaving. Sterilin 90 mm petri dishes (Sterilin LTD, Cambridge) were filled with 25 mL media after autoclaving. Fourteen sterilized seeds were sown in each OryCIM petri dish, and plates were sealed and incubated for three weeks in complete darkness, at 28°C (Figure 6). Contaminated seeds were discarded and *scutellum*-derived *callus* masses growing close to the embryo were selected avoiding smaller root producing *calli* growing from the radicle [55]. All media components were supplied by Duchefa (Duchefa Biochemie BV, The Netherlands) with the exception of casaminoacids (Becton, Dickinson and Company).

Chemical mutagenesis

Callus masses were transferred into three 375 mL NUNC EasyFlasks™ with filter caps for continuous venting (Thermo Fisher Scientific Inc.) containing 96 mL OryCIM liquid media without 2,4-D, but supplemented with 125 mg L⁻¹ L-ascorbic acid and 125 mg L⁻¹ citric acid (Liquid OryCIM) to prevent *callus* oxidation in further *callus* mutagen treatments. *Callus* masses were transferred until the final volume reached 110 mL in each flask. Next, two flasks were immediately mutagenised by adding EMS (0.2% v/v) (Sigma-Aldrich, Madrid, Spain) following the safety instructions provided by the manufacturer's Material Safety Data Sheet (MSDS). No EMS was added to the control flask. Flasks were placed on an orbital shaker (150 rpm) and covered with aluminium foil to avoid light for two hours. After incubation, every *callus* batch was rinsed ten times using 200 mL liquid OryCIM media and removing it after 3 to 4 minutes. After rinsing was complete, *calli* were incubated on modified liquid OryCIM media which was supplemented with 2 mg L⁻¹ 2–4 D and shaken (120 rpm) for two additional days (28°C, darkness).

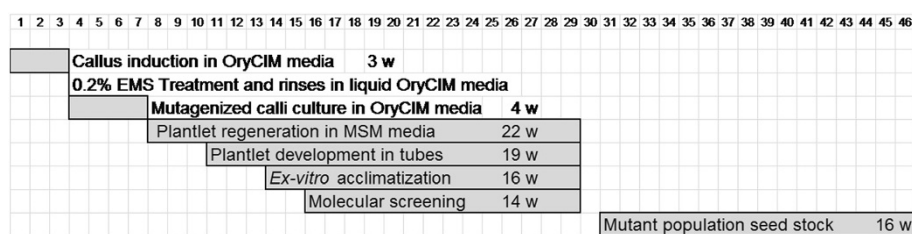


Figure 6 Schedule of the experiment. Schematic representation of TILLING schedule based on callus mutagenesis. The duration of each step (weeks, w) is indicated.

Plantlet regeneration

Calli were partially dried in a laminar flow cabinet on sterile cellulose paper for about 30 minutes before sowing them in solid OryCIM fortified with 4.5 g L⁻¹ Gelrite™ (Duchefa) in 90 mm diameter dish. Fourteen *callus* masses per dish were transferred and cultured for four weeks in darkness at 28°C (Figure 6).

Calli were transferred to regeneration media (MSM) petri dishes (90 mm diameter) and cultured under a 18/6 hours light/dark photoperiod under 70 μmol m⁻² s⁻¹ fluorescent light, and at 28°C until plantlets were fully-formed 30 days later. This MSM media was based on MS [75] standard salts and vitamins, fortified with 1 g L⁻¹ casein hydrolysate, 3 mg L⁻¹ kinetin, 0.5 mg L⁻¹ 6-benzylaminopurine, 0.5 mg L⁻¹ 1-naphthaleneacetic acid, 0.5 g L⁻¹ MES and 30 g L⁻¹ sucrose. The pH was adjusted (5.8) by using 0.5 M HCl solution and 4.5 g L⁻¹ Gelrite™ was added before autoclaving. *Calli* were transferred every three/four weeks to fresh MSM media until the end of the experiment ca. 22 weeks (Figure 6).

Regenerated plantlets were individually numbered and its *callus* mass origin was recorded as soon as they were sub-cultured into culture tubes (17 cm high/2 cm width) filled with 10 mL of root media (RM) [73]. Plants were cultured for three more weeks to enhance rooting and leaf development under a 18/6 hours light/dark photoperiod 70 μmol m⁻² s⁻¹ fluorescent light, at 28°C (Figure 6).

Mutant population seed stock

Rooted plantlets were *ex vitro* acclimatized (Figure 6) as follows. Media was carefully removed from roots using tap water and plants were transplanted into 96-well multi-pots, each pot filled with 35 cm³ of specially designed Floratorf™ peat moss (Floragard Vertriebs, Oldenburg, Germany) - vermiculite (2:1 v/v) substrate that was supplemented with Osmocote™ (The Scotts Company LLC, Ohio, USA) controlled release fertilizer mix [Osmocote Exact™ (15 + 9 + 9 + 3 MgO + micronutrients) 6-month release and Osmocote™ high K (11 + 5 + 15 + 1.2 MgO + micronutrients) 9-month release (1:1), 1gr L⁻¹ of substrate]. Then 1 gr of CaCO₃ per peat litre was added to adjust the substrate to pH 6. Multi-pots were placed in 54 × 31 × 4.6 cm plastic trays with holes ensuring a maximum 1 cm flooding irrigation. Supplementary fertilization was supplied during panicle formation period by adding high ammonium (50% total N) soluble fertilizer (NPK 19 + 6 + 6) supplemented with 4% w/w micronutrients and 4% w/w iron chelate diluted in osmotized water, final electro-conductivity was adjusted to 1000–1200 micro Siemens.

The mutants identified by the TILLING screening were transplanted from multi-pot wells to 4 litre pots to obtain as many seeds as possible, while the rest of plantlets growing in multi-pots were discarded as soon as a

minimum 20 seeds per plant was obtained. Seeds were collected in individually labelled envelopes, and stored as if they were an *M3* mutant population generated by seed mutagenesis (Figure 6).

DNA extraction and sample pooling

About 0.5 cm² of newly formed leaves were sampled from each plant using 96-well sample boxes containing 50–100 μg quartz (Merck KGaA, Darmstadt, Germany) and two 4 mm glass balls (Merck) placed on ice in polystyrene boxes to avoid DNA degradation. Samples were stored at -80°C and the completely frozen material was disrupted for 1 minute using a Mixer Mill (Retsch). Genomic DNA phenol-chloroform extraction: 200 μl buffer 1 (100 mM Tris-HCl pH 8, 50 mM EDTA pH 8, 500 mM NaCl and SDS 20%) was added to each sample and boxes were incubated at 65°C for 30 minutes. Two hundred μl of phenol:chloroform:isoamyl alcohol (24:25:1) was added to every extraction tube and the entire box was vortexed before being centrifuged at 3,000 rpm for 30 minutes at room temperature. Two hundred and twenty μl from the upper phase was transferred to a clean 96-well plate and kept for further analysis. DNA quality and quantity were determined with a combination of electrophoresis gel analysis and gel determination software and the same amount of DNA from 4 distinct plants was pooled together and organized into 96-well format, this being the starting material for TILLING mutant screening.

Target genes selection and primer design

The *ZmACS6* gene is involved in senescence regulation and drought tolerance in maize [49,50]. Several *ACS* genes were found in rice and maize after a BLAST was performed using the *ZmACS6* protein sequence. *OsACS1* was selected as the best candidate after homology studies using CLUSTAL W2 [76]. *OsSGR* has been related to Chlorophyll catabolism [51]. These two genes were selected as target genes.

Primer design was carried out using the Primer 3 program application [77]. A set of primers (*OsSGR* 1–2) was designed to amplify the first two exons of *OsSGR*. Two sets of primers were designed to amplify 2 segments of the *OsACS1* gene: The first segment (*OsACS1* 1–3) was designed to amplify from the first to the third exon, and the second (*OsACS1* 4) was designed to amplify the fourth exon (Figure 3) (Table 2). Eighty plants were sequenced for *OsACS1* and *OsSGR* fragments and compared with Nipponbare japonica cultivar sequences available at NCBI.

Target gene amplification and heteroduplex DNA formation

Fragments of target genes were amplified by polymerase chain reaction (PCR) using 1–4 ng DNA, 200 μM dNTPs

Table 2 Gene specific primers used for the amplification of targeted genes *OsACS1* and *OsSGR* design for target genes amplification

Target gene	Primer name	Nucleotide sequences 5'-3'	Amplicon size (bp)	Amplicon name
<i>OsACS1</i>	ACS1 1-3 F	TAAGCAGCTCGTCCAACCTT	1014	ACS1 1-3
	ACS1 1-3 R	CAGTGCACGGGTACGATCT		
	ACS1 4 F	CTCATCCCCACCCATACTA	1480	ACS1 4
	ACS1 4 R	CCCAAATGTGGGAGTGGTAG		
<i>OsSGR</i>	SGR 1-2 F	TAAGAGATCCGAGGGAGCAG	926	SGR
	SGR 1-2 R	ACAGATGGATGGATGCCAAT		

Primers were designed using Primer3 application and used for the *OsACS1* (Os03g0727600) and *OsSGR* (Os09g0532000) molecular screening of mutations. F: forward. R: reverse. Position of amplicons are shown in Figure 2.

and 0.4 μ M Hispagan genome specific primer sets (Table 2), and 5U Pfu DNA polymerase in 20 μ L 1X Pfu DNA polymerase reaction buffer including 1X final concentration of 2 mM MgCl₂ (Fermentas). The number of cycles, the times and the annealing temperatures were optimized for each specific primer pairs. DNA denaturation and slow re-annealing step (from 95°C to 4°C) processes were added to the end of the PCR amplification thermal cycle programs in order to induce heteroduplex DNA formation within the different amplification products obtained from the pools.

An aliquot of each PCR product (ca. 5 μ L) was checked on a 1% w/v agarose gel and submitted to electrophoresis to verify the efficiency of the PCR amplification and the size of the amplified product.

Fennel crude extract heteroduplex mismatch cleavage detection

The amplified DNA from each pool was subsequently incubated with FCE [67] for heteroduplex mismatch cleavage: 80 ng of heteroduplex DNA, 1 μ L FCE, 20 mM Tris-HCl pH 7.5, 25 mM KCl and 20 mM MgCl₂ (final volume 10 μ L). After 30 minutes incubation at 45°C, the reaction was stopped by adding 2 μ L of stop buffer (20 mM Tris-HCl pH 8, 10 mM EDTA, 12.5% v/v Glycerol, 50% v/v Sybr gold-dimethyl sulfoxide and 0.05% w/v Bromophenol Blue). Digested samples were size-fractionated by polyacrylamide gel electrophoresis (4% acrylamide:bisacrilamide 19:1 in Tris/Borate/EDTA supplemented with 0.008% v/v tetramethylethylenediamine and 0.002% w/v ammonium persulphate) at 300 V and 15–20 mA for 1.5 hours [67]. Results were analysed using a Typhoon 8600 scan (GE Healthcare) with a fluorescein filter (526 nm) for Sybr-Gold stain detection.

Mutant individual identification

Individual plates containing the four individual DNA samples from each positive pool were similarly analysed after mixing (1:1, w/w) with control wild type DNA. PCR product from validated individuals were purified using Microcon™ filters purification kit (Millipore) and

5–40 ng (depending on DNA fragment length) were sequenced on a ABI Prism 3700 (Applied Biosystems) sequencer using Terminator polymerase (Applied Biosystems). In order to detect mosaicism, 8 DNA samples from eight different leaf samples from all 15 identified mutants (120 samples) were extracted and subjected to targeted fragment amplification, mismatch formation, enzymatic digestion gel analysis and sequencing. Furthermore, whenever a mutant plant was detected, plants that had originated from the same *callus* were tracked and the results of the analysis were carefully revised.

Data analysis

The nucleotide sequences were analysed using Chromas Lite 2.1 (Technelysium Pty Ltd) giving the nature and the exact localisation of the mutation within the gene. Mutant amino acid sequences were submitted to the GOR (Garnier-Osguthorpe-Robson) Secondary Structure Prediction application [52] and listed in Table 1.

Mutation frequencies

The mutation rate was calculated by dividing the total number of observed mutations by the total surveyed DNA length. The total surveyed DNA length was calculated by the sum of the screened individuals multiplied by the base pair (bp) sum of amplified fragments. We subtracted 200 bp from each TILLED fragment to account for the difficulty in detecting digested DNA products that migrate in the top and bottom range of the gel like other authors [42].

Additional files

Additional file 1: Table S1. Individual mosaicism and multiple clone regeneration detection. All the independent leaf samples from each mutant individual yield identical results, consequently no individual mosaicism was detected. The incidence of clones regenerated from the same *callus* is represented as the number of TILLED plants originating from the same *callus* related to the number of additional mutant clones detected, being only 1 in all cases.

Additional file 2: Table S2. Total number of wild type (WT), heterozygous mutant (het) and homozygous mutant descendants

obtained from predicted strong effect mutants. Sgr 389 s2 segregation χ^2 test for goodness-of-fit ($\chi^2 = 0.375$) confirms the null hypothesis being less than 0.5991 (2 degrees of freedom, $p = 0.05$). In the case of acs1 398 s4, 228 s1 and 576 s1 mutants, non-germinated seeds have to be considered homozygous mutants in order to predict recessive lethality inheritance ($\chi^2 = 1.500$, $\chi^2 = 1.444$ and $\chi^2 = 0.200$ respectively). For the rest of mutations, the progeny was not studied.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

XS adapted *in vitro* culture media, mutagenesis and regeneration protocols. XS and EL conceived the study and carried out the *callus* mutagenesis. XS directed the *In Vitro* process. NG and RE designed the molecular screening and developed the FCE endonuclease mismatch cleavage detection. XS drafted the manuscript. The final script main contributors were XS, SN, LM and RE. All authors read and approved the final script.

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General discussion

The following discussion aims to:

- Critically examine the results achieved; in relation to our main objective, which was the study of the applicability of transgenic, dihaploid and mutant approaches to Mediterranean rice improvement.
- Discuss these three biotechnological tools in the frame of the current literature.
- Suggest potential future research lines within Mediterranean rice improvement according to the results achieved.

Synthesis and discussion of main results

This Thesis includes three chapters where plant biotechnology techniques have been applied to Mediterranean rice varieties:

- AFLP detection of reverse gene flow (Chapter1).
- Anther culture stabilization and improvement of rice lines (Chapter 2).
- Rice *callus* mutagenesis (Chapter 3).

The table 5 summarizes the literature's scope, our findings and their subsequent consequences and/or improvements.

Table 5. Summarized literature's scope, thesis findings and consequences and/or improvements for all of the three chapters.

Chapter	Literature's scope	Thesis findings	Consequences and/or improvements
Chapter 1: Gene flow	Direct gene flow was considered the main phenomena to calculate security distances between GM rice and conventional rice fields, while reverse flow effects had been ignored.	Reverse gene flow is six times higher than direct gene flow in our conditions. GM weeds results from hybridization through direct and reverse gene flow between red rice and GM rice.	The effects of reverse flow from red weedy rice to GM rice had been ignored, underestimating its effect when calculating security distances between GM and conventional rice fields, which should be recalculated taking reverse flow into account.
Chapter 2: Stabilization through anther cultures	Classical rice line stabilization through mass selection takes over 12 years.	The proposed anther culture protocol adapted to Mediterranean <i>japonica</i> cultivars enables lines stabilization in only 3 years.	Four new rice varieties have been registered and two of them are currently being successfully commercialized. This protocol can reduce 7 years the current stabilization through mass selection protocols used in Mediterranean <i>japonica</i> rice breeding programs.
Chapter 3: Tilling through <i>calli</i> mutagenesis	EMS mutagenesis on seeds was considered one of the best protocols for obtaining rice mutant population for TILLING proposes. CEL-1 was considered to be one of the best mismatch detection enzymes in these protocols	<i>Calli</i> mutagenesis allows bypassing single seed descent since regenerated plantlets from mutagenized <i>calli</i> can be directly screened. The fennel crude extract (FCE) has been proved to be efficient in TILLING protocols.	<i>Calli</i> mutagenesis reduces time, greenhouse resources and the total amount of mutagen needs and wastes. FCE has been proved to be more specific than CEL-1 when detecting EMS-induced mutations. It also reduces TILLING costs.

Chapter 1

Discussion on direct and reverse gene flow

Weedy rice *Oryza sativa f. spontanea* can be defined as any spontaneously and strongly shatterd rice that occurs in cultivated rice fields [80]. While sharing a number of characteristics with other successful weeds, it has unique characteristics such as phenological and morphological similarity to cultivated rice, belonging to the same species as cultivated rice. In tropical regions these weedy forms generally show intermediate traits between the wild rice *Oryza rufipogon* and cultivated rice varieties, originating from natural hybridization followed by selection in man-disturbed habitats [357]. Although few authors maintain that evolution of weedy rice through de-domestication (becoming feral) cannot be excluded [358], it is widely accepted that it can be originated by hybridization in any rice production area where partially compatible wild *Oryza* species coexist with cultivated rice. Several authors, including Xia et al. [80] and Jiang et al. [359], have analysed several weedy rice populations and detected a certain degree of allelic introgression from rice cultivars to coexisting weedy rice. In fact, the gene flow from rice cultivars to weedy rice is well documented and it has been demonstrated that different weedy rice populations exhibit a degree of genetic differentiation. This differentiation is linked to hybridization with the coexisting rice cultivar [80, 359, 360].

Weedy rice was an alarming problem after years of direct seeding in most developed countries [97], becoming one of the most persistent and noxious weeds found in rice-growing ecosystems worldwide [361]. Consequently, first rice gene flow studies in the late 80's aimed to study the pollen-mediated gene flow between cultivated rice and weedy rice relatives [362] and/or wild *Oryza* species [363]. Since the late 90's, a big concern about the possible risks derived from GMO technology arose [364] which is the possibility of pollen-mediated transgene transference from GM rice to weedy rice and wild *Oryza* species originating the so called "super weeds" [139, 140]. The term super weed refers to weeds that acquire advantageous traits such as herbicide

tolerance among others. Firsts GM rice gene flow studies were focused in GM and conventional rice coexistence because of two reasons: i) GM technology was advancing rapidly so it was expected that in a short period of time GM rice would be cultivated widely and coexisting with non-GM rice in the rice production areas and ii) field assays using GM herbicide resistant (HR) lines simplified the detection of pollen mediated gene flow through herbicide treatment over the descendants of non-GM plants [142]. Then, some GM gene flow studies started to include weedy rice in the field trial designs to be able to quantify gene flow from GM plants to them [143].

Cultivating GM rice in open spaces soon became a big concern in different countries and thus, commercial efforts to produce HR rice lines moved from GM technology to mutant technology. In 2002, the first HR mutant lines (CL rice) reached the seed market and scientist soon predicted herbicide resistance transference to weedy rice [140, 143, 259, 260] [141]. Herbicide resistance gene flow over cultivated rice lacks of ecological interest itself, but the rapid transference of the herbicide resistance to weedy rice, as already predicted by experts, was soon reported [262, 365].

In a scenario of transgenic and non-transgenic rice fields coexisting, the reported guidelines to minimize the impact of GM gene flow are: i) the use of different crop species working as a natural barrier, ii) delay in the flowering coincidence and iii) minimum distances between crop fields [146, 366]. In the article entitled "Direct and reverse pollen-mediated gene flow between GM rice and red rice weed", two consecutive field trials were performed to study gene flow in side by side plants between three closely related lines: a GM Senia rice line, conventional Senia and a red rice line from a field where Senia has been cultivated for several years. Six different hybridization paths between these three sexually compatible lines can occur taking into account that each of the three lines can act both as pollen donor and receptor parental (Fig. 14).

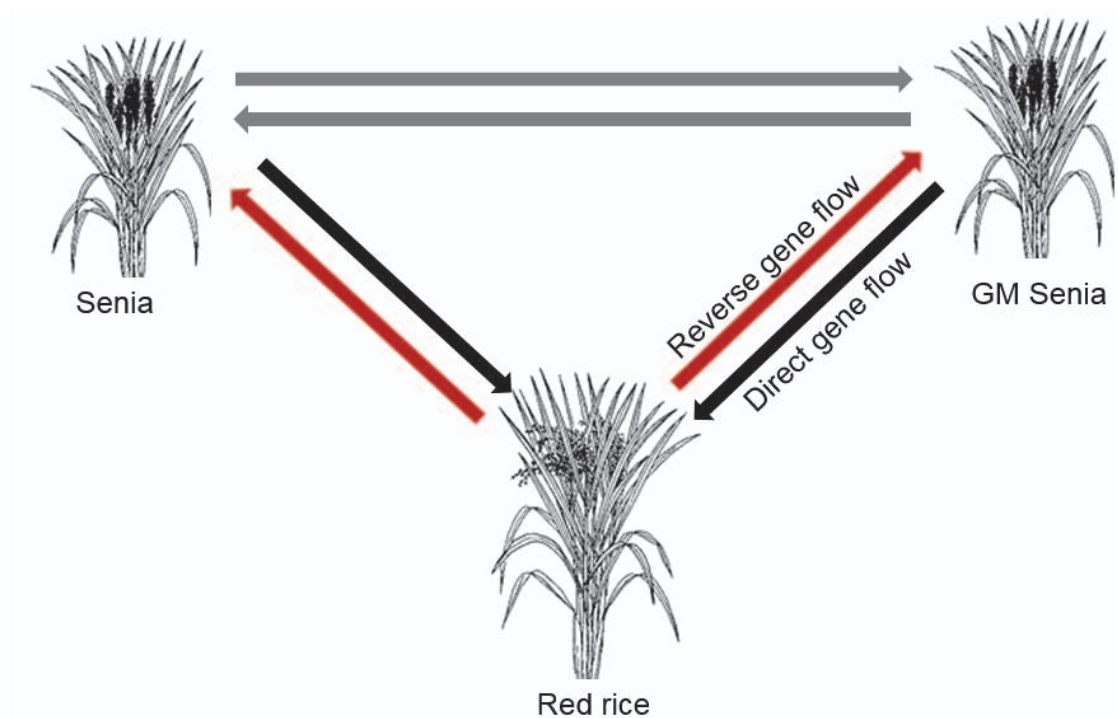


Figure 14. Schematic diagram of different gene flow between the three rice lines used in this study. Grey arrows represent pollen-mediated hybridization between fully sexual compatible Senia and GM Senia plants. Red arrows represent pollen-mediated weedy traits transference. Finally, black arrows represent pollen-mediated GM traits transference to red rice which we could imagine that would be similar to the pollination rate from Senia to red rice.

Studying the gene flow from GM rice to conventional rice is of acute importance as it could help to better establish security distances between GM and conventional rice fields prior to GM rice commercialization. The direct gene flow, which is that from GM rice or from mutant HR lines to red rice, is also relevant in order to study the transference of transgenes to red rice weeds from either GM or HR crops, as it has been already reported that HR weedy red rice appears after only a few years of HR rice cultivation [14, 261, 367, 368]. We could imagine that this direct gene flow rate (from GM Senia to weedy rice) would be quite similar to the gene flow rate that we could find from conventional Senia to red rice [368]. Finally we have also studied what we called the “reverse gene flow” which is the transference of weedy traits from red rice to HR (or any GM) rice, since it could be an alternative way to induce HR (or GM) red rice hybrids emergence. Again, we could imagine that this reverse flow would be quite similar to the gene flow between

eventual GM red rice to either GM and conventional rice. Thus, from the six possible hybridization paths, chapter 1 focused on pollen mediated gene flow from GM to conventional rice, from GM to red rice, but also from red rice to GM rice (reverse gene flow) which, as far as we know, no other study has addressed.

The field design included a pattern of concentric circles in both field trials by placing plants at different distances and a meteorological station placed in the same parcel which recorded wind measurements. This meticulous design made possible to study not only the gene flow frequencies between side by side plants, but also the effects of plant distances and direction and speed of the prevailing wind on gene flow frequencies in Ebro River Delta environmental conditions (Figs. 15 & 16).

Although both field trials quantified the direct and reverse gene flow between GM rice and weedy red rice, only field trial 1 design permitted quantifying the gene flow from GM rice to conventional rice in side by side plants (Fig. 15). Furthermore, both field trials succeeded in studying the effects of distance and the prevailing wind direction and speed in gene flow from GM rice to conventional one, nevertheless, only field trial two-design made possible to study the effect of distance and the prevailing wind direction and speed in gene flow from GM rice to red rice (Fig. 16).

Generally, both field trials demonstrate that in our conditions gene flow was always lower than 0,5%, matching Lu and Yang's review [360] where gene flow in neighboring plants is generally less than 1% in all reviewed reports.

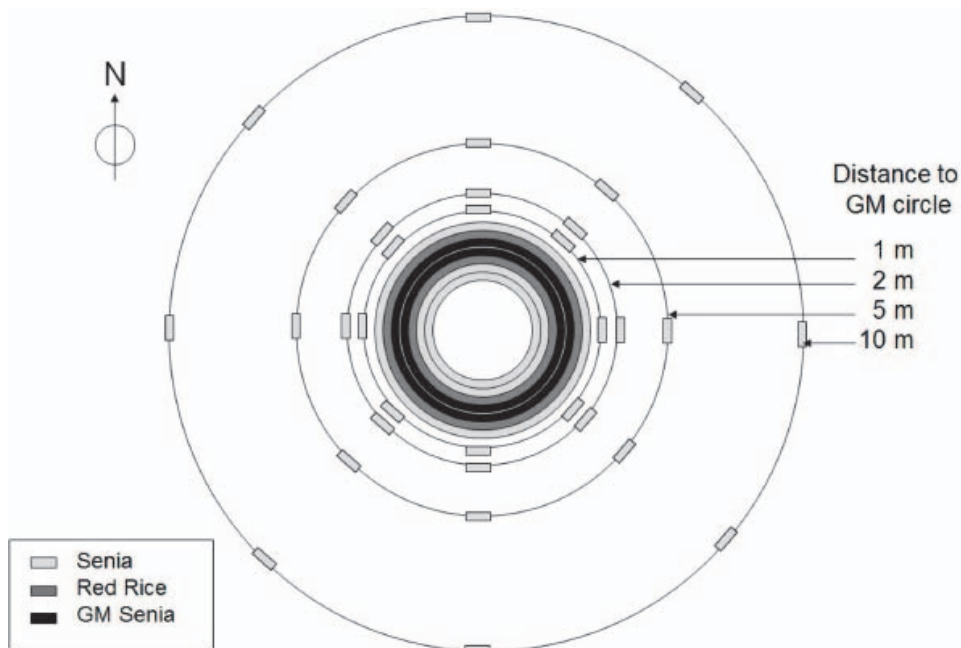


Figure 15. Field trial 1 design: scheme detailing the composition of the circles. One hundred and seven GM Senia plants forming two circles (63 and 44 plants) to evaluate the resulting gene flow. The circular distribution of the plants was designed to maintain the 1:1 plant ratio and to neutralize the effect of the prevailing SW wind. Non-GM Senia plants were also planted at different distances. The results of direct gene flow from the GM Senia to the red rice and Senia plants were published in Messeguer et al 2004.

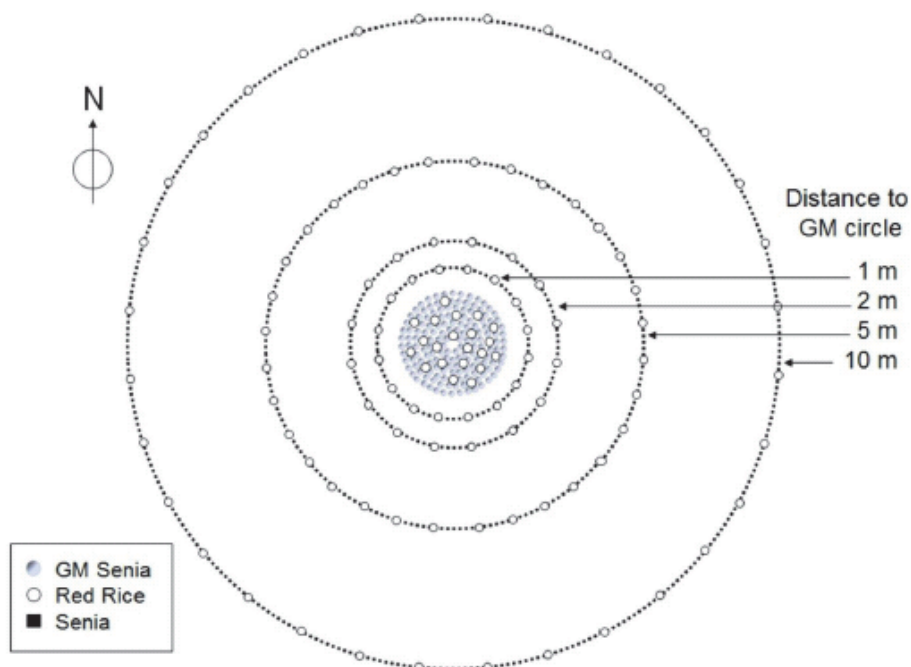


Figure 16. Field trial 2 design: scheme detailing the composition of the circles. One hundred and forty-nine GM Senia plants arranged in seven concentric inner circles formed a 3.5 m diameter transgenic nucleus. Non-transgenic Senia plants were placed at distances 1, 2, 5 and 10m from transgenic nucleus. Twenty-one red rice plants were grown in the transgenic nucleus ($2.3 \text{ plants m}^{-2}$), 16 red rice plants were grown in each of the circles that were 1 and 2 m from the centre, and 32 red rice plants were grown in each of the circles located at distances of 5 and 10 m from the centre.

The central nucleus of field trial 2 design consisted in a 3.5 m diameter circle nucleus of GM Senia plants with interspersed red rice representing a GM rice field with a high red rice infestation (Fig. 16), while the central nucleus in field trial 1 design consisted in side by side concentric circles of conventional, GM and red rice (Fig. 15). Since Senia plants are absent in the central nucleus of field trial 2 design, only field trial 1 could estimate the direct gene flow rate from GM plants to neighboring conventional ones and it was shown to be $0.086\% \pm 0.007$, being similar to results displayed by Chun et al. [369], who have reported a crop to crop gene flow of 0.039% at a distance of 0.5 m, and by Rong et al. [370] who obtained 0.28% gene flow rate at a distance of 0.2 m.

The field trial results showed a significant compatibility asymmetry between GM Senia to Senia gene flow rate ($0.086\% \pm 0.007$) and GM Senia to red rice gene flow rate ($0.036\% \pm 0.001$) (Table 6).

Table 6. Summarized averaged gene flow rates (%) in side by side (neighboring) plants from field trials 1 and 2. The pollen donor and receptor are indicated.

Field trial	Pollen donor	Pollen receptor	Gene flow	Gene flow rate (%) in Neighboring plants
1	GM Senia	Senia		0.086 ± 0.007
1	GM Senia	Red rice	Direct	0.036 ± 0.001
1	Red rice	GM Senia	Reverse	0.222 ± 0.028
2	GM Senia	Senia		Not studied
2	GM Senia	Red rice	Direct	0.137 ± 0.038
2	Red rice	GM Senia	Reverse	0.448 ± 0.056

The asymmetry of the gene flow rate between GM Senia to Senia could be explained by two main reasons:

1. Although the red rice ecotype used in this study was selected from a field where Senia variety had been cultivated for several years, it wasn't fully sexually compatible. Red rice flowered 7 days and 4 days before conventional Senia and GM Senia in field trial 1 and 2 respectively. Consequently the flowering overlap was reduced to only about ten day-period in which cross pollination with the red rice could have taken place [101].
2. Red rice is usually taller than cultivated rice, which probably offers advantages in terms of pollen dispersal [371]. This was confirmed by experimental data, since the height of the red rice lines measured at maturity in both field trials were in fact 15-20 cm taller than GM Senia and conventional Senia (Fig. 17).

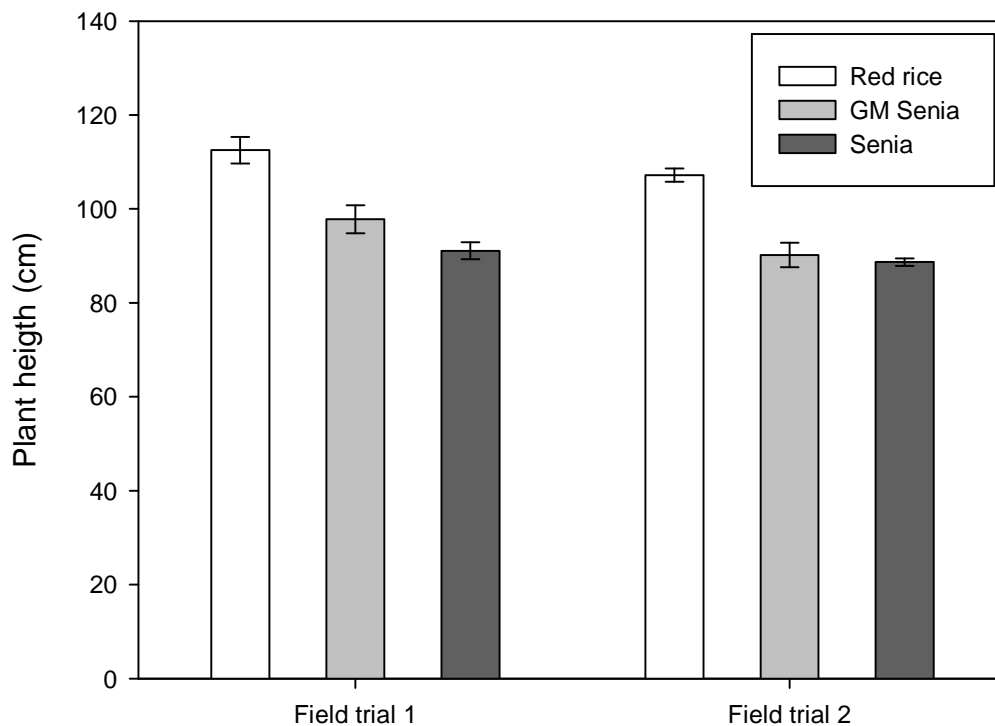


Figure 17. Averaged plant height measurements from filed trials 1 and 2, error bars represent two times standard error.

Both field trial designs permitted estimating the direct gene flow from GM plants to neighboring red rice plants, although it varied substantially between field trials 1 and 2 ($0.036\% \pm 0.001$ and $0.137\% \pm 0.038$ respectively). This variation can be explained by the differences in ratio of plants between the field trial designs. In field trial 1 the GM to red rice ratio was 1:1 which dramatically exceeds any real field red rice infestation. In field trial 2 the proportion of GM to red rice plants was reduced to 7:1, simulating a high red rice infestation (2.3 red rice plants per m^2). Moreover, the most important factor influencing the gene flow rate in neighboring plants could have been the different proportion of transgenic to red rice plants since gene flow is the result of competition between the pollen produced by the plant and that coming from outside.

The concentric circular design in both field trials allowed studying the effects of plant distances and wind direction and speed on gene flow frequencies from GM to conventional plants (Fig. 18). In both field trials, the distance affected dramatically the gene flow frequencies matching Chun et al. [369] results that had previously reported a crop to crop gene flow of 0.0007% at 7 m and Rong et al. [370] that obtained similar results (<0.01%) at a distance of 6.2 m.

Furthermore, a big gene flow variation was detected in accordance to the direction of the prevailing wind. The prevailing Southeast (SE) wind, which is locally called "*vent de baix*", seem to have induced this variation by increasing the gene flow ratio in plants from the opposite sector, the Northwest (NW) sector, at all distances (Fig. 18).

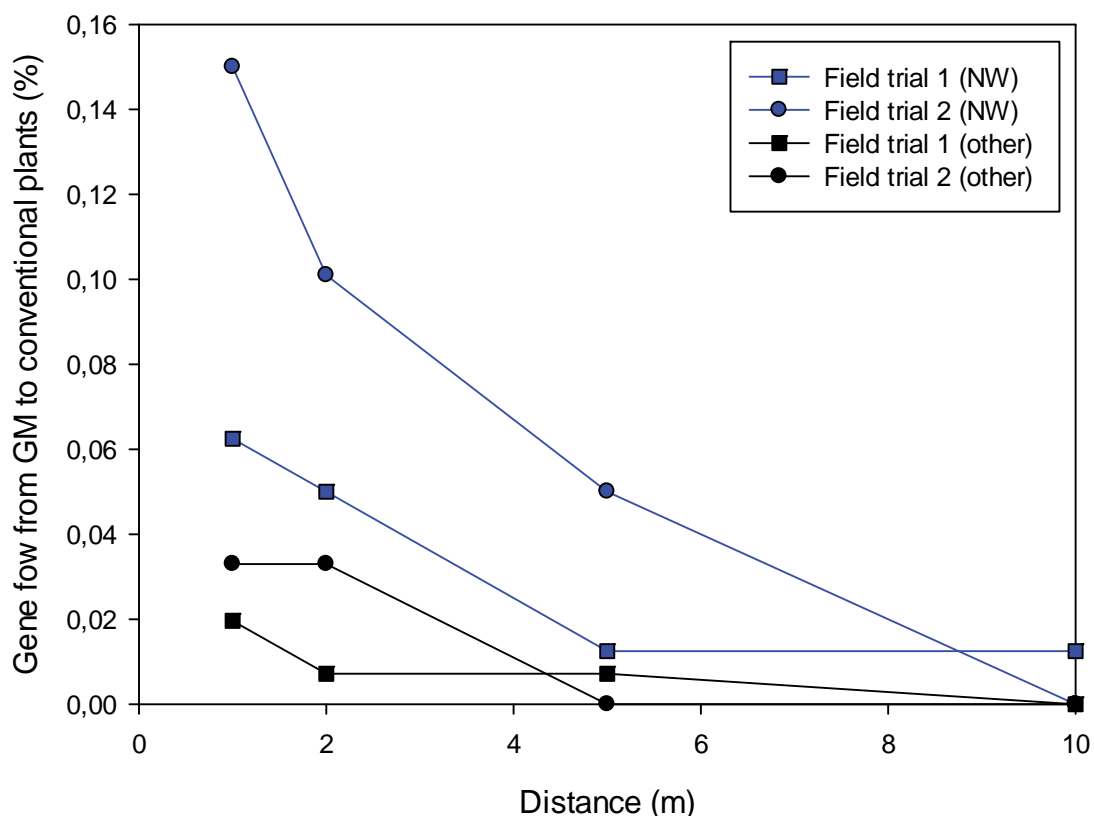


Figure 18. Gene flow rate from GM to conventional plants according to the prevailing wind (in blue) in comparison to other wind directions (in black). Data from field trial 1 (squares) and field trial 2 (circles) are represented in accordance to the distance in meters to the GM source central circles. NW: Northwest sector in both field trials.

Only field trial two-design made possible to study the effect of distance and wind direction and speed in gene flow from GM rice to red rice (Fig. 19). In our study, again, the pollination rate from GM to red rice decreased with distance and was clearly influenced by the direction of the prevailing wind. With respect to the direction of the prevailing wind and other wind directions, direct gene flow rates from GM Senia plants to red rice were very similar to those observed from GM Senia plants to the conventional Senia cultivar (Fig. 19). Most of the hybrids detected in the field trials carried out by Shivrain et al. [261] and Chun et al. [369], suggested that wind speed

and direction affected the flow of GM pollen over weedy rice, although only a few published studies have investigated the influence of distance from the pollen source on outcrossing rates involving red rice [367].

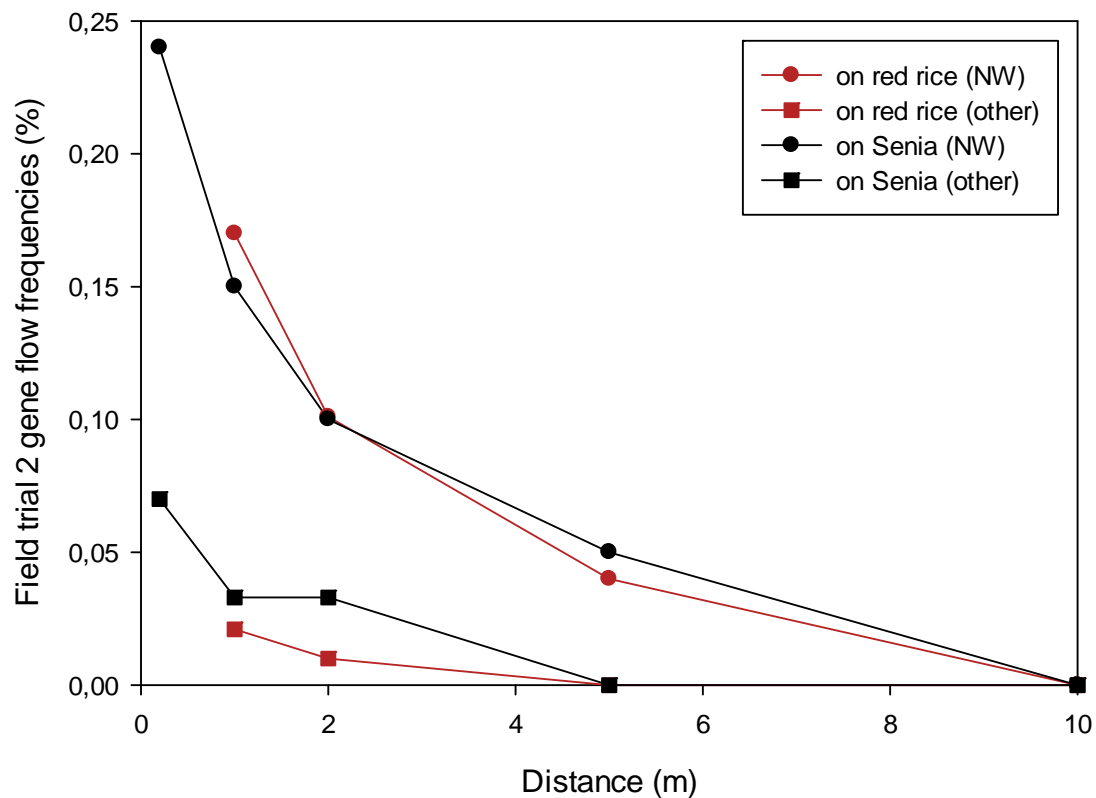


Figure 19. Field trial 2 gene flow rates from GM Senia to conventional Senia plants (black) and red rice plants (red). Circles represent gene flow rates in the direction of the prevailing wind (NW), while squares represent gene flow rates from the other directions according to the distance in meters to the GM central nucleus. NW: northwest sector.

Field trials 1 and 2 design also permitted to detect the reverse gene flow which had generated GM red rice hybrid seeds in GM Senia spikes. These resulting hybrid seeds looked exactly like GM Senia seeds due to maternal inheritance of seed characteristics [372]. The most important phenotypic character that was detectable in reverse flow seedlings was vigour, since the red rice ecotype used in this study grew faster than GM and conventional rice and reverse flow seedlings

are expected to acquire this characteristic. As reported by other authors and confirmed in this study, red rice plants are usually taller and more vigorous than agronomic varieties [373-376].

We therefore initially selected putative reverse flow (PRF) seedlings based on this criteria, and these PRF plants were analysed using AFLPs [377]. Finally these PRF plants were transplanted and grown until seed maturity in order to compare the AFLPs analysis results with a phenotypic seed evaluation in terms of shattering and pericarp colour. In our case, the AFLPs results matched the phenotypic results in all cases.

The reverse gene flow detected in field trials 1 and 2 (0.222 ± 0.028 and 0.448 ± 0.056 respectively) was greater than the direct gene flow (0.036 ± 0.001 and 0.137 ± 0.038 respectively), see table 6. In other words, the reverse gene flow detected was 6.1 times greater than direct gene flow in field trial 1 and 3.3 times greater in field trial 2. Again, the different proportion of GM and red rice plants in field trial 1 and 2 (1:1 and 7:1 respectively) could explain these big differences between field trial 1 and 2.

In our study, differences in height could be detected in the early stages of development and by the flowering stage; some plants were more than 15 cm taller than others. This difference in size could be an advantage for red rice as it would enhance pollen dissemination, nevertheless it can also be a disadvantage for direct gene flow in which pollen grains from GM plants would have to reach stigmas at greater heights due to taller red rice plants.

This result has a limited agronomic relevance because resulting hybrid seeds present no shattering and would be harvested normally. In addition, the proportion of GM to red rice plants in field trial 1 was 1:1, which exceeds any natural occurring red rice infestation, since no commercial field would ever contain such a high proportion of weedy rice because it would not be profitable. Nevertheless, in field trial 2 the proportion of red rice had been reduced to 2.3

plants per m² and although reverse gene flow ratio was reduced in comparison to field trial 1, it was still 3.3 greater than direct gene flow.

Even then, the ecological consequences of reverse gene flow are limited in comparison with those of direct gene flow because non-shattered and non-dormant seeds would be obtained in the first generation due to the maternal inheritance of these characters [372]. These consequences are significantly dependent on agricultural practices and on the presence or absence of natural *Oryza* biodiversity in the cultivation area. In developed countries, where farmers usually cultivate certified seeds, the reverse gene flow from red rice could have only limited ecological consequences, because the hybrid seed would remain in the spike and most of it would be removed during harvesting. There would be only a very small possibility of one seed falling to the soil and becoming a GM weed. Even if there is low occurrence of this phenomenon, it must be taken into consideration in fields used for elite seed production for avoiding any problems of gene flow. However, in underdeveloped or developing countries, and most of the area where there is natural biodiversity of *Oryza*, most farmers do not cultivate certified seeds. Farmers often keep some seeds for planting the following year. In this case, the probability of the proportion of GM red rice weed increasing from year to year is higher and, in consequence, a proper and efficient monitoring plan should be established.

Since GM technology seems to have been displaced by mutant technology in HR rice commercialization and cultivation, potential future research in rice gene flow studies could be to confirm the effect of distances and prevailing wind direction and speed on the gene flow from a mutant HR weedy red rice line (i.e. Clearfield-derived super weed line) to its conventional close related variety. It would be interesting to confirm that gene flow rates from HR red rice to conventional rice are similar to that of the reverse gene flow rate (from non-GM red rice to GM rice) detected in this chapter, which was by far greater than any other gene flow rate.

Gene flow from HR weedy red rice to non-HR weedy red rice could be studied performing a circular design, where GM rice is substituted by a HR red rice and non-HR red rice mix found in a field where the conventional rice of the same variety has been cultivated for years. Thus, few HR red rice plants and non-HR red rice could be interspersed at the same rate (1:1) among a conventional rice central nucleus. This central nucleus could be surrounded by concentric circles of conventional rice with some red rice controls among it. In this way we could compare three different important gene flow rates in neighboring plants while studying the effect of the distance and the prevailing wind direction and speed: i) from HR weedy rice to red rice, ii) from HR weedy rice to conventional rice and iii) from non-HR red rice to conventional rice.

Herbicide treatment would simplify gene flow seeds from HR red rice to either conventional or non-HR red rice. Gene flow markers from non-HR red rice to conventional rice should be obtained through SPNs detection using a next generation molecular technique such as GWAS instead of AFLPs, since more information about red rice syndrome should be obtained. Results from this field trials design, as shown in this study, will permit to increase our knowledge about the HR red rice and non-HR red rice dispersion and gene flow in a rice growing area, and therefore establish guidelines on how to deal with it.

Chapter 2

Applicability of anther culture for double haploid stabilization and improvement selection in Mediterranean rice

An anther culture protocol to obtain Mediterranean *japonica* dihaploid (DH) lines has been described. The Spanish commercial seed register approved four dihaploid lines resulting from these experiments, and currently two of them are being successfully commercialized. Although the anther culture protocol based in Lentini *et al.* [378] was optimized for Mediterranean *japonica* cultivars Hispagran and NRVC 980385, a NRVC 980385 seed stock differing from the original traits in terms of uniformity and height was used to obtain the commercial DH lines. This scientific publication from chapter 2 has not only focused in anther culture procedure as is the common for the majority of articles involving anther culture protocols optimization, but also included detailed descriptions of the greenhouse and field trials selection of the best lines. This information was crucial for selection of DH lines for their successful registration and commercialization.

Many factors can affect the anther-derived *callus* induction such as: i) genotypes of the explants [379], ii) composition of medium [380], iii) culture conditions and methods [381], iv) development stage of the microspores [382], iv) cold pre-treatment of the harvested tillers [383] v) and growth conditions of the donor plants [384]. In this study seeds were sown in winter, from January to March, in a greenhouse heated above 19°C until flowering. Although NRVC 980385 is photoperiod insensitive, the profitable number of spikelets per panicle was less than expected due to a lower total irradiance. Plants sown three to four months in advance of the

normal sowing date in May, had to be ratooned (50 out of the 100 donor plants) in order to obtain a second set of panicles. Ratooning plants and collecting spikes out of season could affect negatively *callus* induction as reported by several authors [378, 380, 385-387], although a higher green plantlet regeneration using ratooned Taipei-309 *indica* rice cultivar was obtained by Guzman and Arias [386].

The microspore developmental stage, crucial for inducing *callus* from anthers, has been correlated to the distance between the atria of the last two leaves in the harvested tillers, though it may vary depending on plant genotype and the environmental conditions in which it is placed [378]. Several authors have studied this correlation to improve anther culture and green plantlet regeneration rates, having each one used different cultivars; for example Chen and Chen [388] recommended collecting tillers with a distance of 5 cm between the atria of the last two leaves in Tainan 5 *indica* cultivar; Herath and Bandara [389] reported 3 to 7 cm using *indica/japonica* hybrids. Our experiments in NRVC 980385 cultivar prior to this present work concluded that a 4 to 8 cm distance between the atria of the last two leaves and the flag leaf had the highest ratio of yellow to slightly green spikelets, which also contained the highest ratio of middle to late uninucleate stage microspores according to Afta et al. [390] and Abassy et al [391]. In the present study, the developmental stage in microspores was determined only in the first panicles since they matched our previous results. Efforts were therefore directed towards sowing as many anthers as possible in order to ensure enough DH lines were yielded. Thus, tillers in booting stage presenting a distance of 4 to 8 cm between the atria of last two leaves were harvested on a daily basis between 8 to 9 am. Nevertheless, the relationship between atria

distances and microspore developmental stages could have changed during the plating process since donor plants were sown out of season and some of them had been ratooned.

Cold pre-treatment of the harvested tillers is used in most of the rice anther culture studies since it has been shown to increase anther-derived *callus* production [385]. The duration and the temperature of the cold pre-treatment may vary between the species or even varieties. Some researchers treated the rice panicles in cold environment during 7 days [392, 393], others [394-397] conducted it within 8 days, and Thuan et al. [398] used 10 days for cold pre-treatment. Lovelin found that the *callus* induction increased with increasing temperatures ($4^{\circ}\text{C} < 8^{\circ}\text{C} < 12^{\circ}\text{C}$) and decreased with increasing duration of the cold pre-treatment (5 d > 9 d > 13 d) in several rice cultivars from India [399]. In wheat anther culture studies, Labbani *et al.* [400] confirmed that duration of cold pre-treatment had a strong effect on the number of embryos produced, developed and formed, while Amssa *et al.* [401] found that cold pre-treatment increased the frequency of endo-reduplication leading to an increase of spontaneously developed DH plants. Duncan and Heberle [402] observed that cold pre-treatment reduced degradation process in anther tissues of Arabidopsis, while long cold pre-treatments enhanced degradation, suggesting that if the duration exceeded 5 to 8 days then the induction frequency of *callus* would have decreased. Finally, they concluded that some of the positive effects of cold pre-treatment include delay of anther wall senescence, increase of symmetric divisions of pollen grains and metabolic changes necessary for androgenesis. Furthermore, Lenka et al. [403] suggested that the possible role of low temperature prior to anther culture may presumably attribute to the elimination of weak or non-viable microspores, while Cho *et al.* [404] and Chen

and Zapata [385, 404] suggested that the delayed anther senescence due to cold pre-treatment might be providing a suitable microenvironment to ensure supply of growth factors to trigger the embryogenesis from microspores. Therefore, to increase the *callus* production from anthers harvested tillers were submitted to a cold treatment, consisting of storing them between 7 to 12 days at 7°C in polystyrene bags at dark, to enhance *callus* induction.

Contamination is quite common in anther culture since spikelets cannot be completely sterilised without affecting anther viability [405]. In our study, the high average contamination rate (26.58%) in the anther derived *callus* induction process could be due to endogenous contamination and soft ethanol disinfection. The soft ethanol sterilisation process applied to the first 41 spikes resulted in a 40% plate contamination, thereby a Lentini's [378] Clorox sterilization process was used to reduce the contamination incidence which was reduced to 22.5%. Nevertheless, no *calli* were obtained from spikes sterilized with Lentini's Clorox protocol. Other authors use mercuric chloride to disinfect anthers [399, 406] although removal of mercuric wastes are more expensive and toxic.

Some authors have succeeded in obtaining rice DH plantlets directly sowing anthers in one-step culture media without transferring to regeneration media so as to save time and resources and to avoid possible contaminations with more transferring steps [407, 408]. Nevertheless, two-step culture media protocols are widely used and described by authors [378] and are by far more efficient in producing DH plantlets. Since the optimal media composition is highly affected by the genetic background of the plants [409], we adapted the protocol of Spanish Hispagan rice

variety, genetically similar to NRVC 980385, assessing N6 and MS salts and vitamins with different hormonal doses in order to induce anther derived *calli* formation and subsequent regeneration. N6 based medium yielded the best results in *calli* formation both in Hispagran and NRVC 980385, which agrees with results from other authors where the application of N6 medium is quite suitable for *japonica* rice varieties, but usually not for *indica* rice cultivars [410]. Furthermore, the N6 medium proposed by Chu has been proven to improve the formation, growth and differentiation of pollen *callus* in rice.

Some of the key modifications used to adapt the anther-derived *callus* induction media for NRVC 980385, formerly used for Hispagran variety, were changes in the concentration of the hormones 2,4-D and naphthaleneacetic acid (NAA). The concentration of 2,4-D was reduced from 2.5 mg L⁻¹ to 2 mg L⁻¹, while kinetin (1 mg L⁻¹) was maintained. These 2,4-D doses are in the range of those used by other authors [411-413]. In contrast, some authors have obtained good *callus* induction results by reducing 2,4-D to even lower concentrations such as 1 mg L⁻¹, although Lovelin [399] reported that increasing the level of 2-4D hormone from 1 mg L⁻¹ to 2 mg L⁻¹ apparently encouraged *callus* yield, but inhibited green plant regeneration. Many other reports mentioned on detrimental side effects of higher 2-4D levels especially in *indica* varieties [380, 414]. Furthermore, Bishnoi et al. [415] reported that reduction of the level of 2,4-D to 0.5 mg L⁻¹ in the *callus* induction medium appeared eventually to be the most effective for anther culture response in *indica/basmati* rice hybrids. Finally, Alemanno and Guiderdoni [416] had good green plantlet regeneration substituting 2,4-D per NAA (1 mg L⁻¹) in the *callus* induction media for a *japonica* variety. In fact, we haven't assessed 2,4-D doses below 2 mg L⁻¹, therefore

it could be interesting to assay lower doses to test its effects on *callus* induction and platelet production of NRVC 980385.

Albinism is a common problem in anther culture [417], this is produced by loss of chlorophyll pigments and incomplete differentiation of chloroplast membranes which is partially influenced by environmental conditions, media composition and culture conditions, but majorly determined by genetic factors [417, 418]. In our study, the proportion of albino plants among anther derived regenerated rice plants was only 14% which is quite low in comparison with other articles. Despite this, Wang *et al.* [419] reported that frequencies of albino shoot forming microspore *calli* range from 5 to 90% in different temperate *japonica* cultivars. Some authors revealed that increased frequency of albino development was observed with a 2mg L⁻¹ 2,4-D concentration [399, 420], which is the one used for NRVC 980385. Even though our success green plantlet regeneration rate, 2,4-D hormone at a lower concentration in the *callus* induction medium could, as reported by other authors, decrease the albino formation. As an exception, Yamagishi *et al.* [379] reported a 36% green plant regeneration using the Nipponbare rice model cultivar and a 4 mg L⁻¹ 2,4-D concentration.

We previously tested 4% maltose in substitution of sucrose as carbon source in *callus* induction media since better results were obtained when applied to *indica* rice cultivars from Iran [421], Nepal [422], Australia [423] and India [424]. Lentini *et al.* [425] reported that using maltose might control the osmotic potential of the cellular environment of *callus* leading to the production of embryogenic *calli* since sucrose could help excised tissues to make more ethylene under *in vitro* conditions resulting in the *callus* browning. Unfortunately, the use of maltose as a carbon source was rapidly discarded in our protocol after different assessments with Hispagan and NRVC 980385 Mediterranean *japonica* cultivars that resulted in null *callus* formation.

Proline was found to increase *callus* induction as reported by Cho and Zapata [404], since it stimulates xylary element formation and provides a readily available source of carbon and reduced nitrogen. We found an optimal dose of 250 mg L⁻¹ for our basal *callus* induction media in both Hispagran and NRVC 980385 cultivars. Although Cho and Zapata just tested a single proline dose of 115.13 mg L⁻¹ proline (1mM) among other media modifications, they found that the medium containing proline was the most effective in promoting *callus* formation [404]. Proline is known to accumulate in rice *callus* culture under saline stress [426]. Nitsch and Godard [427] found that glutamine and proline increased and decreased during the growth phase of pollen embryos in corn and millet which implies that there is both production as well as utilization of these two amino acids. The stimulatory effect of these two amino acids in the development of rice pollen is evident in Cho and Zapata study [404] since the addition of proline and glutamine was required suggesting that these are utilized in the development of isolated pollen. Contrary, Chen *et al.* [4] found that supplements of medium such as glutamine, serine and inositol were not necessary for the culture of isolated pollen of Keng rice cultivar, thus the needed supplements may vary from general to variety-specific ones. Furthermore, other researchers indicate that different materials and explants vary in their requirements for nitrogen sources in the medium. Consequently, the proline (250 mg L⁻¹) together with the casaminoacids (1g L⁻¹) used in the *callus* induction media could be profited as an extra nitrogen source. Finally, the supplied amino acids may be helping the microspores to save energy since they do not need to synthesize them.

When *in vitro* anther cultures are performed, dihaploid plants are mainly homozygous DHs, but heterozygous diploids produced by the somatic tissue can occur, although in rice this is very unlikely [428]. Some authors discard heterozygous diploids using isozyme analyses, RAPD markers, SCARs or SSRs to assess homozygosity [429]. In addition, conventional cytological techniques can be employed to determine the ploidy level of regenerated plants, but ploidy level can be more easily assessed by way of flow cytometry analysis [430].

Although colchicine has been assessed as an antimitotic reagent in order to enhance dihaploid green plantlet regeneration in wheat [431], maize [432] and rice anther culture protocol optimizations [416], no antimitotic was used in this protocol since dihaploid green plantlet regeneration was sufficiently high in our previous media optimization assays. Our results indicated that plants were mainly DH (69%), consisting the other 31% in haploid plants and a single polyploid plant. The DH percentage obtained among green regenerated plantlets (56.4%) is similar to that obtained by Mercy and Zapata [433]. Consequently, flow cytometry was used to discard non-DH plantlets thereby saving time and resources in *in vitro* propagation works. In our case, field experiments resulted in high visual homogeneity in all offspring and no apparent character segregation was observed in any line.

Moreover, this anther culture protocol is now being applied to Mediterranean *japonica* F2 and F5 hybrid lines in order to obtain new commercial varieties. Different cold pre-treatments and colchicine doses are being tested to improve DH green plantlet regeneration. Ploidy determination by flow cytometry is also being optimized to save costs and time.

A first selection of DH lines obtained was carried out when acclimatized plantlets were grown in controlled greenhouse conditions until seed set. Although greenhouse conditions differs by far to the field conditions, important traits like number of tillers per plant, time to heading, sterility or height can be roughly screened under controlled conditions. Only the first 20 DH lines producing seeds were selected to be tested in Experiment 1.

Experiment 1 field trial was performed twice because the DH lines seed set was delayed (Table 7). Consequently, only the first 10 DH lines that produced seeds were assessed in 2008 although it was one month late in season. In addition, due to the low seed availability, the number of individuals per plot was from 50 to 120 plants in Experiment 1 performed in 2008.

Table 7. Field size of different experiments and DH lines assayed during 2008, 2009 and 2010 field trials. Arrows indicate the selection process. Registered lines are highlighted in bold letters.

Experiment	Field size	2008 assessed lines	2009 assessed lines	2010 assessed lines
1	Small	DH1 to DH10	DH3, DH8, DH9 DH11 to DH20	
2	Medium		DH3, DH8, DH9	DH3, DH8, DH9 DH12, DH16, DH18
3	Large			DH3, DH8, DH9

Apparently, the number of tillers per plant was strongly increased in DH3, DH8 and DH9 lines being 43%, 70% and 58% tiller number increase respectively. However, no significant increases in this trait were obtained from subsequent experiments. It was suspected that this apparent increased tiller formation was due to the fact that in 2008 Experiment 1 NRVC 980385 controls had more plants per plot and it decreased the border effect in comparison to DH line plots. Ten more lines were assessed next year in 2009 Experiment 1 while selected DH3 DH8 and DH9 lines were assayed again. This time Experiment 1 was performed in season and using 120 plants per line, same as controls.

Selection criteria was initially mainly focused on reduced height, enhanced tillering and enhanced yield, although all other weekly measures data records such as different height measurements, grain/spike phenological stage, uniformity, pests/diseases, plant shape, fungal lesions on leaves, flag leaf width, length and angle, fungal infection in spikes, days to heading, spike position and exertion and late flowering ratio were taken into account.

Six lines were selected from Experiment 1, three from the 2008 Experiment 1 field trial, which were considered to be the most interesting in terms of height reduction and yield having no evident bad results in other characters. Three more lines were selected from the 2009

Experiment 1 which demonstrated insufficient height reduction but which had interesting yield results. These six lines were assessed on medium scale field trials (Experiment 2) and from these, the three lines obtained in 2008 experiment were assessed in real conditions large scale trials (Experiment 3) as they had obvious height reduction, high homogeneity and acceptable productivity. Nevertheless, the DH12 line had an exceptional and unexpected yield result.

Although rice production and yield assays should be ideally repeated using replicates and control replicates prior to Spanish register submission, four lines (DH3, DH8, DH9 and DH12) were submitted and successfully approved. Since the Spanish registration process takes two years, certified seed multiplication scheme has been conducted in parallel in all lines to further select the lines that would be finally commercialized while preparing sufficient seed stock. The shortest DH3 line and the most productive DH12 line are currently being successfully commercialized since they maintain NRVC 980385 quality traits, high germination capability (even in low temperatures) and low nitrogen requirements, while presenting unexpected advantages. DH3 presents reduced plant height, shorter flag-leaf to spike distance (half the distance), wider and bigger spikes, while DH12 has higher productivity but less height reduction, although it presents thicker tillers that prevents lodging. Thus, the field assays demonstrate that producing new rice lines through anther culture is a feasible and efficient technique. Despite this, the protocols can and should be optimized for a far more general use in Spanish varieties and to increase the number of green plantlets obtained at the end of the anther culture process.

Chapter 3

Applicability of rice *calli* mutagenesis for rapidly obtaining TILLING mutant populations

A new rice mutagenesis protocol based on rice *calli* mutagenesis has been proposed, which reduces the time and resources for producing rice mutant populations. Specifically, the plants regenerated from mutagenized *calli* were directly used to detect individual mutation using Targeting Induced Local Lesions IN Genomes (TILLING). The TILLING process was also modified in order to reduce costs by using a fennel crude extract (FCE) to cleave mismatches instead of the commercial CEL-1 mismatch cleavage enzyme. Furthermore, ACS1 and SGR9 senescence related genes were screened for mutants that could have acquired a delayed senescence phenotype.

The most used protocol to produce chemically-induced mutant populations consists in using a mutagenic solution to incubate germinating seeds. After the mutagenic treatment, the seeds (*M1* population) are rinsed to remove the mutagenic reactive and grown until seed set. The first generation (*M1*) produced directly from the mutagenic treatment cannot be screened because the majority of the generated mutations are somatic and consequently not transmitted to the progeny [434]. To solve this problem, the *M1* mutant population has to be grown and then self-fertilized. The mutations in *M1* sexual structures can produce whole mutant *M2* descendants, thereby avoiding any ambiguities caused by mosaicism. The resulting *M2* progeny can be screened for mutations.

Instead of performing a chemical mutagenesis using germinating seeds, we've explored a new mutagenesis approach incubating the mutagen agent with *scutellum*-derived *callus* masses containing primary, embryogenic and non-embryogenic *calli* [435]. In most cases, the embryo-derived rice *callus* regeneration is only generated from a few cells. Thus, the regenerated *M1*

plantlets from mutant *calli* could be screened directly without waiting for a self-pollinated M2 population (Fig. 20).

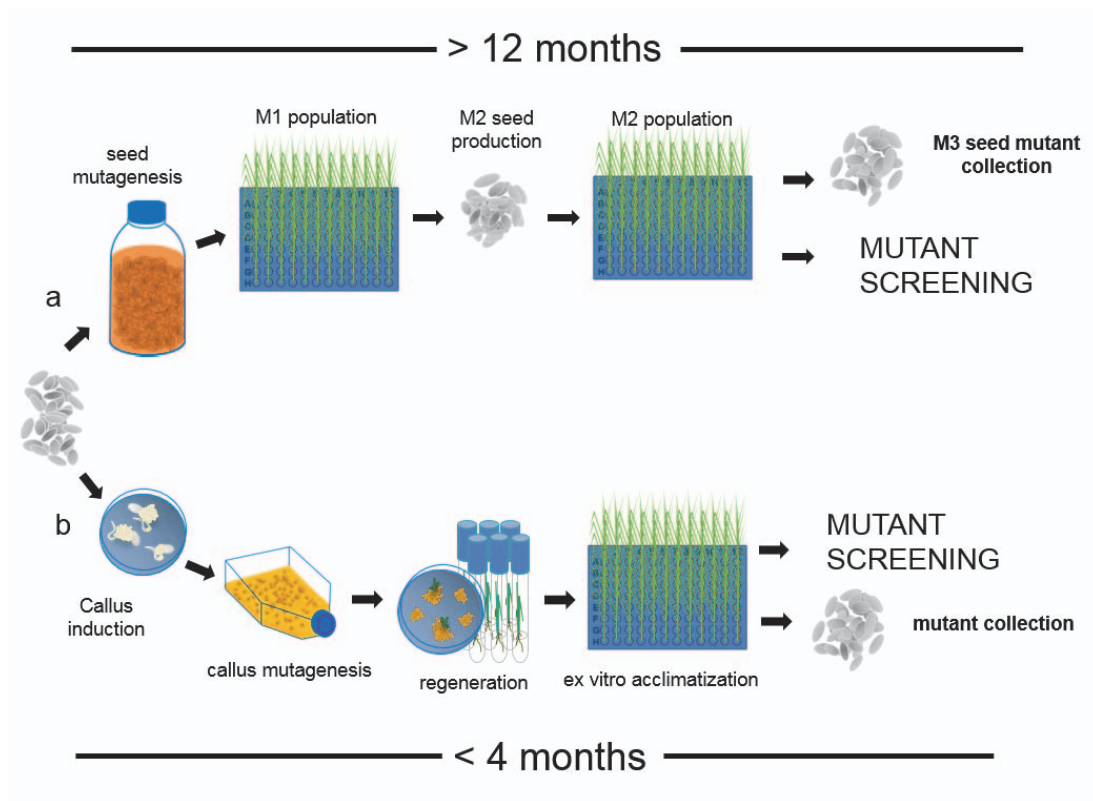


Figure 20. Diagram comparing *callus* mutagenesis and seed mutagenesis protocols in rice TILLING. (a) In the basic TILLING method, seeds are mutagenized, the resulting M1 plants are self-fertilized and the M2 generation of individuals is used to prepare DNA samples for mutational screening while their seeds are inventoried. (b) In the proposed rice TILLING protocol through *callus* mutagenesis, *calli* are induced, mutagenized, and the regenerated plants provide DNA for molecular screening of mutations. The steps represented cover from *callus* induction (b) or seed imbibition (a) to DNA extractions for molecular screening of mutations. Duration of each step is indicated between black bars.

Root *callus* was the only *callus* mass to be discarded as it is unable to regenerate plants and is easily identified as it grows separately from the *scutellum*-derived *callus* masses. The cost and time consuming embryogenic *callus* selection used in transformation protocols was bypassed in order to make *callus* mutagenesis feasible through an easy and quick manner. Consequently, a mutant population was successfully obtained by mutagenizing and regenerating embryogenic *calli* contained in the whole *scutellum*-derived *callus* masses.

In plants, chemical mutagenesis protocols use seeds under germination process so the mutagen can be absorbed by the germinating embryos. Thus, the mutagen molecules can reach the meristematic region where the germ cells are contained. However, the mutagen has to reach the target meristematic cells and induce mutations after crossing the cell wall, and the plasmatic and nuclear membranes. Moreover, the seed protecting layers reduce the seed mutagenesis efficiency since only few molecules can reach the nucleus of germ line cells contained in the embryo. To overcome this, other alternatives such as pollen, microspore or single zygotic cells mutagenesis have been performed. Pollen mutagenesis has been assayed in maize [436-438], although these mutagenic treatments are difficult to perform in field conditions and subsequent pollination requires an acceptable pollen viability. Therefore, no rice pollen mutagenesis attempts have been reported to date as rice pollen lifetime is too short, and manual pollination is a lot more complicated than in maize. Iftikhar and Mumtaz [439] mutagenized oilseed brassicas microspores using EMS for genetic improvement through a mutation breeding programme. Suzuki *et al.* [440] and Satoh *et al.* [441, 442] in turn, reached high mutagenesis rates when treating single zygotic cells in recently fertilized rice spikelets by using N-methyl-N-nitrosourea; however, a low cell survival was obtained and the resulting seeds (*M1*) had to be grown, fertilized and harvested until the *M2* population was ready for screening. Recently, suspension-cultured cell mutagenesis using EMS has been reported [443], regenerated mutant plants were self-pollinated in order to obtain 302 *M2* lines for phenotypic analysis in field conditions which achieved high mutagenesis rates.

EMS [444], a high potency mutagen [445] commonly used in plant mutagenesis [446, 447] mainly induces C-to-T changes resulting in C/G to T/A substitutions [448]. In fact, EMS induces a strong biased alkylation of guanine residues forming O6-ethylguanine, which can pair with thymine but not with cytosine. Through subsequent DNA repair, the original G/C pair can then be replaced with A/T [448-451]. Other mutagens such as N-methyl nitrosourea (NMU), diepoxybutane (DEB) or sodium azide had been also used in plant mutagenesis. NMU is a

monofunctional alkylating agent causing single-strand DNA breaks during interphase stages, which is thought to induce an error-prone interference of recombinative repair and replication in damaged basic repeats of large tandem repeat arrays [452]. MNU had been used to obtain rice mutant populations by Suzuki *et al.* [440] and Satoh *et al.* [441, 442]. DEB is capable of producing alkali-labile sites in DNA and forming inter- and intrastrand cross-links [453]. Although the exact mode of action of DEB and the premutagenic lesions are not well understood, it has been observed to induce chromosome breaks and deletions in rice [454], although point mutations and small insertions/deletions were also observed by Wu *et al.* [455]. Finally, sodium azide is an inhibitor of catalase and peroxidase enzymes, increasing the frequency of chromosomal aberrations. However, in barley it has been shown to induce substitutions comprising transitions and transversions [456]. It has been described as a potent respiration inhibitor which is metabolized *in vivo* to the mutagenic compound azido alanine [457] that predominantly leads to base transitions [456, 458]. Different sodium azide doses had been assessed in rice, although mutation frequencies estimations were only phenotypic [442, 459, 460].

In this study EMS was selected as the mutagenic agent for mutating uncoated embryogenic cells thus facilitating the mutagen action. This statement is supported by our study, where less than 200 μl of pure EMS were needed to generate a 6912 rice plants mutant population with a sufficiently high mutation density by using a low EMS concentration (0.2%) and a short incubation time (2 hours) in comparison to other studies, where bigger amounts of EMS and longer times have to be used to incubate the germinating seeds [451, 461-463]. Furthermore, due to the low amount of EMS needed, the contaminated wastes derived from the mutagenic treatment and rinses was highly reduced in comparison to the EMS seed mutagenesis protocols. Moreover, the regeneration rate of the mutagenized *callus* was similar to that for non-mutagenized control *calli*, suggesting that it is still possible to increase the dose and exposition time in order to obtain even higher mutation densities if needed. Nevertheless, we expect that

EMS dose and incubation time shouldn't be increased over 0.4% and 18 hour respectively, since Chen *et al.* [443] obtained a high mortality and high mutagenesis rate when using 0.4% and 0.6% concentrations and 18 to 22 h exposure times in cell suspension cultures.

Although the mutation frequencies are estimated scoring the bp changes per bp screened, authors commonly consider that mutations at the beginning and the end of every amplicon are not detectable because the cleaved fragments are too small to be considered. Consequently, authors systematically discount 200 bp of every amplicon when scoring the mutation frequencies [461]. One mutation in every 1Mb was obtained by Wu *et al.* [455], 1 in 300 Kb by Till [461] and 1 in 135 Kb was reported by Suzuki [440] using seed mutagenesis protocols. In our study, the total number of mutations identified was divided by the sum of the screened individuals multiplied by the bp sum of amplified fragments. In order to be able to compare with other authors, we have also discounted 200 bp from each fragment. Our results confirm that a high mutagenesis rate (1 mutation in every 451 Mb) has been achieved when compared to other studies.

Only less than 20% of total *calli* obtained from 1,200 Hispagran seeds was used for mutagenesis. Although the capability for obtaining and regenerating embryogenic *callus* depends on the cultivar and culture media used [435], it would have been possible to mutagenize the same amount of *calli* using less than 200 *callus* forming seeds sown in less than 20 petri dishes of callus induction media (less than 500 ml of OryCIM callus induction media). Callus mutagenesis and rinses were performed in a cabin fume hood to fully protect operators from EMS gases, although callus material wasn't completely protected from fungal or bacterial contamination even by working next to a Bunsen burner. Consequently, in order to ensure that contamination-free material is obtained from a single mutagenesis procedure, it would be highly recommendable that one use flask replicates containing different batches of *callus* masses.

A fresh fennel crude extract (FCE), which previously showed a strong mismatch endonuclease activity, was used to efficiently screen the mutant population and turned out to be highly indicative for the screening of EMS mutations, since it cleaved A/C and T/G mismatches preferentially, matching them with the most likely substitutions induced by EMS. Contrarily, the commercial CEL-1 purified enzyme cleaved C/C mismatches preferentially [434] being less specific for TILLING EMS-mutagenized plant populations. Nevertheless, the endonucleases responsible of the FCE mismatch cleavage activity are still unknown since it is still under study. Furthermore, the FCE 3'-5' exonuclease activity and other aspects imply the need for specific incubation conditions [464]. Till *et al.* [461] used CEL-1 cleavage enzyme and a selection of those primer combinations that allow for a high pooling degree, being able to till eight-plant pool samples. In fact, in our previous experiments, selecting those genes and primer pairs that were easier to amplify in terms of size and reproducibility allowed the FCE mismatch cleavage detection of a single mutant individual from a 16-plant pool (1:16). Nevertheless, in this published FCE cleavage experiment the genes and primers combinations were not selected in terms of efficiency and reproducibility, but in agronomical interest of mutants in senescence-related genes. OsACS1 and OsSGR9 genes were selected since mutations in those genes can lead to a senescence delay enhancing the final grain fill. Consequently, the DNA was pooled in a 1:4 ratio in order to increase the technical sensitivity thereby avoiding false negative results.

The screening of the mutants started just when the first regenerated plantlets batch was acclimatized, and it was stopped as soon as sufficient interesting mutants had been detected in senescence related genes OsACS1 and OsSGR9. Consequently, although the mutant population consisted in 6912 individuals, only 2400 DNA samples were fourfold pooled and analysed.

OsACS1 is a 1-aminocyclopropane-1-carboxylate synthase gene involved in plant survival under flood (low O₂) conditions related to long term ethylene production and stem elongation [465], although it is also involved in senescence and fruit ripening delay [466]. Delayed senescence and

salinity tolerance have been reported in *ZmACS6* [467, 468] closely related to the *OsACS1* gene, while partial sterility in other ACS gene mutants has been reported in maize [468]. In our study, strong effect *ACS1* mutants such as 152s3, 398s4 and 576s1 were partially sterile and no mutant homozygotes were obtained. A 25% of the flowers were sterile, 50% produced heterozygous *ACS1* mutant descents and the remaining 25% were *ACS1* homozygous wildtype descents, this leads us to suspect that those mutations became lethal in homozygosis.

OsSGR9 (Stay Green Rice 9) is an ubiquitin-protein ligase which acts as a senescence-inducible chloroplast stay-green protein that regulates chlorophyll degradation [469-472]. Up-regulation of *SGR9* increases chlorophyll breakdown during senescence in rice [470] while down-regulating mutations can delay this chlorophyll degradation, although some authors consider that mutations in *OsSGR* result in non-functional chlorophyll accumulation [469, 473].

Eight different tillers of the mutant plants obtained using this *callus* mutagenesis protocol were sampled again in order to discard the mosaicism that would be expected in a seed mutagenized M1 population. These *callus* mutagenized regenerated plants could be considered similar to an M2 population obtained from regular seed mutagenesis since mosaicism was not detected in any of the mutant individuals. In addition, all mutants were unique; no clones were detected in the mutant population after screening the whole population and upon revision of all the results involving any plant that had shared the same *callus* of origin.

In conclusion, the *callus* mutagenesis protocol described here saved time, resources and the amount of mutagen and wastes needed for a common seed mutagenesis. Furthermore FCE was more specific than CEL-1 for EMS-induced mutations while reducing TILLING costs since the expensive commercial CEL-1 was substituted by simply using a crude extract of fresh fennel.

Future perspective

The availability of a complete rice genome sequence has opened up large opportunities not only for rice but for the plant community as a whole. In addition, next-generation sequencing has become a powerful and economic tool, while publicly available sequencing information are facilitating the precise comparison of genome sequences between cultivars and complement the gene prediction by mapping the RNA-Seq reads to the genome [474]. The International Rice Informatics Consortium (IRIC) [131] have recently centralized information access to rice research data. This fully available 3000 rice varieties SNPs database with phenotype and variety Information integrates the SNP genotyping data from the so called 3,000 Rice Genomes Project [132] and the phenotype and passport data for the 3,000 rice varieties from the International Rice Information System. Together with *Gramene* [130] and *Oryzabase* [475] databases, they provide computational tool to facilitate rice improvement via discovery of new gene-trait associations and accelerated breeding.

The use of mutant resources for high throughput gene discovery [476] is another area of increased research focus. These mutant resources greatly aid in the functional analysis of all the rice genes. Moreover, a new reverse genetic technique described by Tsiai *et al.* [477] combines TILLING [461] with next-generation sequencing. This new mutant reverse genetics detection approach is replacing the mismatch cleavage detection to a more powerful deep search for mutations in targeted loci. This method is sensible enough managing pools of 64 or 96 individuals, while maximum pulling of samples in mismatch cleavage detection is far lower. Furthermore, the method is immediately applicable to TILLING mutant populations available in multiple crops. It should also be applicable to any search for rare SNPs or mutations in a complex sample.

Mutant populations would be relegated to be used only for forward genetics phenotypic screenings since a new Targeted genome engineering method based on the bacterial CRISPR

(clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) type II prokaryotic adaptive immune system has emerged as a breakthrough method for genome engineering [478-480]. The system is based on the Cas9 nuclease and an engineered single guide RNA (sgRNA) that specifies a targeted nucleic acid sequence. The ability to reprogram CRISPR/Cas endonuclease specificity using customizable small noncoding RNAs has set the stage for novel genome editing applications [481-483]. Interestingly, four independent groups have shown that the CRISPR/Cas9 system can introduce biallelic or homozygous mutations directly in the first generation of rice and tomato transformants, highlighting the exceptionally high efficiency of the system in these species [484-487]. This new tool will permit to efficiently introduce any site-specific mutation to any plant variety.

The research has moved from a single-gene based approach to more holistic genome (and proteome) wide translational research and rice will become a model species for plant omics research in the near future. The advance of the rapid, high throughput and cheaper technologies about whole genome resequencing and single molecular sequencing permit us to study the genome and transcriptome of a population rather than individual. The advances in rice omics have provided us with more detailed DNA variation patterns, large amount of functional genes, transcription patterns and protein expression profiling, which have throw highlight on the genetic basis of the development of important agronomic traits. The core collection of rice germplasm being genetically representatives, could be extensively used as the uniform panel of genetic materials for omics research. The achievement of several transgenic crops and discovery of large-scale marker data sets indicates that breeders can design to pyramid the most favourable alleles into one variety. With more and more genes controlling the important agronomic traits cloned and functionally annotated, the molecular design breeding or synthetic biology will make more progress. Consequently, the more desired varieties are being developed by these high-tech approaches [151].

Rice research is immersed in an exciting era and it is expected that the information generated from such massive studies would pave the way for designing of better yielding crop plants.

Conclusions

1. The pollen-mediated gene flow between GM and conventional rice cultivars (direct gene flow) exist and has been quantified, being low and strongly influenced by plants distance and sexual compatibility, but also by the prevailing wind speed and direction.
2. The pollen-mediated gene flow between weedy red rice and GM rice (reverse gene flow) exists and has been quantified and compared to direct gene flow using fully sexual compatible lines.
3. Although the detected reverse gene flow is higher than direct gene flow, its consequences are limited because non-shattered and non-dormant seeds would be obtained in the first generation due to the maternal inheritance of the weedy characters.
4. Rigorous red rice eradication would be needed to avoid reverse gene flow and the consequent arising of GM weedy red rice plants.
5. A fast and efficient anther culture protocol have been adapted to Spanish rice varieties to obtain new dihaploid lines for commercial proposes.
6. Producing new Spanish rice lines through anther culture is a feasible and efficient technique that allows us to obtain new commercial rice lines ready for registration in less than four years.
7. Rice embryo-derived *calli* mutagenesis can represent a significant advantage in terms of time-saving, greenhouse space and work during the generation of mutant plant populations.

8. This effective chemical mutagenesis protocol ensures high mutagenesis rates thereby saving in waste removal costs and the total amount of mutagen needed thanks to the reduced mutagenesis volume reduction.

9. The use of a fennel crude extract instead of commercial CEL-1 mismatch cleavage enzyme represents an important cost reduction, being more specific to detect the most provable mutations when using EMS mutagen in TILLING experiments.

10. A fast and efficient TILLING protocol using rice *callus* mutagenesis has been developed to boost mutation breeding rice improvement.

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