PROTEOMIC AND TRANSCRIPTOMIC APPROACHES TO ABIOTIC STRESS TOLERANCE IN RICE (*Oryza sativa* L.)

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Dedicated to
my dear family,

especially to Pedro, my mother and my sister;
to the memory of my father
and grandparents

‘…Caminante, son tus huellas
el camino y nada más;
caminante, no hay camino,
se hace camino al andar.

Al andar se hace camino
y al volver la vista atrás
se ve la senda que nunca
se ha de volver a pisar.

Caminante no hay camino
sino estelas en la mar...’

Extracto de um poema de Antonio Machado
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ABSTRACT

Cereals are the most important food source, and rice (*Oryza sativa* L.) alone is the staple crop for more than half of the world's population. Environmental stresses limit plant growth and productivity, and in the case of rice, drought has become the most serious constraint to yield potential in all agro-climatic zones. The challenge for the next generations is thus to achieve a sustainable rice production with less arable land, reduced inputs of agrochemicals (fertilizers and pesticides) and above all, with limited water supply, given the increasing scarcity of water resources at global level. In this context, understanding the molecular mechanisms that plants in general, and rice in particular, cope with water scarcity is primordial to obtain varieties with improved stress tolerance. The main aim of this thesis was therefore to get novel insights into the molecular mechanisms involved in the abiotic stress tolerance, with special emphasis on water stress. We characterised six rice genotypes bred at IRRI with contrasting responses (tolerance or sensitivity) to drought, salinity and low temperature. The proteomic analysis of the rice embryo – a good model to address water-deficit tolerance since it withstands severe water loss at the final stage of seed maturation – disclosed significant features regarding stress tolerance. Hence, hierarchical and multidimensional scaling analyses revealed that sensitive varieties were closely related, while tolerant genotypes were more divergent. The higher distance between tolerant genotypes, probably reflects an adaptation to more diverse environmental conditions, conferring to these varieties a higher plasticity to face stressful challenges, as compared to sensitive ones. Furthermore, we report for the first time a difference in the phosphorylation status of LEA Rab21 (a late embryogenesis abundant protein described to be associated with drought tolerance) in the seed embryos of genotypes adapted to diverse environmental conditions. The results indicated that the protein was more strongly phosphorylated in the embryos of the sensitive varieties than in the embryos of the tolerant ones. The differences found in phosphorylation status of Rab21 are proposed to be related to stress

tolerance. On the other hand, we characterised a 20-kD rice protein that cross-hybridised with an antibody raised against the maize DBF1 DRE-binding protein. This transcription factor belongs to the AP2/ERF superfamily, whose members have important functions in the transcriptional regulation of both plant developmental and stress responses. The expression patterns exhibited by the 20-kD protein in rice were quite similar to those described for maize DBF1, being both induced by water-deficit, salinity and ABA. Moreover, the 20-kD rice protein was also induced by cold, contrary to that reported for maize DBF1. The anti-DBF1 antibody proved to recognise proteins with identical molecular mass and pI in both maize and rice embryos, suggesting a cross-hybridization with homologous proteins. The high basal levels of the 20-kD polypeptide in rice vegetative tissues of 'PSBRc1'- a drought-tolerant genotype - in pre-stress conditions, whereas it was strongly induced by water-deficit in a sensitive one, seems to indicate that the protein may take part in 'stress-anticipating' mechanisms in the tolerant genotype. In addition, we identified by cDNA-AFLP several clones that were differentially expressed under water-stress conditions either in a drought-tolerant genotype, and/or in a sensitive one. All cDNA clones presented significant homology to rice genes (e.g., a calcium-binding EF hand family protein and a C-type cyclin), and most of them pointed to a major function in cellular signal transduction. The expression profiles of the water-stress responsive genes were further analysed after seedling exposure to salinity and cold stress treatments, across genotypes with differential responses to abiotic stress. The overall comparison of gene expression indicated that the water-deficit responsive genes also responded to salinity and cold, in at least one of the compared genotypes. Six of the identified genes suggest an involvement in stress tolerance and can therefore be included in future assays aiming for crop improvement. The results taken together provided some novel insights into the complex molecular mechanisms integrating the abiotic stress response/tolerance in rice.

RESUMO

Os cereais são uma das principais fontes alimentares, sendo o arroz (*Oryza sativa* L.) um alimento de primeira necessidade para mais de metade da população mundial. Os stresses ambientais limitam o crescimento das plantas e a produtividade agrícola, embora no caso concreto do arroz, a secura se tenha convertido na principal ameaça à produtividade praticamente em todas as zonas agro-climáticas. Um dos grandes desafios para o futuro, no que diz respeito à produçao de arroz, consiste em garantir uma produtividade elevada, dispondo de áreas de cultivo cada vez mais reduzidas, recorrendo a um menor uso de agroquímicos (fertilizantes e pesticidas), mas acima de tudo, utilizando menos água, devido à necessidade de assegurar a sustentabilidade dos recursos hídricos. Neste contexto, a compreensão dos mecanismos moleculares que contribuem para que as plantas em geral, e o arroz em particular, possam fazer face a situações de escassez de água, é fundamental para a obtenção de futuras variedades com um certo nível de tolerância à secura. Assim, este trabalho de doutoramento teve como objectivo principal a aquisição de novos conhecimentos acerca dos mecanismos moleculares subjacentes à tolerância ao stress abiótico em plantas, com especial ênfase na tolerância ao stress hídrico. No presente trabalho, caracterizou-se a nível molecular seis genótipos de arroz oriundos do IRRI, tolerantes e sensiveis a três tipos fundamentais de stress abiótico i.e. secura, salinidade e baixa temperatura. A análise proteómica do embrião de arroz – um modelo apropriado na abordagem da tolerância ao stress hídrico, dada a sua capacidade de sobrevivência à forte perda de água no período final da maturação da semente – evidenciou aspectos importantes no que respeita à tolerância ao stress ambiental. Nomeadamente, as análises de escala hierárquica e multidimensional revelaram uma relação estreita entre as variedades sensíveis, enquanto que a existente entre os genótipos tolerantes demonstrou ser bastante mais divergente. A relação mais distante entre as variedades tolerantes reflecte muito provavelmente a adaptação a factores

ambientais mais diversos, podendo estar na base de uma maior plasticidade na resposta a condições adversas por parte destas variedades, quando comparadas com as sensíveis. Por outro lado, descreve-se pela primeira vez neste trabalho, uma diferença no grau de fosforilação da proteina 'Rab21' em embriões de sementes de cultivares adaptadas a condições ambientais muito diversas. Esta proteína é particularmente abundante na fase tardia da embriogénese e foi descrita como estando associada à tolerância ao stress hídrico. Os resultados parecem demonstrar que a proteína 'Rab21' se apresenta mais fortemente fosforilada nos embriões das variedades sensíveis do que nos embriões das variedades tolerantes. Desta forma, propomos que as diferenças encontradas no grau de fosforilação da proteína 'Rab21' estejam relacionadas com a tolerância ao stress ambiental. Também se procedeu à caracterização molecular de uma proteína de arroz com uma massa molecular aparente de 20 kD, reconhecida por um anticorpo criado contra uma proteína de milho designada por 'DBF1'. Esta proteína de milho pertence à família dos factores de transcrição do tipo 'AP2/ERF', cujos membros desempenham funções importantes na regulação transcricional do desenvolvimento da planta e da resposta ao stress ambiental. Os padrões de expressão exibidos por esta proteína de 20 kD em arroz, foram muito semelhantes aos descritos para a proteína 'DBF1' em milho, sendo ambas induzidas por stress hídrico, salinidade e ácido abscíssico (ABA). Salientamos ainda que esta proteína de arroz evidenciou indução por frio, ao contrário do descrito para a proteína 'DBF1' em milho. O anticorpo 'anti-DBF1' reconheceu proteínas de embrião com uma massa molecular aparente e ponto isoeléctrico idênticos em milho e arroz, sugerindo assim tratar-se de proteínas homólogas. A proteína de arroz de 20 kD apresentou níveis altos de expressão basal na variedade tolerante à secura, em condições prévias ao stress, enquanto que demonstrou ser fortemente induzida em condições de stress hídrico, na variedade sensível. Este perfil de expressão sugere que a proteína possa estar envolvida em mecanismos que antecipam o stress hídrico na variedade tolerante. Por

último, foram ainda identificados vários genes através da técnica de 'cDNA-AFLP', com uma expressão diferencial em condições de stress hídrico, quer no genótipo tolerante à secura, quer no sensível. Todos os clones de cDNA isolados apresentaram uma homologia significativa com genes do arroz. Damos como exemplo uma proteína de união ao cálcio e uma ciclina do tipo C. A maioria dos genes identificados indicou um envolvimento em mecanismos de transdução de sinal celular. A expressão dos genes que responderam ao tratamento de stress hídrico, foi também analisada em orgãos vegetativos, em resposta à salinidade e baixa temperatura. A comparação global da expressão génica entre variedades tolerantes e sensíveis aos diferentes tipos de stress abiótico, indicou que os genes induzidos por stress hídrico também apresentaram uma resposta à salinidade e ao frio, pelo menos num dos genótipos comparados. Os dados sugerem um papel importante na tolerância ao stress abiótico para seis dos genes de arroz identificados, que poderão vir a ser assim explorados em programas de melhoramento desta espécie. No seu conjunto, os resultados obtidos neste trabalho contribuem com novas perspectivas sobre os complexos mecanismos moleculares que integram a resposta e a tolerância ao stress abiótico no arroz.

LIST OF ABBREVIATIONS

ABA	Abscisic acid
ABRE	ABA-responsive element
AP	Alkaline Phosphatase
AP2	APETALA2
bp	base pair(s)
BSA	Bovine Serum Albumin
CBB	Coomassie Brilliant Blue
cDNA-AFLP	complementary DNA-Amplified Fragment Length Polymorphism
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
cv.	cultivar
DAP	Days After Pollination
DBF1	DRE-binding factor/protein 1
DIG	Digoxigenin
DNase	Deoxyribonuclease
dNTP	deoxy-nucleotide triphosphate
DRE	Drought-responsive element
DTT	Ditiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EF-	alpha-helix loop (E); second alpha-helix loop (F)
EREBP	Ethylene responsive element binding protein
ERF	Ethylene responsive factor
h	hour
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
IRRI	International Rice Research Institute
kD	kilodalton
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LEA	Late Embryogenesis Abundant
M	Molar
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-Flight mass spectrometry
min	minute
M-MLV RT	Moloney Murine Leukemia Virus Reverse Transcriptase
M*r*	Molecular mass
MS	Mass spectrometry
MV	Modern variety
MW	Molecular weight
ORF	Open Reading Frame
PAGE	Polyacrylamide gel electrophoresis
PBS buffer	Phosphate-buffered saline
PEG	Polyethylene glycol
p*I*	Isoelectric point
PMSF	Phenylmethyl sulfonyl fluoride

Rab	Responsive to abscisic acid
RNase	Ribonuclease
RT-PCR	Reverse transcription-polymerase chain reaction
s	second
SDS	Sodium Dodecyl Sulfate
SOD	Superoxide dismutase
ssp.	subspecies
TBE buffer	Tris-borate-EDTA
TDF	Transcript-derived Fragment
TF	Transcription factor
TIGR	The Institute for Genomic Research
TMEV	TIGR Multiple Experiment Viewer
Tukey 'HSD' Test	Tukey 'honestly significant difference' test
vs.	*versus*
W	Watt
1-DE	One-dimensional gel electrophoresis
2-DE	Two-dimensional gel electrophoresis
% Vol.	normalised spot volume

CONTENTS

Chapter I

GENERAL INTRODUCTION

AND AIMS OF THE THESIS

I. Abiotic stress tolerance in plants: cellular and molecular mechanisms

1. A brief perspective on plant responses to abiotic stress

Environmental stresses e.g., drought, high salinity and extreme temperatures influence negatively plant growth thus contributing for major crop losses worldwide (Grover *et al*., 2001; Wang *et al*., 2003; Vinocur and Altman, 2005; Sreenivasulu *et al*., 2007; Nakashima *et al*., 2009). Drought followed by salinity is the most significant environmental constraint to rice yield potential in all agro-climatic zones (Gorantla *et al*., 2007; Bernier *et al*., 2008). Moreover, in the particular case of rice, abiotic stress affects more severely crop productivity than biotic stress (Hossain, 1996).

Despite the different meanings of 'drought' across meteorologists (lack of rainfall), farmers, agronomists (yield compromised by limited water), physiologists or molecular biologists (water withdraw, desiccation, exposure to strong osmotica), all them refer to insufficient water availability (Passioura, 2007). On the other hand, plant water deficit relates to water loss by evapotranspiration that exceeds water uptake, thus lowering the water potential of the cells (Close, 1996; Bray, 1997; Verslues *et al*., 2006). Most important, disruption of plant water status occurs not only during drought, but also at low temperatures and in the presence of high concentrations of salts in the soil (Bohnert and Jensen, 1996; Close, 1996; Bray, 1997; Verslues *et al*., 2006). Hence, the ice formed at freezing temperatures on the plant surface, apoplast, xylem or in the soil, causes a large chemical potential gradient, making water to move out the protoplasm towards the ice. On the other hand, high salt concentrations in the soil results in cell dehydration by osmosis due to a lower chemical potential in the solutions outside than inside the cells (Close, 1996; Verslues *et al*., 2006).

Plant growth under water deficit is limited due to photosynthetic decline (Chaves and Oliveira, 2004). Indeed, stomatal closure reduces water loss at the same time that reduces leaf carbon fixation (Chaves and Oliveira, 2004). On the other hand, abiotic stresses are closely related to the generation of reactive oxygen species (ROS), responsible for the denaturation of functional and structural proteins (Zhu, 2001; Sung *et al*., 2003; Chinnusamy *et al*., 2004; Vinocur and Altman, 2005). Therefore plants must respond and adapt to stress conditions to continue their growth and complete the life cycle (Bray, 1997; Wang *et al*., 2003; Vinocur and Altman, 2005; Tran *et al*., 2007). Stress responses are controlled by complex molecular regulatory pathways that lead to changes in gene expression resulting in cellular, physiological and biochemical modifications (Bray, 1997; Wang *et al*., 2003; Vinocur and Altman, 2005; Yamaguchi-Shinozaki and Shinozaki, 2005; Langridge *et al*., 2006; Shinozaki and Yamaguchi-Shinozaki, 2007; Sreennivasulu *et al*., 2007).

Plant adapting responses to cope with water deficit may be divided into stress avoidance and stress tolerance mechanisms (Bray, 1997; Vinocur and Altman, 2005) (Fig. 1). Stress avoidance [(1)] help plants to maintain high leaf water potential during drought by extracting more water from the soil through deeper roots, or by reducing water loss through stomatal closure (Tripathy *et al*., 2000; Verslues *et al*., 2006; Bernier *et al*., 2008). Stress avoidance at the whole-plant organism level involves morphological and physiological changes to cope with water deficit (e.g., increased root/shoot ratio), whereas at cellular level it may involve the maintenance of cell turgor by the accumulation of compatible solutes (Bray, 1997; Verslues *et al*., 2006) (Verslues *et al*., 2006). However, tolerance responses allow plants cells to maintain turgor and volume and to continue metabolism, even

[(1)] According to Levitt (1980), water stress avoidance and tolerance are stress *resistance* mechanisms, in opposition to stress *escape* mechanisms. Stress escape is achieved e.g., by earlier flowering to complete life cycle before water stress occurs.

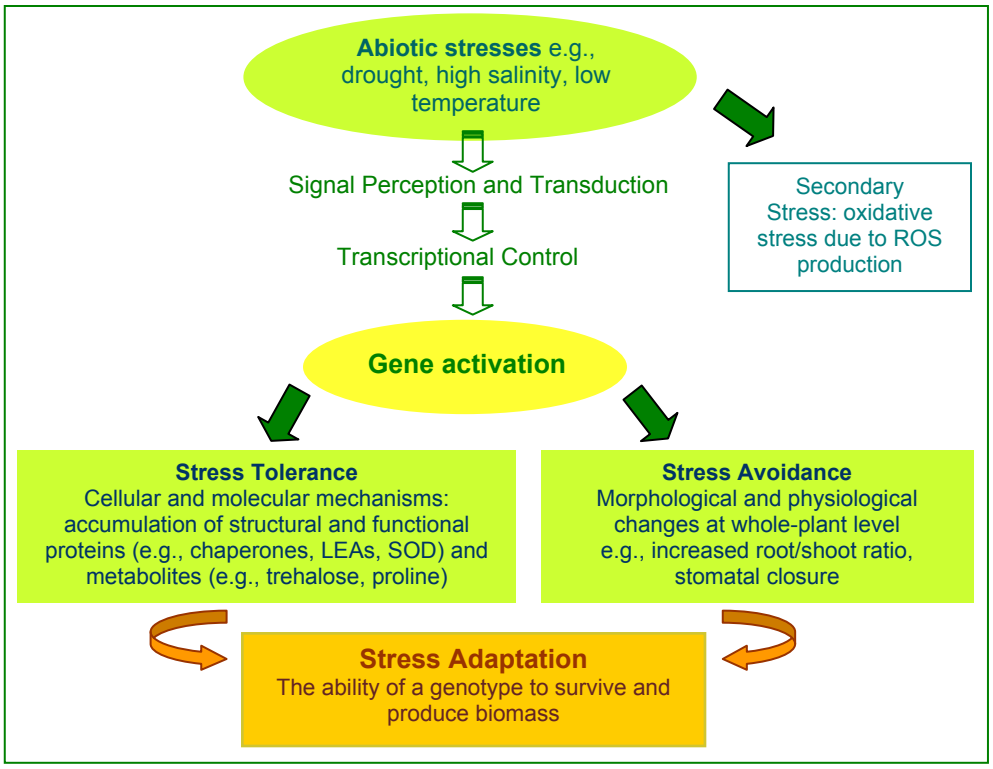

Figure 1. Plant responses leading to abiotic stress adaptation (adapted from Vinocur and Altman, 2005). Abiotic stresses are often interconnected and associated to the generation of ROS. These stresses contribute for cellular damage and disruption of homeostasis. Cellular signalling transduction pathways convert physical stresses into biochemical responses. The control of stress gene expression is crucial to activate specific genes responsible for the protection of the cellular machinery and repair of the damage caused to membranes and proteins. The correct coordination of whole gene activation machinery leads to plant stress adaptation, either through stress tolerance and/or stress avoidance mechanisms. Both are stress-responsive mechanisms.

at a low water potential (Tripathy *et al*., 2000; Verslues *et al*., 2006). Plant stress tolerance is achieved by molecular and biochemical modifications that protect the cell membrane and the integrity of proteins, besides involving the repair of any cellular damage (Bray, 1997; Vinocur and Altman, 2005). Hence, a large set of gene products with a protective and/or damage repair function accumulates in the cell e.g., heat shock proteins, proteinase inhibitors, enzymes involved in oxygen-radical scavenging and detoxification (Bonhert and Jensen, 1996; Bray, 1997; Vinocur and Altman, 2005) (Fig. 1).

Desiccation tolerance [(2)] is widespread in the plant kingdom, including ferns and mosses, besides pollen and seeds of most angiosperms (Bradford and Chandler, 1992; Hoekstra *et al*., 2001). Drought and desiccation tolerance are correlated with the presence of considerable amounts of non-reducing di- and oligosaccharides, some amino acids and specific proteins, such as late embryogenesis proteins (LEAs) and heat shock proteins (HSPs) (Hoekstra *et al*., 2001). Tolerant species seem to present higher levels of compatible solutes when compared to sensitive ones (Bohnert and Jensen, 1996). Drought tolerance at cellular level is based on structural stabilization by preferential hydration, whereas desiccation tolerance involves the replacement of water by molecules that also form hydrogen bonds (Fig. 2). Furthermore, desiccation-tolerant cells are capable of rehydrating successfully (Hoekstra *et al*., 2001).

The decrease in cellular volume due to water stress results in a high number of interactions at molecular level, responsible for protein denaturation and membrane fusion (Hoekstra *et al*., 2001). Proline, trehalose and oligosaccharides, among other compatible solutes can prevent such adverse molecular interactions (Fig. 2). Because of their preferential exclusion from the surface of proteins, compatible solutes help proteins to keep hydrated and to maintain their folded conformation (Fig. 2). Since preferential exclusion is thermodynamically unfavourable, the surface area of proteins is minimal and the folded conformation is the most frequent (Hoekstra *et al*., 2001). However, in the presence of preferentially bound co-solvents, the protein denaturated state is the most common (Fig. 2). In extreme dehydration conditions i.e. desiccation, only sugars can structurally and functionally preserve proteins and membranes, whereas most of the other compatible solutes are unable to do it (Hoekstra *et al*., 2001).

[(2)] 'Drought tolerance' refers to tolerance to moderate dehydration/water loss, while 'desiccation tolerance' refers to further dehydration, or excessive water loss (Hoekstra *et al*., 2001).

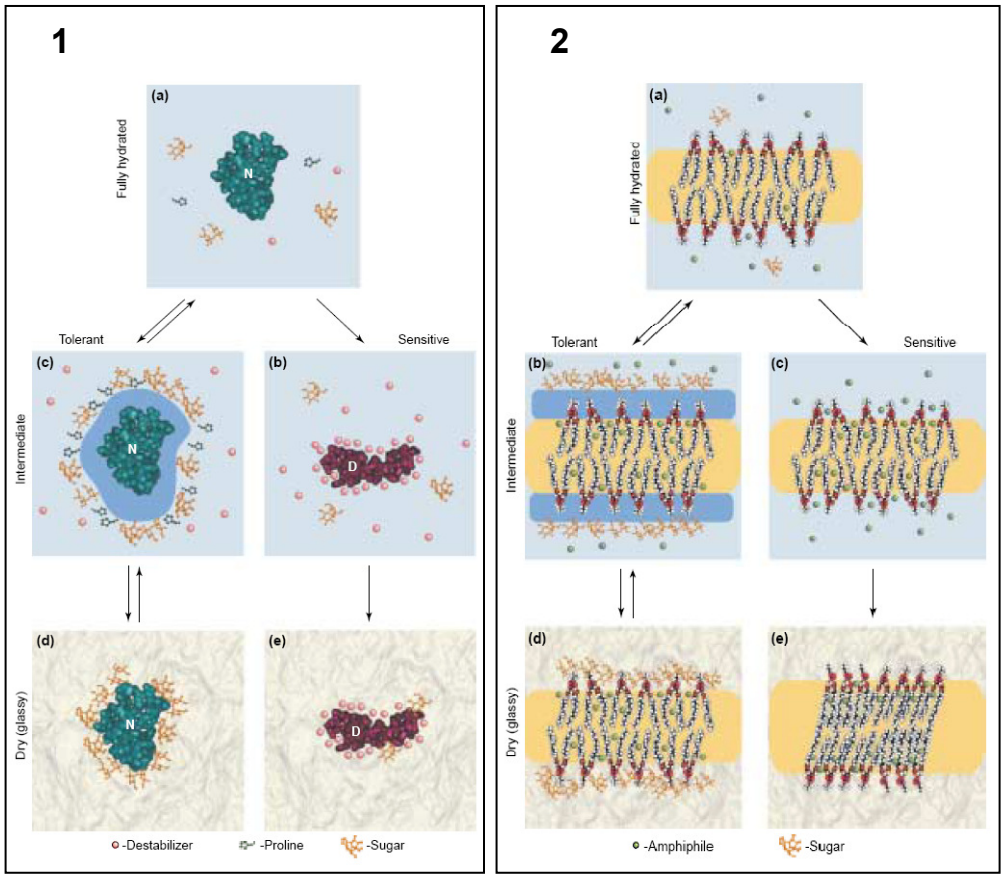

Figure 2. Mechanisms of protein and membrane structure stabilization during water stress in tolerant and sensitive cells (Hoekstra *et al*., 2001). In fully hydrated cells **(1a; 2a)**, the folded/native (N) form of a protein **(1)** is termodynamically favourable and membrane lipids **(2)** are in an undisturbed liquid crystalline state. During water loss the probability of cytoplasmic solutes to interact with the protein surface increases due to molecular crowding. In tolerant cells **(1c)** preferential exclusion of compatible solutes from the protein surface dominates over preferential binding, causing a preferential hydration state of the protein (indicated by the blue ring around the protein), which maintains proteins in their native conformation.

In sensitive cells **(1b)**, the lack of compatible solutes (e.g., proline and sugars) causes preferential binding to dominate over preferential exclusion, leading to protein unfolding and denaturation. On the other hand, the concentration of cytoplasmatic amphiphilic compounds increases upon water loss, causing membrane disturbance. During drought, the presence of preferentially excluded solutes in tolerant cells **(2b)**, keeps membrane surface preferentially hydrated (indicated by the blue bands) whereas in sensitive cells **(2c)**, the absence of these solutes may compromise the maintenance of the spacing between the phospholipid molecules.

(Legend continues on following page)

Legend Figure 2 *(Continued from previous page)*

In more severe conditions (desiccation), sugar molecules in tolerant cells replace water via hydrogen bonding, thus stabilizing the native protein structure in the dried/glassy cytoplasm **(1d)**, and replace water in the hydration shell of the membranes **(2d)**, maintaining the spacing between phospholipid molecules and allowing the bilayer to remain in the liquid crystalline phase. In sensitive cells, unfolded proteins **(1e)** become 'fixed' in the dried cytoplasm, whereas the removal of water from the hydration shell in the absence of sugars, results in the packing of phospholipid molecules **(2e)**, leading to the transition into the gel phase.

The reversibility of the processes occurring during dehydration and rehydration is indicated by arrows in both directions.

2. Cereal seeds, ABA and LEA proteins: three major components to address drought tolerance

2.1. Cereal seeds as a model to study drought tolerance

Embryo development initiates from a single-celled zygote by extensive mitotic division and differentiation followed by cell expansion (Bartels *et al*., 1996). Meanwhile, storage proteins, lipids and polysaccharides are deposited in the endosperm to supply the growing embryo with sugars and amino acids once environmental conditions favour germination (Finnie *et al*., 2002). At the final stage of seed maturation, characterised by the acquisition of desiccation tolerance (and entry into dormancy), the embryo reaches its lowest water content (Bartels *et al*., 1996; Vicente-Carbajosa and Carbonero, 2005). The embryos of cereal seeds sustain reductions of about 80% of their initial fresh weight, whereas such severe desiccation kills the starchy endosperm cells (Jensen *et al*., 1996). Rice mature embryos readily tolerate water contents below 5% (Bradford and Chandler, 1992). Comparative studies between the drought response in vegetative tissues and the acquisition of desiccation tolerance in seeds, demonstrated that *ABF* and *DREB2A* – two transcription factors known to be involved in the drought stress responses in vegetative tissues (Shinozaki and Yamaguchi-Shinozaki, 2000) – also participate in desiccation tolerance in seeds (Sreenivasulu *et al*., 2007). Therefore, understanding the mechanisms involved in desiccation

tolerance in seeds can contribute to elucidate about drought tolerance in plant vegetative tissues (Bartels *et al.*, 1996; Jensen *et al*., 1996; Campalans *et al*., 1999; Cooper *et al*., 2003; Sreenivasulu *et al*., 2007).

2.2. ABA and its role in seed maturation and abiotic stress responses

The phytormone ABA plays a dominant role during the embryogenesis by supressing precocious embryo germination, and inducing the expression of genes involved in the accumulation of storage compounds and acquisition of desiccation tolerance (White *et al*., 2000; Vicente-Carbajosa and Carbonero, 2005). Seed development and maturation is determined by an ABA/gibberellins (GAs) balance, also involving other hormones such as cytokinins (White *et al*., 2000; Vicente-Carbajosa and Carbonero, 2005). Besides regulating plant developmental processes (e.g., seed maturation, dormancy and germination) ABA acts as major signalling molecule in the abiotic stress response (Chinnusamy *et al*., 2008; Wasilewska *et al*. 2008; Melcher *et al.*, 2009; Myazono *et al*., 2009). High levels of the phytohormone under non-stressful conditions inhibit plant growth, whereas under stress conditions promote the activation of many genes that together increase stress tolerance (Bray, 2002; Finkelstein *et al*., 2002; Xiong and Zhu, 2003).

ABA receptors and other implications

In the past decades and despite exhaustive work, the information on ABA receptors was based on circumstancial evidence and it was only recently that these molecules have been reported. The identification of three putative ABA receptors i.e. a RNA-binding flowering-time control protein (FCA) (Razem *et al*., 2006), a Mg-chelatase H subunit (Shen *et al*., 2006) and a putative G protein coupled receptor (GCR2)[3] (Liu *et al*., 2007), provided a major step forward in understanding ABA signalling processes. Different

[3] It is mentioned as 'putative' because there is some controversy about if it is really a G protein coupled receptor, instead of a bacterial lanthionine synthetase homologue. See Johnston *et al*. (2007) Science 318: 914.

subcellular localizations of the receptors (nucleus, chloroplast and plasma membrane) suggested ABA to act simultaneously and independently at multiple sites in the cell (Hirayama and Shinozaki, 2007). These receptors were somehow controversial since they could not be related neither to positive nor negative regulators of ABA signalling (Ma *et al*., 2009; Park *et al*., 2009). Nevertheless, in 2009 a new family of START proteins, the PYRABACTIN RESISTANCE PYR/PYL/RCAR proteins, was identified in *Arabidopsis* as ABA receptors, acting via SnRK2 kinases (activated by ABA) and PP2C phosphatases (negative regulators of ABA) (Ma *et al*., 2009; Park *et al*., 2009; Nishimura *et al*., 2010). Despite that most ABA responses are transcriptionally regulated, recent discoveries also point to epigenetic regulation through histone modifications and cytosine DNA methylation (reviewed by Chinnusamy *et al*., 2008). Other surprising new findings report the presence and role of ABA in human granulocytes revealing a close parallel between the ABA-signalling mechanisms in plants and in mammals, which suggests an ancient origin for ABA and its action mechanisms (Bruzzone *et al*., 2007; Sturla *et al*., 2009).

2.3. LEA proteins: intriguing abundant proteins in the seed embryos

2.3.1. *An approach to LEAs' classification*

Late embryogenesis abundant (LEA) proteins accumulate extensively in in the latter stages of seed maturation disappearing following germination (Galau *et al*., 1986). On the other hand, the expression of LEAs is often ABA-dependent and can be induced by drought (Gomez *et al*., 1988), salinity (Mundy and Chua, 1988) or cold stress in vegetative tissues (Hajela *et al*., 1990; Lang and Palva, 1992). LEA proteins can be classified into several families and/or groups based on their primary sequences and expression pattern similarities (Dure, 1989; Bray, 1994; Close, 1997; Dure, 1997; Cuming, 1999; Bray, 2000). They were initially found in cotton (Dure *et al*., 1981; Galau *et al*., 1986; Dure, 1997) and wheat seeds (Grzelczak *et al*.,

1982) but they have been characterised in a wide range of plant species including gymnosperms (Dure, 1997). LEA proteins were first classified by Dure into families based on their similarity to LEA cotton (*Gossypium hirsutum*) proteins, which in turn followed the original names of the clones derived from a cDNA library labelled 'D' e.g., D-7, D-11, D-19, D-29, D-34, D-95 and D-113 (Baker *et al*., 1988; Dure, 1997). Other authors distribute LEAs among six major groups instead of the D-families (Bray, 1994; Cuming, 1999; Bray *et al*., 2000). The classification of LEA proteins is contradictory existing consensus only for three groups of LEAs: Group 1 (D-19), Group 2 (D-11, also known as dehydrins) and group 3 (D-7) (Wise, 2003).

A computational method called 'POPP' (Protein or Oligonucleotide Probability Profile) allows the comparison of proteins based on the similarities of their peptide compositions (consensus POPP) rather than on sequence similarities (Wise, 2002). According to POPP classification, LEAs can be grouped into superfamilies that align mainly with LEA protein groups 1, 2 and 3 (Wise and Tunnacliffe, 2004) (Table I). The super-families (SF) designation introduced further refinement in the categorization of LEA proteins e.g., the separation of Group 2 into two subgroups: group 2a comprising SF1 and SF10, and Group 2b including SF3. Such apparent complexity suggests important clues on the function of LEAs. For instance, group 2a includes proteins that are expressed in the late embryogenesis (SF10), but does not include proteins associated with cold tolerance (SF1); group 2b includes proteins broadly related to cold tolerance together with others clearly not accumulated during late embryogenesis (SF3). More dramatic is the disappearance of groups 4 and 5 under POPP classification, being the members of these groups redistributed into LEA groups 2 and 3 (Wise and Tunnaclife, 2004).

Table I. ***Correspondence of LEA typical groups to POPP superfamilies*** (Wise and Tunnacliffe, 2004)

LEA group	Sequence motif	LEA superfamily	Consensus POPP[a]
1a	GGQTRREQLGEEGYSQMGRK	4	+E, +G, +EG, +GE, +GG, +KG, +QE, +RK, +GGE, +KGG
1b		6	+E, +G, +DE, +EG, +ES, +GG, +GQ, +RE, +RK, +ARE, +DES, +REG
2a	DEYGNP (Y domain)	1	+G, −L, +EK, +GG, +GT, +EKL, +IKE, +KEK, +KIK, +KKG, +KLP, +LPG
2a	EEKK (K domain)	10	−F, +G, −I, +AG, +EK, +GG, +GQ, +KE, +SS, +EKL, +GAG, +IKE, +KEK, +KLP, +LPG, +SSS
2b	S_n (S segment)	3	−F, −I, −L, −R, −W, +DK, +EK, +KK, +KL, +LP, +TH, +EKK, +KEK, +KLP, +LPG
3a	TAQAAKEKAGE	2	+A, −C, +E, −F, −I, +K, −L, −P, +AE, +AK, +EK, +ET, +GE, +GK, +KE, +AAE, +AKD, +EKA
3b		5	+A, −I, +K, −L, −P, +Q, +T, −V, +AA, +AQ, +EK, +KE, +KT, +QA, +QQ, +QS, +QT, +TQ, +AAK, +AQA, +EKT, +QAA, +TQQ
6	?	7	+A, −F, −L, +AA, +AE, +MQ, +QS, +VA, +AAA, +GVA, +QSA, +SAA

'+' and '-' indicate significant over- or under-representation of a peptide

2.3.2. *The Dehydrin/RAB (responsive to abscisic acid) family*

Dehydrins belong to LEA group 2 (D-11 family), accumulating during the late embryogenesis and/or in response to drought, low temperature, high salinity and ABA treatments (Close, 1997). The rice *Rab21* gene was the first rab cDNA reported (Mundy and Chua, 1988), whereas the maize *Rab17* was the first rab genomic clone (Vilardell *et al*., 1990). A unique feature of dehydrins is the presence of one or several copies of a highly conserved lysine-rich 15 amino acid consensus (EKKGIMDKIKEKLPG), usually referred as the K segment (Close, 1996) (Fig. 3). Some dehydrins also contain a tract of 7 to 9 serine residues (the S-segment), followed sometimes by a stretch of residues rich in lysine. The Y segment, an N-terminal conserved domain of seven amino acids (T/VDEYGNP), constitutes another distinct domain typical of dehydrins (Fig. 3). According to that, the nomenclature of dehydrins can be written in the '$Y_nS_nK_n$ shorthand' (Close, 1996), depending 'n' on the number of repeats of each segment. The K-segment can be repeated up to 11 times in cold induced dehydrins! Dehydrins contain in most cases regions or domains (the Φ segment) that are rich in glycine and polar amino acids (especially threonine) tandemly repeated between the K-segments (Fig. 3). However, there are contradictory cases where the Φ

segments are either rich in other types of amino acids, such as proline and alanine, or simply do not appear in tandem repeats (Close, 1997).

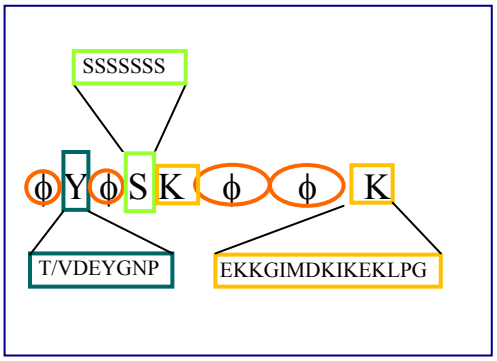

Figure 3. Conserved domains in dehydrin proteins. Typical YSK_2 dehydrin domains (blue, green and yellow, respectively).

Dehydrins include most of the Rab (Responsive to abscisic acid) and COR (cold regulated) proteins (Nylander *et al*., 2001; Lee *et al*., 2005). The rice *rab21/16A* dehydrin gene was found to make part of a single locus together with *rab16B*, *rab16C* and *rab16D* (Mundy and Chua, 1988; Yamaguchi-Shinozaki *et al*., 1989). These four genes were tandemly arrayed in a locus of approximately 30 kbp, and had slightly different expression patterns in response to osmotic stress (Yamaguchi-Shinozaki *et al*., 1989). Two additional putative members of this multigene family have been proposed i.e. *Rab16E* and *Rab16F* (Lee *et al*., 2005). Other rice dehydrins responsive to abiotic stress have been reported, namely the osmotic stress-responsive *Rab25* (Kusano *et al*., 1992) and *WSI724* genes (Takahashi *et al*., 1994), the low temperature induced *LIP5* and *LIP9* genes (Aguan *et al*., 1991), and the drought- and cold- inducible *OsDhn1* dehydrin (Lee *et al*., 2005). The genetic relatedness between these best-characterised rice dehydrins is presented in Figure 4.

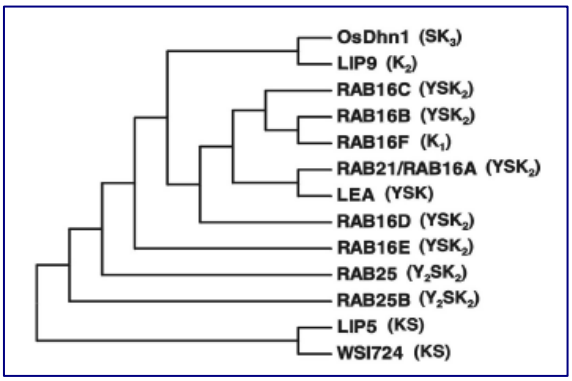

Figure 4. Phylogenetic analysis of rice dehydrins (Lee *et al*., 2005). YSK motifs are indicated in parenthesis

2.3.3. *Putative functions of LEAs*

LEA and LEA-like proteins are found in organisms other than plants, such as nematodes (*Caenorhabditis elegans, Steinernema feltiae* and *Aphelenchus avenae*) and bacteria (*Deinococcus radiodurans, Bacillus subtilis* and *Haemophylus influenzae*) (Wise and Tunnacliffe, 2004). The presence of LEAs in desiccation-tolerant organisms like nematodes supports an important role in desiccation tolerance (Goyal *et al*., 2005a). Although they are expected to play a protective role during dehydration their precise function remains unclear. They are proposed to protect cellular structures by acting as a hydration buffer, sequestering ions, protecting other proteins (or membranes), or renaturing unfolded proteins (Wise and Tunnacliffe, 2004; Grelet *et al*., 2005; Lee *et al*., 2005; Chakrabortee *et al*., 2007). Other less common and putative functions have been proposed by Wise and Tunnacliffe (2004) based on the 'POPP' (Protein or Oligonucleotide Probability Profile) computational method (Table II). The relevance of this method relies on the ability to querry a database of proteins with known functions, entering proteins of unknown functions (e.g., LEAs) but sharing similar protein profiles. Significant matches obtained for LEA proteins were used to deduce putative functions (Table II) (Wise, 2003; Wise and

Tunnacliffe, 2004). Other functional and structural characteristics of LEAs have been recently revised by Battaglia *et al*. (2008).

Table II. ***Putative functions for LEA proteins*** (Wise and Tunnacliffe, 2004). Protein hits for each POPP group/superfamily were annotated into a list of representative keywords. These keywords represent mechanisms or structural elements that LEAs share with proteins of similar peptide composition or profiles.

LEA Group	LEA Superfamily	Keywords and phrases	Putative function(s)
1a	4	Histone H4, chromosomal protein, nuclear protein, methanofuran, DNA binding	DNA binding nuclear protein
1b	6	dsRNA binding, DNA gyrase, (DNA) breakage, CLP, ATP binding	Nucleic acid unwinding or nucleic acid repair Molecular chaperone
2a	1	(DNA) break, ATP binding, DNA topoisomerase, protein biosynthesis, topoisomerase, repair	DNA unwinding or repair
2a	10	Nuclear protein, DNA binding, transcription regulation, intermediate filament, keratin, chaperone, homeobox, coiled coil, HMG box domain, cytoskeletal	DNA-binding nuclear protein; regulation of transcription? Cytoskeleton
2b	3	Coiled coil, nuclear protein, histone H1, chaperone, tropomyosin, filament, (DNA) break, DNA topoisomerase	DNA unwinding or repair, Cytoskeleton, Ca^{2+} binding, Molecular chaperone
3a	2	Chaperone, coiled coil, tropomyosin, stress, filament, phosphorylation, elongating factor, neurofilament, actin binding, cytoskeleton, rotamase	Molecular chaperone, Cytoskeleton, Ca^{2+} binding
3b	5	Coiled coil, histone H1, filament, nuclear protein, neurofilament, antigenic, flagella, HAMP domain, synuclein, peptidoglycan anchor, DNA binding, hsp70	Chromatin-associated protein, Filament, Kinase or phosphatase?
6	7	GroEl protein, nuclear protein, histone H1, chaperonin, DNA binding, HAMP domain, synuclein, transcription regulation	Molecular chaperone, Chromation-associated nuclear protein; transcription factor?

Abbreviations: CLP: an ATP-dependent plant chaperone; HMG: high mobility group (a DNA binding domain); HAMP: group of histidine kinases, adenylyl cyclases, methyl binding proteins and phosphatases.

Particular cases

The dehydrin Rab17 (also known as dehydrin 'DHN1') is strongly phosphorylated in maize mature embryos, localizing either in the nucleus or cytoplasm (Goday *et al.*, 1994; Jensen *et al*., 1998; Riera *et al.*, 2004). CK2 phosphorylates Rab17 both *in vitro* and *in vivo*, with phosphorylation occurring at the serine cluster region of the protein (Plana *et al*., 1991; Goday *et al*., 1994; Riera *et al*., 2004). It was found that transgenic plants overexpressing Rab17 presented a stronger delay in germination under osmotic stress conditions, as compared with lines overexpressing a mutated version of Rab17 in the CK2 consensus site (mRab17). Furthermore, the lines overexpressing Rab17 presented higher phosphorylation status of the protein than mRab17 lines, suggesting a major implication of CK2 in the phosphorylation of Rab17 (Riera *et al*., 2004). In the model presented by Riera *et al*. (2004) Rab17 associates with CK2β regulatory subunits in the cytoplasm, moving to the nucleus in this complex form. Once in the nucleus, the complex CK2β/Rab17 disrupts and CK2β subunits associate with CK2α (catalytic) subunits to form the holoenzyme capable of phosphorylating Rab17 (Fig. 5). The authors thus proposed that Rab17 would have a role in embryo growth arresting in water stress conditions mediated by the phosphorylation status of the protein (Riera *et al*., 2004). After being phosphorylated inside the nucleus, Rab17 could move again into the cytoplasm to play its still unknown function.

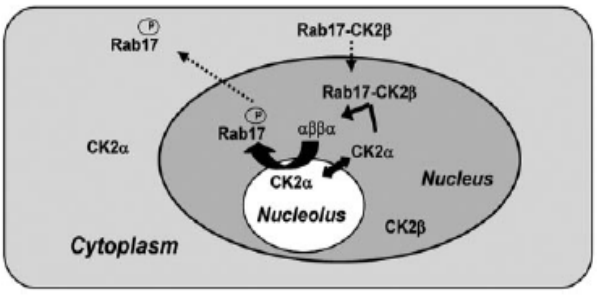

Figure 5. Model for nuclear/cytoplasmic trafficking of maize Rab17 (Riera *et al*., 2004).

Grelet *et al*. (2005) demonstrated that LEAs may protect other proteins during desiccation. The authors verified that the mitochondrial LEA (group 3) from *Pisum sativum* (PsLEAm) could protect *in vitro* two other proteins during drying. The protein conferred significant protection of enzyme activity, probably by helping to preserve the native structure and correct folding of the enzymes (Grelet *et al*., 2005).

Dehydrins are intrinsically disordered proteins (Mouillon *et al*., 2006), which means they don't have stable tertiary structure under physiological conditions, although they exert specific functions in biological processes (Mouillon *et al*., 2006). This apparent contradiction suggests that dehydrins may have a configuration highly flexible to resist unspecific collapse and aggregation. Mouillon and collaborators (2006) proposed that the conserved segments of dehydrins would play their biological function acting as beads on a string, recognising specific targets instead of promoting tertiary structure.

A study involving the AavLEA1 protein from the nematode *Aphelenchus avenae* supports the hypothesis that LEA proteins act as anti-aggregants, by behaving as ''molecular shields'' during water stress (Goyal *et al*., 2005 a,b). In desiccation conditions, the AavLEA1 protein is specifically cleaved into discrete, smaller polypeptides, suggesting that the cleavage allows rapid and maximal availability of active molecules to the dehydrated animal (Goyal *et al*., 2005a). More recently, Chakrabortee *et al*. (2007) showed that the AavLEA1 protein when co-expressed in a human cell line is able to prevent aggregation of a wide range of proteins both *in vitro* and *in vivo.* Among them two proteins associated with neurodegenerative diseases.

3. Rice (*Oryza sativa* L.) ecosystems and varieties with contrasting responses to abiotic stress

3.1. Rice Ecosystems

Rice can be grown in four major environments: irrigated, rainfed lowland, upland and deepwater rice (Mackill *et al*., 1996). Irrigated rice is the most common ecosystem and comprises 56.9% of the global rice area. It is followed by the rainfed lowland with 30.9%, whereas the upland and deepwater ecosystems only account for 9.4% and 2.8%, respectively (IRRI World Rice Statistics). In South America and Asia, irrigated rice occupies nearly 50% to 60% of ricelands, while in Europe, Australia and North America (in USA) registers 100%, contrasting to 22.8% in Africa. In this last continent, the upland and rainfed lowland are the dominant rice cultures (32.6% and 35.4%, respectively), whereas in South America, the upland ecosystem justifies 46.7% (IRRI World Rice Statistics).

In the rainfed lowland ecosystem rice grows in levelled, bunded fields shallowly flooded with rainwater. The soil surface is flooded at least part of the crop cycle (contrary to an upland) and the maximum sustained flooding depth is less than 50 cm (unlike a deepwater) (Mackill *et al*., 1996). The rainfed lowland environment can be divided into other subecosystems according to their hydrological conditions e.g., rainfed (shallow) favourable, rainfed drought-prone, and rainfed drought/submergence-prone ecosystem (Fig. 6) (Fischer *et al*., 2003). In the rainfed lowland favourable subecosystem, though water in the field cannot be completely controlled, rainfall is usually adequate for crop growth (Mackill *et al*., 1996). However, in Asia the rainfed lowland drought-prone is the dominant environment where short rainy seasons may alternate with longer but more erratic rainfall seasons (Mackill *et al*., 1996).

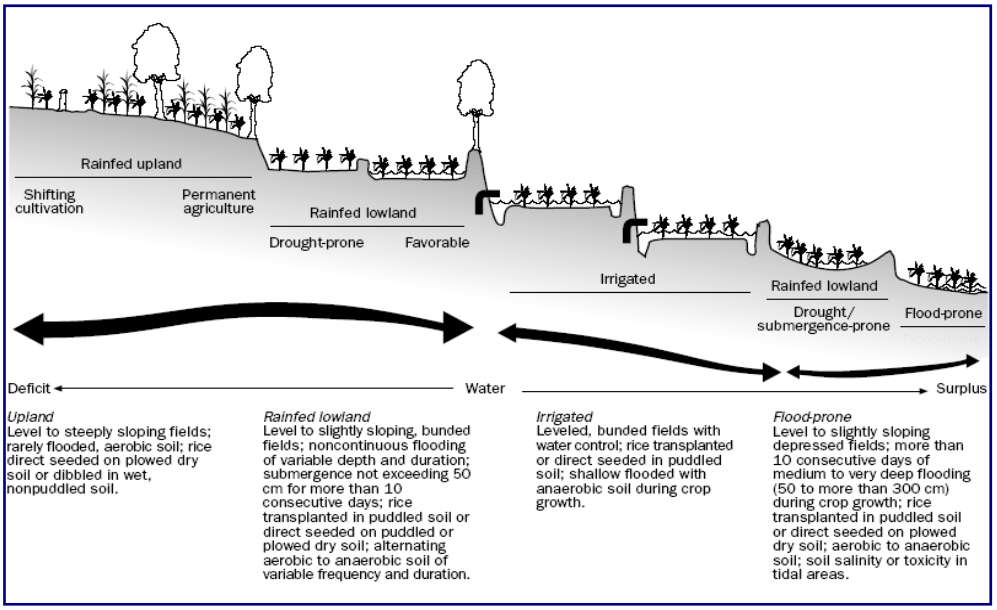

Figure 6. Rice ecosystems (Fischer *et al*., 2003).

In the rainfed upland ecosystem water does not accumulate in the field due to soil drainage and/or uneven land distribution, contrasting with irrigated fields where rice grows under complete water control in bunded (paddy) fields (Bernier *et al*., 2008). Upland rice is generally the lowest-yielding ecosystem, while irrigated rice is the most productive (Khush, 1997). In the latter ecosystem rice production takes place on well-drained, fertile soils that are not submitted to drought (or flooding), contrary to uplands where little or no fertilizer is applied, being at the same time highly prone to drought (Bernier *et al*., 2008). More recently, improved upland rice varieties with higher yield potential were obtained in the Philippines (at IRRI), as well as in several other Asian countries and in Brazil (Bernier *et al*., 2008). It is the so-called 'aerobic rice' that combines the characteristics of aerobic adaptations of traditional upland varieties with the high yield potential of irrigated lowland genotypes, therefore decreasing water requirements in rice production (Bouman, 2001).

3.2. Considerations about rice varieties bred at IRRI

In 1966, the International Rice Research Institute (IRRI) released 'IR8' [4] the first modern high-yielding semidwarf variety, triggering the Green Revolution in the Philipinnes and rest of Asian tropical countries (Estudillo and Otsuka, 2002; Khush and Virk, 2005). Until 2005, about 60% of the world rice area was planted with IRRI-bred varieties or their progenies (Khush and Virk, 2005). More than half of the varieties released by IRRI in the Philippines until 2000 were for irrigated areas, whereas the rest were distributed to other environments e.g., uplands, saline or cool elevated areas (Sandiwà-IRRI, 2002).

The first-generation of modern varieties (MVs) included lines with higher yield than traditional varieties, being released from the middle 60s to the middle 70s. They included 'IR' series from 'IR5' to 'IR34' bred at IRRI, and 'C4' series bred at the University of the Philippines. The second-generation MVs were designed to ensure yield stability by incorporating resistance to multiple pests and diseases (5). They were released between the mid-1970s and mid-1980s, incorporating 'IR' series from 'IR36' to 'IR62' (Estudillo and Otsuka, 2002). In 1975, the national Philippine Seed Board decided to name varieties irrespective of the institution involved in their breeding. However, the prefix 'IR' was maintained until 1988 (Khush and Virk, 2005). Between the mid-80s to the late 90s, the third-generation MVs incorporated better grain quality and stronger host-plant resistance, including 'IR' series from 'IR64' to 'IR72', and 'PSBRc' series [6] from 'PSBRc1' to 'PSBRc74'. (Estudillo and Otsuka, 2002). The fourth-generation includes those varieties released

[4] 'IR' means *International Rice*. Usually there is no relationship between the IR designation of the variety and the IR cross number from which it has been selected (e.g., 'IR28' from the cross number IR2061).

(5) The most common diseases are blast, bacterial blight, grassy stunt and tungro, whereas the most destructive insects are brown plant hopper, green leaf hopper, and stem borers.

[6] In 1990, the **P**hilippine **S**eed **B**oard started to name the rice varieties with their initials i.e. **PSB Rc** (**Ric**e).

in the Philippines from the late 90s to 2007, and a new prefix - NSIC Rc (National Seed Industry Council Rice) replaced the PSBRc designation since 2002. Despite that IRRI substituted the designation of previously fixed breeding and elite rice lines in 2006 (e.g., 'PSBRc1' for 'IRRI 101') (Zeigler *et al*., 2006), the primary designation was adopted in the present work.

3.3. Standard Evaluation System for Rice

IRRI evaluates rice characters either related to agronomic traits or crop damage caused by biotic and abiotic stress, according to the Standard Evaluation System for rice (SES) (Inger Genetic Resources Center, 1996; IRRI, 2002). The system uses a scale that divides the total range of possible phenotypic expressions into a number of defined classes. The SES scale usually consists of five digits (1, 3, 5, 7 and 9) in which number 1 corresponds to high stress resistance, 3 to moderate resistance or tolerance, 5 between tolerance and susceptibility, 7 to susceptibility, and finally 9, to high stress susceptibility. Salt and drought stresses are commonly evaluated during vegetative growth, whereas cold stress during both vegetative and maturation stages. Agronomic traits such as seedling and vegetative vigour (e.g., tillering ability, plant height, leaf senescence, root length) are the most common characters used to estimate salt and cold stress injuries. Drought injury is the most difficult to assess, and leaf rolling and drying indexes can be used to estimate drought sensitivity/tolerance (Singh and Mackill, 1991).

3.4. Rice varieties with contrasting responses to drought, salinity and low temperature used in this study

3.4.1. *'PSBRc1' and 'IR64'*

Research on germplasm improvement in uplands seeks to overcome major abiotic constraints to yield, like drought stress, nutrient availability, acidity and soil erosion, besides biotic adverse effects. Resistance donors are usually traditional varieties from drought-prone environments (Singh and

Mackill, 1991). Mass screening for drought tolerance in upland conditions during dry seasons has been conducted at IRRI for at least 20 years (IRRI, 1991). For more details on field screening under drought conditions see De Datta *et al*. (1988), IRRI (1991) and Fischer *et al*. (2003).

In 1990, the IRRI-bred line '10147-113-5-1-1-5' (popularly named 'Makiling') was released for acid upland areas in the Philippines under the 'PSBRc1' designation (IRRI, 1991; Sandiwà-IRRI, 1999). The variety resulted from complex crosses between parents from different origins i.e. West African, Indonesian, Philippine and from USA (Table III). Besides drought tolerance, the 'PSBRc1' variety possesses good grain quality with intermediate levels of amylose (IRRI, 1991).

The IRRI-bred line 'IR18348-36-3-3' released in the Philippines in 1985 under the 'IR64' designation would become the widest-grown rice variety in the world (Khush and Kirk, 2005). The genealogy of this variety is complex including donors from China, Korea, Indonesia, India, Thailand, Vietnam, Philippines and USA (Table III). 'IR64' is a high-yielding and semidwarf variety that combines important agronomic traits e.g., resistance to the most important diseases and insects with desirable cooking/eating characteristics as intermediate amylose (and gelatinisation temperature) and soft gel consistency. The quality of the grain is considered superior as compared to other IR varieties (Khush and Virk, 2005). 'IR64' was released for irrigated and rainfed lowland areas (Khush and Virk, 2005) and it has a sensitive behaviour under drought-stress conditions (Liu *et al*., 2006; Lafitte *et al*., 2007).

3.4.2. '*IR52724-2B-6-2B-1-1' and 'IR29'*

Rice is one of the most widely grown crops in coastal areas worldwide, despite being considered moderately sensitive to salinity (Gregorio *et al*., 1997; Senadhira *et al.*, 2002). On the other hand, soil salinisation is common in the ricelands of the arid and semi-arid areas in the tropics, and it is a

major constraint to rice production in temperate areas (Lee *et al*., 2003). Furthermore, salinity is undoubtedly the most widespread and prevalent problem in irrigated agriculture (Zeng *et al*., 2003). Salinization rarely occurs isolated, as many saline soils are also prone to submergence or drought. Thus, breeding rice for saline environments should cover multiple stress tolerance traits (Gregorio *et al*., 2002). Efficient and practical screening methodologies to evaluate salinity tolerance at the seedling, vegetative and reproductive stages were reported by Gregorio *et al*. (1997).

The IRRI-bred rice line 'IR52724-2B-6-2B-1-1' was tested in the field for salt tolerance, presenting a good grain yield in both saline and non-saline conditions (Senadhira *et al*., 2002). The traditional variety Pokkali originary from the coastal areas of India has been used as the salt tolerant donor in the crosses of this line (see Table III). The 'IR52724-2B-6-2B-1-1' rice line was used as a standard salt tolerant genotype (among others provided by IRRI) in the assessment of tolerance of potential donor landraces in the coastal saline areas of Bangladesh (Lisa *et al*., 2004). Despite that it was not released in the Philippines, this breeding line was selected as a standard salt tolerant genotype in the present work.

Most IR varieties have high levels of amylose, but consumers in tropical and subtropical Asia prefer rice with lower contents (Khush and Virk, 2005). The 'IR29' variety (IRRI-bred line 'IR2061-464-4-14-1') was released in the Philippines in 1974 as a special glutinous rice [7] (Khush and Virk, 2005). It is a high-yielding variety suitable for irrigated and rainfed lowland areas (Khush and Virk, 2005). However, 'IR29' has been used extensively as a contrasting sensitive control in salt tolerance screenings (Gregorio and Senadhira, 1993; Gregorio *et al*., 1997; Senadhira *et al*., 2002; Lee *et al*., 2003; Zeng *et al*., 2003; Mohammadi-Nejad, 2008).

[7] Glutinous rice has negligible amounts of amylose (i.e., 1-2%), or no amylose, and high amounts of amylopectin. Amylose (20%) and amylopectin (80%) are the two components of starch. Amylopectin is responsible for the sticky quality of rice.

3.4.3. *'PSBRc96 'and 'IR58'*

Low temperature is a major constraint to rice production, particularly in temperate areas of China, Japan, Korea, United States (California) and European countries. Cold is also a serious constraint in tropical areas where rice can be grown at high altitudes in mountainous regions e.g., Philippines and Indonesia (IRRI, 1979; Mackill *et al*., 1996; Andaya and Mackill, 2003). It was estimated that about 7 million hectares could not be planted with modern high yielding varieties in South and Southeast Asia (including both temperate and tropical areas) due to low temperatures (IRRI, 1979; Andaya and Mackill, 2003). The success in breeding varieties for cold tolerance depends on the use of cold-tolerant donor parents from diverse origins, and appropriate breeding and screening methods (IRRI, 1979). For more details on screening for cold tolerance see Vergara *et al*. (1976), IRRI (1979), Li *et al*. (1981), Mackill *et al*. (1996), and Andaya and Mackill (2003).

The *indica* rice subspecies is more sensitive to low temperature than *japonica* subspecies (Andaya and Mackill, 2003). The IRRI line 'IR61608-3B-20-2-2-1-1' - a *japonica* variety - was released for cool and high-altitude areas in the Philippines with the designation of 'PSBRc96' (IRRI, 2000). This variety has good eating qualities and it is resistant to insect pests and diseases, besides improved tolerance to low temperature (IRRI, 2000). The 'Dobongbyeo' Korean variety was the cold tolerant donor and it was crossed with the West African upland *japonica* variety 'Moroberekan'. The F_1 hybrids were then crossed to an IRRI-bred line (Table III).

The IRRI-bred line 'IR9752-71-3-2' released in the Philippines in 1983 under the designation of 'IR58' is a semidwarf high-yielding cultivar resistant to blast and suitable for both irrigated and rainfed lowland areas (Khush and Virk, 2005), though evaluated as a cold sensitive genotype (Table III).

Table III. ***Main characteristics of the rice varieties provided by IRRI***

	PSBRc1	IR64[8]	IR52724-2B-6-2B-1-1	IR29	PSBRc96	IR58
Parentage	KN-1B-361-1-8-6/IR1750-F5 B-3// BPI76*9/Dawn	IR5657-33-2-1/IR2061-465-1-5-5	Mahsuri// IR11418-19-2-3/Pokkali	IR833-6-2-1-1//IR 1561-149-1/IR1737	IR32429-47-3-2-2// Dobongbyeo/ Moroberekan	IR28/ Kwang-Chang-Ai// IR36
Year of release (MV generation)	1990 (MV3)	1985 (MV3)	Not released; tested on field in 2002	1974 (MV1)	2000 (MV4)	1983 (MV2)
Abiotic stress tolerance;most appropriate ecosystem	Drought tolerant; good for acid upland	Drought susceptible; irrigated lands	Salt tolerant; saline areas	Salt susceptible; irrigated and rainfed lowlands	Cold tolerant; cool elevated areas	Cold susceptible; irrigated lands
Average Yield (wet season) kg/ha	2392	3852	3000	3020	3675	3857
Rice type	Indica	Indica	Indica	Indica	Japonica	Indica
Maturity[1]	121 days	117 days	130 days	116 days	NA	106 days
Plant height [1(	104 cm	103 cm	Semidwarf	97 cm	Semidwarf	86 cm
Tillering ability[1]	45 tillers	14 tillers	NA	14 tillers	NA	15 tillers
Grain amylose content[1]	NA	Non-glutinous; intermediate (23.2%)	NA	Glutinous (1%)	NA	Non-glutinous; High content (26.2%)
Brown plant-hopper biotype 1[2]	MS	Tolerant	NA	Tolerant	NA	Tolerant
Green leaf-hopper[2]	NA	Tolerant	NA	Tolerant	NA	Tolerant
White-backed plant-hopper [2]	NA	MS	NA	HS	NA	Susceptible
Yellow stem borer[2]	MR	MS	NA	MS	NA	MS
Leaf blast[3]	Resistant	MS	NA	Tolerant	NA	Tolerant
Bacterial blight biotype 1[3]	NA	Highly Resistant	NA	Highly Resistant	NA	Highly Resistant
Bacterial blight biotype 2[3]	NA	MS	NA	MS	NA	MS

NA (Not available data)
[1] Agronomic traits
[2] Crop damage (insects)
[3] Crop damage (diseases)
MS (Moderately susceptible); **MR** (moderately resistant); **HS** (highly susceptible)

[8] To consult further details on grain quality and nutrient value of 'IR64', see Vasconcelos (2003).

4. LITERATURE CITED

Aguan K, Sugawara K, Suzuki N, Kusano T (1991) Isolation of genes for low-temperature-induced proteins in rice by a simple subtractive method. Plant Cell Physiol 32: 1285-1289.

Andaya VC, Mackill DJ (2003) Mapping of QTLs associated with cold tolerance during the vegetative stage in rice. J Exp Bot 54: 2579-2585.

Baker JC, Steele C, Dure LS (1988) Sequence and characterization of 6 LEA proteins and their genes from cotton. Plant Mol Biol 11: 277-291.

Bartels D, Furini A, Ingram J, Salamini F (1996) Responses of plants to dehydration stress: a molecular analysis. Plant Growth Regul 20: 111-118.

Battaglia M, Olvera-Carrillo Y, Garciarrubio A, Campos F, Covarrubias AA (2008) The enigmatic LEA proteins and other hydrophilins. Plant Physiol 148: 6-24.

Bernier J, Atlin GN, Serraj R, Kumar A, Spaner D (2008) Breeding upland rice for drought resistance. J Sci Food Agric 88: 927-939.

Bohnert HJ, Jensen RG (1996) Strategies for engineering water-stress tolerance in plants. Trends Biotechnol 14: 89-96.

Bouman BAM (2001) Water-efficient management strategies in rice production. International Rice Research Notes 26.2, Los Baños, Philippines, pp 17-22.

Bradford KJ, Chandler PM (1992) Expression of 'dehydrin-like' proteins in embryos and seedlings of *Zizania palustris* and *Oryza sativa* during dehydration. Plant Physiol 99: 488-494.

Bray EA (1994) Alterations in gene expression in response to water deficit. In AS Basra, ed, Stress-Induced Gene Expression in Plants. Harwood Academic Publishers, India, pp 1-23.

Bray EA (1997) Plant responses to water deficit. Trends Plant Sci 2 (2): 48-54.

Bray EA, Bailey-Serres J, Weretilnyk E (2000) Responses to abiotic stresses. In BB Buchanan, W Gruissem, RL Jones, eds, Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, Rockville, MD, pp 1158-1203.

Bray EA (2002) Abscisic acid regulation of gene expression during water deficit stress in the era of the *Arabidopsis* genome. Plant Cell Environ 25: 153-161.

Bruzzone S, Moreschi I, Usai C, Guida L, Damonte G, Salis A, Scarfì S, Millo E, De Flora A, Zocchi E (2007) Abscisic acid is an endogenous cytokine in human granulocytes with cyclic ADP-ribose as second messenger. Proc Natl Acad Sci 104 (14): 5759-5764.

Campalans A, Messeguer R, Goday A, Pagès M (1999) Plant responses to drought, from ABA signal transduction events to the action of the induced proteins. Plant Physiol Biochem 37 (5): 327-340.

Chakrabortee S, Boschetti C, Walton LJ, Sarkar S, Rubinsztein DC, Tunnacliffe A (2007) Hydrophilic protein associated with desiccation tolerance exhibits broad protein stabilization function. Proc Natl Acad Sci 104 (46): 18073-18078.

Chaves MM, Oliveira MM (2004) Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. J Exp Bot 55: 2365-2384.

Chinnusamy V, Shumaker K, Zhu JK (2004) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. J Exp Bot 55(395): 225-236.

Chinnusamy V, Gong Z, Zhu J-K (2008) Abscisic acid-mediated epigenetic processes in plant development and stress responses. J Integr Plant Biol 50(10): 1187-1195.

Close TJ (1996) Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins. Physiol Plant 97: 795-803.

Close TJ (1997) Dehydrins: a commonality in the response of plants to dehydration and low temperature. Physiol Plant 100: 291-296.

Cooper B, Clarke JD, Budworth P, Kreps J, Hutchison D, Park S, Guimil S, Dunn M, Luginbuhl P, Ellero C, Goff SA, Glazebrook J (2003). A network of rice genes associated with stress response and seed development. Proc Natl Acad Sci 100 (8): 4945-50.

Cuming AC (1999) LEA Proteins. In PR Shewry, R Casey, eds, Seed Proteins. Kluwer Academic Publishers, The Netherlands, pp 753-780.

De Datta SK, Malabuyoc JA, Aragon EL (1988) A field screening technique for evaluating rice germplasm for drought tolerance during the vegetative stage. Field Crops Res 19: 123-134.

Dure L III, Greenway SC, Galau GA (1981) Developmental biochemistry of of cotton seed embryogenesis and germination: XIV. Changing mRNA populations as shown by *in vitro* and *in vivo* protein synthesis. Biochem 20: 4162-4168.

Dure L III, Crouch M, Harada J, Ho T-HD, Mundy J, Quatrano R, Thomas T, Sung ZR (1989) Common amino acid sequence domains among the LEA proteins of higher plants. Plant Mol Biol 12: 475-486.

Dure L III (1997) Lea proteins and the desiccation tolerance of seeds. In BA Larkins, IK Vasil, eds, Cellular and Molecular Biology of Plant Seed Development. Kluwer Academic Publishers, The Netherlands, pp 525-543.

Estudillo JP, Otsuka K (2002) 'Lessons from three decades of green revolution in the Philippines'. Paper presented at the workshop on 'Green Revolution in Asia and its Transferability to Africa,' sponsored by FASID and held in Tokyo, December 8-10, 2002, pp 1-51.

Finkelstein RR, **Gibson SI** (2002) ABA and sugar interactions regulating development: cross-talk or voices in a crowd? Curr Opin Plant Biol 5(1): 26-32.

Finnie C, Melchior S, Roepstorff P, Svensson B (2002) Proteome analysis of grain filling and seed maturation in barley. Plant Physiol 129: 1308-1319.

Fischer KS, Lafitte R, Fukai S, Atlin G, Hardy B (2003) Breeding rice for drought-prone environments. International Rice Research Institute, Los Baños, Philippines, 98 p.

Galau GW, Hugues DW, Dure L III (1986) Abscisic acid induction of cloned cotton late embryogenesis abundant (LEA) messenger RNAs. Plant Mol Biol 7: 155-170.

Goday A, Jensen A, Culiañez-Macià FA, Albà MM, Figueras M, Serratosa J, Torrent M, Pagès M (1994) The maize abscisic acid-responsive protein Rab17 is located in the nucleus and interacts with nuclear localization signals. Plant Cell 6: 351-360.

Gomez J, Sanchez-Martinez D, Stiefal V, Rigau J, Puigdomenech P, Pagès M (1988) A gene induced by the plant hormone abscisic acid in response to water stress encodes a glycine-rich protein. Nature 334(6179): 262-264.

Gorantla M, **Babu PR**, **Lachagari VB**, **Reddy AM**, **Wusirika R**, **Bennetzen JL**, **Reddy AR** (2007) Identification of stress-responsive genes in an *indica* rice (*Oryza sativa* L.) using ESTs generated from drought-stressed seedlings. J Exp Bot 58: 253-265.

Goyal K, Pinelli C, Maslen SL, Rastogi RK, Stephens E, Tunnacliffe A (2005a) Dehydration-regulated processing of late embryogenesis abundant protein in a desiccation-tolerant nematode. FEBS Lett 579: 4093-4098.

Goyal K, Walton LJ, Tunnacliffe A (2005b) LEA proteins prevent protein aggregation due to water stress. Biochem J 388: 151-157.

Gregorio GB, Senadhira D (1993) Genetic analysis of salinity tolerance in rice (*Oryza sativa* L.). Theor Appl Genet 86: 333-338.

Gregorio GB, Senadhira D, Mendoza RD (1997) Screening rice for salinity tolerance. IRRI Discussion paper Series No.22. International Rice Research Institute, Los Baños. Laguna, Philippines, 30 p.

Gregorio GB, Senadhira D, Mendoza RD, Manigbas NL, Roxas JP, Guerta CQ (2002) Progress in breeding for salinity tolerance and associated abiotic stresses in rice. Field Crops Res 76: 91-101.

Grelet J, Benamar A, Teyssier E, Avelange-Macherel MH, Grunwald D, Macherel D (2005) Identification in pea seed mitochondria of a late-embryogenesis abundant protein able to protect enzymes from drying. Plant Physiol 137:157-167.

Grover A, Kapoor A, Lakshmi S, Agarwal S, Sahi C, Katiyar-Agarwal S, Agarwal M, Dubey H (2001) Understanding molecular alphabets of the plant abiotic stress responses. Curr Sci 80: 206-216.

Grzelczak ZP, Sattolo MH, Hanley-Bowdoin LK, Kennedy TD, Lane BG (1982) Synthesis and turnover of proteins and mRNA in germinating wheat embryos. Can J Biochem 60: 389-397.

Hajela RK, Horvath DP, Gilmour SJ, Thomashow MF (1990) Molecular cloning and expression of cor (cold-regulated) genes in *Arabidopsis thaliana*. Plant Physiol 93: 1246-1252.

Hirayama T, Shinozaki K (2007) Perception and transduction of abscisic acid signals: keys to the function of the versatile plant hormone ABA. Trends Plant Sci 12(8):343-351.

Hoekstra FA, Golovina EA, Buitink J (2001) Mechanisms of plant desiccation tolerance. Trends Plant Sci 6 (9): 431-436.

Hossain M (1996) Recent developments in the asian rice economy: challenges for rice research. In RE Evenson, RW Herdt, M Hossain, eds, Rice Research in Asia: Progress and Priorities. International Rice Research Institute, Philippines, pp 17-33.

Inger Genetic Resources Center (1996) Standard evaluation system for rice, 4th edition. IRRI, Los Baños, Philippines, pp 1-52.

IRRI (1979) Report of a rice cold tolerance workshop. Los Baños, Philippines, 139 p.

IRRI (1991) Program Report for 1990: Upland Rice Ecosystem Research Program, Los Baños, Philippines, pp 98-118.

IRRI (2000) Program Report for 2000: Irrigated Rice Ecosystem Research Program. Los Baños, Philippines, p 7, *on World Wide Web URL:* www.irri.org/publications/program/pdfs/00ProgramReport/Irrigated.pdf

IRRI (2002) Standard Evaluation System for Rice (SES), *on World Wide Web URL:* www.knowledgebank.irri.org/ses/SES.htm

IRRI World Rice Statistics (2004-2006) Rice ecosystem, item 30: distribution of rice crop area, by environment, *on World Wide Web URL:* http://beta.irri.org/statistics/index.php?option=com_content&task=view&id=413&Itemid=192

Jensen AB, Busk PK, Figueras M, Albà MM, Peracchia G, Messeguer R, Goday A, Pagès M (1996) Drought signal transduction in plants. Plant Growth Regul 20: 105-110.

Jensen AB, Goday A, Figueras M, Jessop AC, Pagès M (1998) Phosphorylation mediates the nuclear targeting of the maize Rab17 protein. Plant J 13: 691-697.

Khush GS (1997) Origin, dispersal, cultivation and variation of rice. Plant Mol Biol 35: 25-34.

Khush GS, Virk PS (2005) IR varieties and their impact. International Rice Research Institute. Los Baños, Philippines, 163 p.

Kusano T, Aguan K, Abe M, Sugawara K (1992) Nucleotide sequence of a rice *rab16* homologue gene. Plant Mol Biol 18:127-129.

Lafitte HR, Yongsheng G, Yan S, Li Z-K (2007) Whole plant responses, key processes, and adaptation to drought stress: the case of rice. J Exp Bot 58: 169-175.

Lang V, Palva ET (1992) The expression of a rab-related gene, *rab18*, is induced by abscisic acid during the cold acclimation process of *Arabidopsis thaliana* (L.) Heynh. Plant Mol Biol 20(5): 951-962.

Langridge P, Paltridge N, Fincher G (2006) Functional genomics of abiotic stress tolerance in cereals. Brief Funct Genomic Proteomic 4: 343-354.

Lee K-S, Choi W-Y, Ko J-C, Kim T-S, Gregorio GB (2003) Salinity tolerance of *japonica* and *indica* rice (*Oryza sativa* L.) at the seedling stage. Planta 216: 1043-1046.

Lee SC, Lee MY, Kim SJ, Jun SH, An G, Kim SR (2005) Characterisation of an abiotic stress-inducible dehydrin gene, *OsDhn1*, in rice (*Oryza sativa* L.). Mol Cells 19(2): 212-218.

Levitt J (1980) Responses of plants to environmental stresses. Vol II, Water, radiation and other stresses. Academic Press, New York.

Li TG, Visperas RM, Vergara BS (1981) Correlation of cold tolerance at different growth stages in rice. Acta Bot Sinica 23: 203-207.

Lisa LA, Seraj ZI, Elahi CMF, Das KC, Biswas K, Islam MR, Salam MA, Gomosta AR (2004) Genetic variation in microsatellite DNA, physiology and morphology of coastal saline rice (*Oryza sativa* L.) landraces of Bangladesh. Plant and Soil 263: 213-228.

Liu JX, Liao DQ, Oane R, Estenor L, Yang XE, Li ZC, Bennett J (2006) Genetic variation in the sensitivity of anther dehiscence to drought stress in rice. Field Crops Res 97: 87-100.

Liu X, Yue Y, Li B, Nie Y, Li W, Wu W, Ma L (2007) A G protein-coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid. Science 315: 1712-1716.

Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E (2009) Regulators of PP2C phosphatase activity function as abscisic acid sensors. Science, 324: 1064-1068.

Mackill DJ, Coffman WR, Garrity DP (1996) Rainfed lowland rice improvement. International Rice Research Institute, Los Baños, Philippines, 242 p.

Melcher K, Ng L-M, Zhou XE, Soon F-F, Xu Y, Suino-Powell KM, Park S-Y, Weiner JJ, Fujii H, Chinnusamy V, Kovach A, Li J, Wang Y, Li J, Peterson FC, Jensen DR, Yong E-L, Volkman BF, Cutler SR, Zhu J-K, Xu HE (2009) A gate–latch–lock mechanism for hormone signalling by abscisic acid receptors. Nature 462 (7273): 602-608.

Miyazono K-i, Miyakawa T, Sawano Y,Kubota K, Kang H-J, Asano A, Miyauchi Y, Takahashi M, Zhi Y, Fujita Y, Yoshida T, Kodaira K, Yamaguchi-Shinozaki K, Tanokura M (2009) Structural basis of abscisic acid signalling. Nature 462 (7273): 609-614.

Mohammadi-Nejad G, Arzani A, Rezai AM, Singh RK Gregorio GB (2008) Assessment of rice genotypes for salt tolerance using microsatellite markers associated with the *saltol* QTL. African J Biotechnol 7(6): 730-736.

Mouillon JM, Gustafsson P, Harryson P (2006) Structural investigation of disordered stress proteins. Comparison of full-length dehydrins with Isolated peptides of their conserved segments. Plant Physiol 141: 638-650.

Mundy J, Chua NH (1988) Abscisic acid and water-stress induce the expression of a novel rice gene. EMBO J 7(8): 2279-2286.

Nakashima K, Ito Y, Yamaguchi-Shinozaki K (2009) Transcriptional regulatory networks in response to abiotic stresses in *Arabidopsis* and grasses. Plant Physiol 149: 88-95.

Nishimura N, Sarkeshik A, Nito K, Park S-Y, Wang A, Carvalho PC, Lee S, Caddell SF, Cutler SR, Chory J, John R. Yates JR, Schroeder JI (2010) PYR/PYL/RCAR family members are major in-vivo ABI1 protein phosphatase 2C-interacting proteins in Arabidopsis. Plant J 61(2): 290-299.

Nylander M, Svensson J, Palva ET, Welin BV (2001) Stress-induced accumulation and tissue-specific localization of dehydrins in *Arabidopsis thaliana*. Plant Mol Biol 45: 263-279.

Park SY, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow TF, Alfred SE, Bonetta D, Finkelstein R, Provart NJ, Desveaux D, Rodriguez PL, McCourt P, Zhu JK, Schroeder JI, Volkman BF, Cutler SR (2009) Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. Science 324: 1068-1071.

Passioura J (2007) The drought environment: physical, biological and agricultural perspectives. J Exp Bot 58: 113–117.

Plana M, Itarte E, Eritja R, Goday, A, Pagès M, Martinez MC (1991) Phosphorylation of maize Rab17 protein by casein kinase 2. J Biol Chem 266: 22510-22514.

Razem FA, El-Kereamy A, Abrams SR, Hill RD (2006) The RNA-binding protein FCA is an abscisic acid receptor. Nature 439: 290-294.

Riera M, Figueras M, López C, Goday A, Pagès M (2004) Protein kinase CK2 modulates developmental functions of the abscisic acid responsive protein Rab17 from maize. Proc Natl Acad Sci 101(26): 9879–9884.

Sandiwà-IRRI (1999) A rare breed indeed, pp 2-3, *on World Wide Web URL:* www.irri.org/publications/sandiwa/pdfs/Sandiwa12.pdf

Sandiwà-IRRI (2002) IRRI enhances Philippine development, pp 12-13, *on World Wide Web URL:* www.irri.org/publications/sandiwa/pdfs/Sandiwa23.pdf

Senadhira D, Zapata-Arias FJ, Gregorio GB, Alejar MS, de la Cruz HC, Padolina TF, Galvez AM (2002) Development of the first salt-tolerant rice cultivar through indica/indica anther culture. Field Crops Res 76: 103-110.

Shen Y-Y, Wang X-F, Wu F-Q, Du S-Y, Cao Z, Shang Y, Wang X-L, Peng C-C, Yu X-C, Zhu S-Y, Fan R-C, Xu Y-H, Zhang D-P (2006) The Mg-chelatase H subunit is an abscisic acid receptor. Nature 443: 823-826.

Shinozaki K, Yamaguchi-Shinozaki K (2000) Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. Curr Opin Plant Biol 3: 217-223

Shinozaki K, Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance. J Exp Bot 58: 221-227.

Singh BN, Mackill DJ (1991) Genetics of leaf rolling under drought stress. *In* Rice Genetics II. Proceedings of the Second International Rice Genetics Symposium 14-18 May 1990. IRRI, Manila, Philippines, pp 159-166.

Sreenivasulu N, Sopory SK, Kishor PBK (2007) Deciphering the regulatory mechanisms of abiotic stress tolerance in plants by genomic approaches. Gene 388: 1-13.

Sturla L, **Fresia C**, **Guida L**, **Bruzzone S**, **Scarfi S**, **Usai C**, **Fruscione F**, **Magnone M**, **Millo E**, **Basile G**, **Grozio A**, **Jacchetti E**, **Allegretti M**, **De Flora A**, **Zocchi E**.(2009) LANCL2 is necessary for abscisic acid binding and signaling in human granulocytes and in rat insulinoma cells. J Biol Chem 284(41): 28045-28057.

Sung DY, Kaplan F, Lee KJ, Guy CL (2003) Acquired tolerance to temperature extremes. Trends Plant Sci 8 (4): 179-187.

Takahashi R, Joshee N, Kitagawa Y (1994) Induction of chilling resistance by water stress, and cDNA sequence analysis and expression of water stress-regulated genes in rice. Plant Mol Biol 26: 339-352.

Tran LS, **Nakashima K**, **Shinozaki K**, **Yamaguchi-Shinozaki K** (2007) Plant gene networks in osmotic stress response: from genes to regulatory networks. Methods Enzymol 428: 109-28.

Tripathy JN, Zhang J, Robin S, Nguyen TT, Nguyen HT (2000) QTLs for cell-membrane stability mapped in rice (*Oryza sativa* L.) under drought stress. Theor Appl Genet 100: 1197-1202.

Vasconcelos MW (2003) A biotechnological strategy to improve iron content in the endosperm of *elite* rice varieties. PhD Thesis. ITQB-UNL, Oeiras, Portugal.

Vergara BS, Visperas RM, Coffman WR, Villareal RL, Bacalangco E (1976) Screening of the rice germplasm for low temperature tolerance at different stages of growth. SABRAO J 8:99-104.

Verslues PE, Agarwal M, Katiyar-Agarwal S, Zhu J, Zhu J-K (2006) Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. Plant J 45: 523-539.

Vicente-Carbajosa J, Carbonero P (2005) Seed maturation: developing an intrusive phase to accomplish a quiescent state. Int J Dev Biol 49: 645-651.

Vilardell J, Goday A, Freire MA, Torrent M, Martínez MC, Torné JM, Pagès M (1990) Gene sequence, developmental expression and protein phosphorylation of RAB-17 in maize. Plant Mol Biol 14: 423-432.

Vinocur B, Altman A (2005) Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. Curr Opin Biotechnol 16: 123-132.

Wang W, Vinocur B, Altman A (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta 218: 1-14.

Wasilewska A, Vlad F, Sirichandra C, Redko Y, Jammes F, Valon C, Frei dit Frey N, Leung J (2008) An update on abscisic acid signaling in plants and more. Mol Plant 1(2): 198-217.

White CN, Proebsting WM, Hedden P, Rivin CJ (2000) Gibberellins and seed development in maize. I. Evidence that gibberellin/abscisic acid balance governs germination *versus* maturation pathways. Plant Physiol 122(4):1081-1088.

Wise MJ (2002) The POPPs: clustering and searching using peptide probability profiles. Bioinformatics 18 (Suppl 1): S38-S45.

Wise MJ (2003) LEAping to conclusions: A computational reanalysis of late embryogenesis abundant proteins and their possible roles. BMC Bioinformatics 4: 52.

Wise MJ, **Tunnacliffe A** (2004) POPP the question: what do LEA proteins do? Trends Plant Sci 9(1): 13-17.

Xiong L, Zhu JK (2003) Regulation of abscisic acid biosynthesis. Plant Physiol 133: 29-36.

Yamaguchi-Shinozaki K, Mundy J, Chua NH (1989) Four tightly linked rab genes are differentially expressed in rice. Plant Mol Biol 14(1): 29-39.

Yamaguchi-Shinozaki K, Shinozaki K (2005) Organization of cis-acting regulatory elements in osmotic- and cold-stress responsive promoters. Trends Plant Sci 10: 88-94.

Zeigler RS Mackill, DJ, Capilit, GLS (2006) Designation of IRRI breeding lines. IRRN (International Rice Research Notes) 31(1): 40-42.

Zeng L, Poss JA, Wilson C, Draz A-SE, Gregorio GB, Grieve CM (2003) Evaluation of salt tolerance in rice genotypes by physiological characters. Euphytica 129: 281-292.

Zhu JK (2001) Plant salt tolerance. Trends Plant Sci 6(2): 66-71

II. Aims of the thesis

The main goal of this work was to obtain new clues on the molecular mechanisms involved in abiotic stress tolerance in rice, with special emphasis on water stress. We focused our analysis in the differences (and similarities) involved in the stress response/adaptation between rice varieties bred at IRRI for drought-prone uplands, saline soils and cool-elevated areas (i.e. stress tolerant genotypes), and three other varieties released for favourable environmental conditions (i.e. stress sensitive genotypes). For that purpose we adopted the following strategies:

1) Proteomic approaches to:

- compare the embryo proteome of the rice genotypes with differential stress adaptation, by means of two-dimensional gel electrophoresis (2-DE). The main aim was the identification by MS of rice proteins with a putative role in drought tolerance. Since it was previously described that the Rab17 protein was strongly phosphorylated in the maize mature embryo, one of the main objectives was to evaluate the phosphorylation status of the homologous protein Rab21 in the embryo of rice genotypes adapted to different environmental conditions, to further elucidate about its role in abiotic stress tolerance.

- characterise rice protein(s) that could cross-hybridise with an antibody raised against the maize DBF1 transcription factor, through one- and two-dimensional gel electrophoresis. The main aim was to identify the rice homologous protein and determine its involvement in stress tolerance.

2) Transcriptomic approach using the cDNA-AFLP methodology to:

- identify drought-responsive genes, and further analyse if their expression could be also influenced by high salinity, low temperature and ABA, attempting to find genes with a critical role in abiotic stress tolerance.

Goday A, Sánchez-Martinez D, Gómez J, Puigdomènech P, Pagès M (1988) Gene expression in developing *Zea mays* embryos: regulation by abscisic acid of a highly phosphorylated 23- to 25-kD group of proteins. Plant Physiol 88: 564-569.

Kizis D and Pagès M (2002) Maize DRE-binding proteins DBF1 and DBF2 are involved in *rab17* regulation through the drought-responsive element in an ABA-dependent pathway. Plant J 30(6): 679-689.

Mundy J, Chua NH (1988) Abscisic acid and water-stress induce the expression of a novel rice gene. EMBO J 7(8): 2279-2286.

Vilardell J, Goday A, Freire MA, Torrent M, Martínez MC, Torné JM, Pagès M (1990) Gene sequence, developmental expression and protein phosphorylation of RAB-17 in maize. Plant Mol Biol 14: 423-432.

Chapter II

Comparison of the embryo proteome in rice (*Oryza sativa* L.) varieties with contrasting abiotic stress tolerance

Farinha AP [1,3], Irar S [1], Oliveira E [2], Oliveira MM [3] and Pagès M [1].
Comparison of the embryo proteome in rice (*Oryza sativa* L.) varieties with contrasting abiotic stress tolerance (under revision to be submitted).

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AP Farinha declares to have participated actively in this work, by performing the experimental design, all the laboratory work (including 2-DE, image and data analysis, Western-blots) and manuscript writing.

Abstract

Cereal embryos sustain severe water-deficit at the final stage of seed maturation. The molecular mechanisms underlying the acquisition of desiccation tolerance in seed embryos are similar to those displayed during water-deficit in vegetative tissues. The genetic variation among six rice genotypes adapted to diverse environmental conditions was analysed at the proteome level, in order to get further clues on the mechanisms leading to water-stress tolerance. Mass spectrometry analysis allowed the identification of 30 proteins, mostly involved in stress tolerance (e.g., late embryogenesis abundant proteins) and nutrient reservoir activity. Although the major role of seed storage proteins is related to energy supply to germinating embryos, it is likely that some of these proteins may also participate in the embryo desiccation tolerance. The differences found in the accumulation of a glutelin type-B 2 isoform suggest a protective function against water-deficit in the seed embryo. Hierarchical clustering and multidimensional scaling analyses revealed a close relationship between the stress-sensitive genotypes, whereas the stress-tolerant varieties were more distantly related. Besides important qualitative and quantitative changes in embryo proteins across the distinct varieties, we also found differences at post-translational level. Here, we report for the first time a difference in the phosphorylation status of LEA Rab21 in the seed embryos of genotypes adapted to diverse environmental conditions. Hence, the results indicated that the protein was more strongly phosphorylated in the embryos of the sensitive varieties than in the embryos of the tolerant ones. The differences found in phosphorylation status of Rab21 are proposed to be related to stress tolerance.

Comparison of the embryo proteome in rice (*Oryza sativa* L.) varieties with contrasting abiotic stress tolerance

1. Introduction

Cereals are the most important food source, and rice (*Oryza sativa* L.) alone is the staple crop for more than 3 billion people i.e. more than half of the world's population (Langridge *et al*., 2006; Nguyen, 2008). Global rice production must increase nearly 40% to feed the predicted 5.0 billion consumers out of a total of 8.3 billion people that are expected by 2030 (FAO, 2002; Khush, 2005). The challenge for the next generations is thus to achieve a sustainable rice production with less arable land, reduced inputs of agrochemicals (fertilizers and pesticides) and above all, with limited water supply (Khush, 2005; Zhang, 2007). Environmental stresses limit plant growth and productivity, and in the particular case of rice, drought has become the most serious constraint to yield potential in all agro-climatic zones, including irrigated lands due to the rising water demand for competing uses (Gorantla *et al*., 2007; Pandey *et al*. 2007). Nonetheless, the salinization of soil and water across many areas of the planet is turning into an increasing threat to crop productivity (Vinocur and Altman, 2005; Tester and Bacic, 2005).

The embryos from orthodox seeds (Roberts, 1973) and the vegetative tissues of resurrection plants (e.g., *Craterostigma plantagineum*) withstand water contents that can be lower than 5% (0.05 g H_2O/g dry weight), making them interesting models to address desiccation tolerance (Bartels and Salamini, 2001; Hoekstra *et al*., 2001; Caramelo and Iusem, 2009). Cereal embryos are able to survive to severe water loss at the final stage of seed maturation, whereas the starchy endosperm undergoes programmed cell death (Jensen *et al*., 1996). Desiccation tolerance in seed embryos always attracted the attention of researchers, in part due to the similarity to water deficit responses in vegetative tissues (Bray, 1997). Indeed, the late embryogenesis abundant (LEA) proteins not only accumulate extensively in

the cells of seed embryos during the late phase of maturation, but are also induced in vegetative organs in response to water deficit and ABA, besides to salinity and cold conditions (Galau *et al*., 1986; Gomez *et al*., 1988; Mundy and Chua, 1988; Hajela *et al*., 1990; Lang and Palva, 1992). Although their precise function in drought/desiccation tolerance is still not totally clear, it has been shown that LEAs have a role in the prevention of protein aggregation, and in the protection of other proteins by preserving their enzymatic activity during dehydration (Wise and Tunnacliffe, 2004; Goyal *et al*., 2005a; Grelet *et al*., 2005; Chakrabortee *et al*., 2007). On the other hand, the existence of LEAs in desiccation-tolerant organisms other than plants (e.g., nematodes), reinforces their role in protecting cells against the damage caused by dehydration (Goyal *et al*., 2005b).

Abscisic acid (ABA) is simultaneously a key hormone in the regulation of seed maturation and in the adaptation to abiotic stresses, in particular drought and high salinity (Leung and Giraudat, 1998; Wasilewska *et al*., 2008). Transcriptome analysis of the regulation of barley seed maturation by ABA revealed that different signalling pathways take place between the embryo and the endosperm (Sreenivasulu *et al*., 2006). In the barley embryo, ABA participates in the acquisition of desiccation tolerance via ABA-responsive element (ABRE) binding factors, while in the endosperm it regulates the synthesis of storage products (mainly starch) (Sreenivasulu *et al*., 2006). Comparative studies on the transcriptome of maturing embryos and germinated radicles of *Medicago truncatula* seeds supported further evidence of a partial overlap between the regulatory machinery controlling desiccation tolerance in embryo seeds, and drought tolerance in vegetative organs (Buitink *et al*., 2006). The regulatory genes up-regulated in the embryos during maturation, and in radicles after the re-establisment of desiccation tolerance, coincided with transcription factors homologous to those typically expressed in abiotic/drought stress conditions (e.g., AREB-like, Myb and DREB2) (Buitink *et al*., 2006). Thus, both ABA-dependent and -independent signalling pathways seem to be involved in the acquisition of

desiccation tolerance in *Medicago* seeds. Most interestingly, Buitink and co-workers (2006) verified that the re-establishment of desiccation tolerance in *Medicago* seeds evoked the quiescent state prior to germination. Despite that seed embryogenesis and acquisition of desiccation tolerance are tightly linked, being reasonable to argue that the acquisition of tolerance in embryos depends on developmental programs, desiccation tolerance can be achieved by seed embryos even if severe alterations occur during embryogenesis (Wehmeyher and Vierling, 2000; Golovina *et al*., 2001; Buitink *et al*., 2006).

Proteomic approaches may be helpful in the identification of functional proteins responsible for the survival and adaptation of organisms to their surrounding environment (Agrawal and Rakwal, 2006). On the other hand, comparative proteome analyses offer a unique dynamic view of the proteome, more challenging than the mere cataloguing of proteins (Thelen and Peck, 2007). In this work, we focused on the rice embryo proteome, aiming to get further knowledge on the molecular mechanisms beyond water stress tolerance. Until now, rice embryo comparative proteome studies provided important information on the changes occurring during seed germination (Kim *et al*., 2009) and demonstrated their usefulness in pedigree analyses (Xie *et al*., 2006; Wang *et al*., 2008). However, there is none comparative proteomic report on the rice embryo addressing drought/abiotic stress tolerance. Hence, we decided to compare the embryo proteome of rice varieties adapted to different abiotic stress conditions, and possessing contrasting stress responses. Given that water deficit occurs at cellular level not only during drought, but also after salinity and low temperature injuries, it was appealing to monitor protein profiles across genotypes adapted to these stress conditions to get further clues on water-stress tolerance. After comparing the embryo proteome of varieties bred at the International Rice Research Institute (IRRI, Philippines) for different environments (e.g., drought-prone, salinity and cool-elevated areas), important differences were observed in proteins commonly involved in stress tolerance/response (e.g.,

LEAs), besides changes in proteins typically found in seed organs, like storage proteins. One of the most interesting differences between tolerant and sensitive varieties was observed in the LEA protein OsRab21, at post-translational level. The protein was apparently more strongly phosphorylated in the embryos of the sensitive varieties as compared to the embryos of the tolerant ones. The differences across the different genotypes at the whole-proteome level, and in the expression of individual proteins such as Rab21, will be further discussed.

2. Materials and Methods

2.1. Plant material and protein extraction

Seeds of drought tolerant (ssp. *indica* cv. 'PSBRc1'), drought sensitive (ssp. *indica* cv. 'IR64'), salt tolerant (ssp. *indica* breeding line 'IR52724-2B-6-2B-1-1'), salt sensitive (ssp. *indica* cv. 'IR29'), cold tolerant (ssp. *japonica* cv. 'PSBRc96') and cold sensitive (ssp. *indica* cv. 'IR58') rice cultivars were provided by Dr. Glenn B. Gregorio from IRRI (Los Baños, Philippines). The hulls from dried seeds were removed and mature embryos were manually separated from the endosperm with scalpel, immediately frozen in liquid nitrogen and kept at -80°C until protein extraction.

Total protein extracts were prepared by grinding the dried embryos with mortar and pestle in liquid nitrogen. For 1-DE, embryo protein extracts were solubilized in 50 mM Tris-HCl pH 8.0, 10 mM NaCl, 1% (v/v) SDS, 5% (v/v) β-mercaptoethanol, with protease inhibitors (1mM PMSF, 50 μM leupeptin, 1 μM pepstatin, 10 μM E-64, 10 μg mL $^{-1}$ aprotinin); for 2-DE, proteins were solubilized in 8M Urea, 2M Thiourea, 4% (w/v) CHAPS, 40mM Tris-HCl pH 8.0 (Lysis Buffer) containing protease inhibitors in the same concentrations as previously mentioned, and a protease-free DNaseI-RNaseA mixture. Protein extracts were centrifuged at 10.000$_X$g for 20 min at 4°C until supernatant was completely clear. For dephosphorylation assays, protein extracts were prepared with 50 mM Tris-HCl pH 8.0 and 10 mM NaCl

containing protease inhibitors, and then treated with calf intestine alkaline phosphatase (Roche) at 37°C for 4h, as previously described (Plana *et al*., 1991). For 2-DE analyses, dephosphorylated protein extracts were desalted prior to isoelectric focusing (IEF) with spin columns (Pierce), following the manufacturer's instructions. Protein concentrations were determined using the Bio-Rad Protein Assay, and Bovine Serum Albumine (BSA) as standard. Equal protein loading was confirmed by staining proteins with CBB R-250, after SDS-polyacrylamide gel electrophoresis (PAGE).

2.2. Two-dimensional gel electrophoresis

Total protein extracts were prepared from 100 mg of embryos in 1 mL of Lysis Buffer (see section 2.1). One hundred milligrams corresponds approximately to 200 embryos (the exact number of rice embryos depended on the genotype). Prior to IEF, 1 mg of sample protein was diluted in rehydration solution containing 8M Urea, 18 mM Tris-HCl pH 8.0, 4% (w/v) CHAPS, 0.5% (v/v) IPG (immobilized pH gradient) buffer in same range as the IPG strip (i.e. pH 3-11), 1.6% (v/v) DeStreak Reagent (GE Healthcare Amersham Biosciences) and 0.002% (w/v) Bromophenol Blue. Rice total embryo proteins were loaded onto 18-cm long IPG strips, pH 3-11 NL (non-linear) (Immobiline DryStrips, GE Healthcare Amersham Biosciences).

Strips containing protein samples were rehydrated for 6 hours at room temperature, followed by 6.5 h at 30V. IEF was performed at 500V for 1h, 1000V for 1h, followed by 8000V for 7h, using the Ettan^{TM} IPGphor^{TM} Isoelectric Focusing System (GE Healthcare Amersham Biosciences). Prior to second dimension, IPG strips were equilibrated with 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (v/v) SDS, a trace of Bromophenol Blue and 10 mg mL $^{-1}$ DTT for 15 min, followed by a second equilibration step with the same buffer containing 25 mg mL $^{-1}$ iodoacetamide for further 15 min, with gentle shaking. For the second dimension, IPG strips containing the focused proteins were applied on 12% (v/v) SDS-polyacrylamide Laemmli gels (26x20x0.1cm) and electrophoresis was performed at 2.5

W/gel for 30 min then 100 W total until run was completed (between 4-5 h) using the Ettan DALT*six* System (GE Healthcare Amersham Biosciences). 2-DE gels were run in triplicate (technical replicates) and three independent extraction events were performed from different seed stocks (biological replicates) for each varietiy. Gels were then stained with CBB R-250.

2.3. Image and data analysis

The stained gels were scanned with an ImageScanner desktop instrument, using the LabScan application (GE Healthcare Amersham Biosciences). Gel TIFF (Tag Image File Format) image analysis was performed using the ImageMaster™ 2D Platinum 5.0 Software (GE Healthcare Amersham Biosciences). Gel replicates were used to obtain synthetic gel images (with averaged positions, shapes and optical densities) for each cultivar. After automatic spot detection, manual spot editing was carried out. Spots matching in at least ⅔ of the gels were included in synthetic gel images. These gel images thus included only highly reproducible spots. In order to compare the embryo proteome in the distinct rice varieties, automatic gel matching was established among the corresponding averaged synthetic gels, selecting the 'PSBRc96' cultivar as reference. Careful visual inspection was performed again to confirm correct spot matching.

To evaluate protein expression differences among different gels, normalized spot volumes (also referred as relative volumes) were used instead of raw spot volumes. These values were calculated by the ImageMaster software and provided in percentage. Each value obtained represented the ratio of a given spot volume ($Vol._S$) to the sum of all spot volumes detected in the gel with 'n' spots ($\sum_{S=1}^{n} Vol._S$). This normalization procedure is particularly useful for correcting gel-to-gel variations due to uneven protein loading, staining, or differences obtained during image acquisition, thus minimizing experimental variations.

Furthermore, all normalized spot volumes (% Vol.) were adjusted a second time, by calculating correction constants as described by Hajduch *et al*. (2005). The reason of performing this second normalization was to ensure an accurate quantitative comparison between protein amounts of different embryo proteomes. Since the mean number of detected spots varied across the different genotypes, this could contribute to overestimate the differences in the quantities (i.e. relative volumes) of matching proteins. The correction constants (C) were average values of individual constants obtained for each embryo proteome in relation to other five. The individual constants were calculated by dividing the sum of all spot volumes in a certain variety ($\sum_{S=1}^{n} \text{Vol.}_S$) by the sum of all spot volumes in that variety plus the sum all spot volumes in the compared variety. An example of how an averaged correction constant was obtained for variety 1 (e.g., 'PSBRc1') after all pairwise combinations with the other five, is given below:

$$C\,(\text{Variety 1}) = \text{Mean}\left(\frac{\sum_{S=1}^{n} \text{Vol.}_S \text{ Variety 1}}{\sum_{S=1}^{n} \text{Vol.}_S \text{ Variety 1} + \sum_{S=1}^{n} \text{Vol.}_S \text{ Variety p}} \right)$$

$$p = 2,3,4,5,6$$

The sum of spot volumes in each variety was obtained from 2-DE synthetic gel images data set. After calculating C (i.e. 0.512, 0.547, 0.591, 0.518, 0.440 and 0.392 for 'IR58', 'PSBRc96', 'IR29', IR52724-2B-6-2B-1-1, 'IR64' and 'PSBRc1' varieties, respectively), individual normalized spot volumes of each replicate were then multiplied by this factor, and then by a scaling factor of 2 to restore the initial range of values.

2.3.1. Statistical analyses

Coefficients of correlation (*r*) and variation (CV) were determined after pairwise comparisons between gel replicates running the same protein sample (technical/analytical) or different protein samples obtained from a same variety (biological). All spot volumes matching between technical replicates (the mean number for each variety is depicted in Fig. 2) were considered for calculating *r* and CV, whereas a mean of 140 spots covering all spot volume ranges was used to determine biological variation. Correlation between gel replicates was further analysed by statistical analysis (Student's *t*-test, $P < 0.01$).

Significant differences in protein abundance between individual spots or between a set of spots matching in two varieties were validated by Student's *t*-test analysis ($P < 0.05$), using the statistical software package SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). The differences in the means were calculated based on data from three replicates. Prior to any statistical analyses, normalised spot volumes were log standardized, as the log transformation improves normality (Karp *et al*., 2005). All normalized spot volumes were exported and $\log_{10}$ transformed using the statistical software package SPSS.

For hierarchical clustering and multidimensional scaling analyses we selected 83 spots expressed in all varieties. The means of log normalised spot volumes from three replicates were used. The TIGR MultiExperiment Viewer software (TMEV version 3.1) (Saeed *et al*., 2006) was used for hierarchical clustering, whereas multidimensional scaling was performed using the Proxscal algorithm (version 1.0, Leiden University, The Netherlands) available in the statistical software package SPSS. The average linkage method and Euclidean distances were used in hierarchical clustering, and for multidimensional scaling analysis, a proximity matrix based on Euclidean distances was calculated.

2.4. Western-blot analysis

Rice embryo proteins were separated by 1-DE and 2-DE for Western-blot analyses. Embryo protein extracts were separated by 1-DE on 15% (v/v) SDS-PAGE Laemmli gels (8x7.3x0.1cm) at 30mA (15mA/gel, constant current) until the tracking dye reached the bottom of the gel. For 2-DE Western-blot analysis, protein isoelectric focusing was performed as described in section 2.2. When separating proteins onto 7-cm IPG strips (NL pH 3-11) during the first dimension, total protein extracts (100 µg) were diluted on 125 µl final volume. The protein samples were then run on 12% (v/v) SDS-PAGE gels (8x7.3x0.1cm) using the Mini-Protean 3 System (Bio-Rad), at 25V for 10 min and then 120 V until the dye front reached the bottom gel. When separating proteins onto 18-cm IPG strips (NL pH 3-11) the procedure was similar, except that 200 µg of total proteins were loaded on IPG strips, and the separated onto large-format gels, as described in section 2.2. The proteins separated either by 1-DE or 2-DE were blotted onto nitrocellulose membranes (Schleicher and Schuell, 0.45 µm) using a Trans-blot Semidry Transfer Cell (Bio-Rad). A polyclonal rabbit antiserum raised against Rab17, a group 2 LEA protein from maize (Goday *et al*., 1988; Vilardell *et al*., 1990) was used to cross-react with rice proteins. This primary antibody was diluted 1:2000, whereas anti-rabbit IgG, Horseradish Peroxidase-linked (GE Healthcare Amersham Biosciences) was used as the secondary antibody at a dilution of 1:15000. Western-blot detection was performed using the ECL Western blotting detection reagents and analysis system (GE Healthcare Amersham Biosciences), following the manufacturer's instructions.

2.5. Protein identification by mass spectrometry

Proteins were identified either at the Structural and Biological Mass Spectrometry Unit, IIBB-CSIC IDIBAPS (Barcelona, Spain), or at the Proteomics Platform of Barcelona Science Park, University of Barcelona (a member of ProteoRed network). Protein spots of interest were manually

excised from 2-DE gels, digested with trypsin and identified by MALDI-TOF mass spectrometry (MS). Whenever a protein was not conclusively identified by this method, nanoflow liquid chromatography coupled to electrospray ionization quadropole time-of-flight tandem mass spectrometry (nano-LC ESI-Q-TOF MS/MS) was used. Only in the particular case of OsLEA Rab21, *de novo* sequencing had to be performed.

Protein digestion was accomplished as described by Irar *et al*. (2006), with minor modifications. Briefly, protein spots were reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide and proteolitically cleaved with trypsin (Promega, Madison, WI, USA) overnight, at 37°C. The digested peptides were extracted with ACN-TFA (acetonitrile-trifluoroacetic acid), evaporated to dryness and re-dissolved in 5 µL of MeOH:H_2O (1:2, v/v) and 0.5% TFA, for the MALDI-TOF MS analysis. MALDI-TOF MS was performed using a MALDI-TOF Voyager DE™ PRO (Perspective Biosystems/Applied Biosystems) instrument in the reflectron positive ion mode. Spectra were externally mass calibrated using a standard peptide mixture. For the analysis, 0.5 µL of the peptide extract and 0.5 µL of matrix (5 mg mL^{-1} α-cyano-4-hydroxy cinaminic acids) were loaded onto the MALDI plate. When ions corresponding to known trypsin autolytic peptides (m/z 842.5099, 1045.5642, 2211.1046) were detected at adequate intensities, an automatic internal calibration of the spectra was performed.

The nano-LC ESI-Q-TOF-MS/MS analysis was performed using an ESI-Q TOF Global mass spectrometer (Waters-Micromass, Manchester, UK). In this case, samples were resuspended in 10 µL 10% (v/v) formic acid solution and 4 µL of peptide extract were injected for chromatographic separation in reverse-phase capillary C_{18} column (75 µm of internal diameter and 15 cm length, PepMap column, LC Packings). The eluted peptides were ionized via coated nano-ES needles (PicoTip™, New Objective). A capillary voltage of 1800-2200 V was applied together with a cone voltage of 80 V. The collision in the collision-induced dissociation (CID) was performed at 25-35 eV with

argon as collision gas. Data were generated in PKL file format and were submitted for database search using the software package 'MASCOT'.

The software 'Protein Prospector' version 3.4.1 (UCSF Mass Spectrometry Facility, University of California) has been used to identify proteins from MALDI-TOF MS analyses, besides 'MASCOT'. On the other hand, the 'SEQUEST' software (Thermo-Instruments, Spain) was used for preliminary protein identification from the tandem mass spectra analysis followed by manual sequence data confirmation. Sequence searching was performed on SwissProt and non-redundant NCBI databases using protein full range of molecular mass and p*I*. No species restriction was applied and when an identity search produced no significant matches, the homology mode was used.

For peptide matching, we accepted one miss cleavage maximum and peptide modifications by oxidation of Met and carbamidomethylation of Cys. The peptide mass and fragment tolerance was 200 ppm and 0.25 Da, respectively. For MASCOT searching, individual ions scores > 49 indicated identity or extensive homology ($P < 0.05$). Besides significant ($P < 0.05$) MASCOT score, the number of matched peptides and protein sequence coverage were also taken into consideration for protein identification.

3. Results

3.1. Comparison of the embryo proteome profile between stress-tolerant and -sensitive varieties

The aim of this work was to compare the embryo proteome of six rice varieties adapted to distinct environmental conditions. Total embryo proteins from rice varieties bred at IRRI with proven tolerance and sensitivity to drought, salinity and cold were separated by 2-DE along a p*I* range between 3 and 11, onto large-format gels. Similar patterns of accumulation were observed across the distinct varieties with no remarkable spot overlapping and even distribution between 14,000 and 66,000 Daltons (D) (Fig. 1).

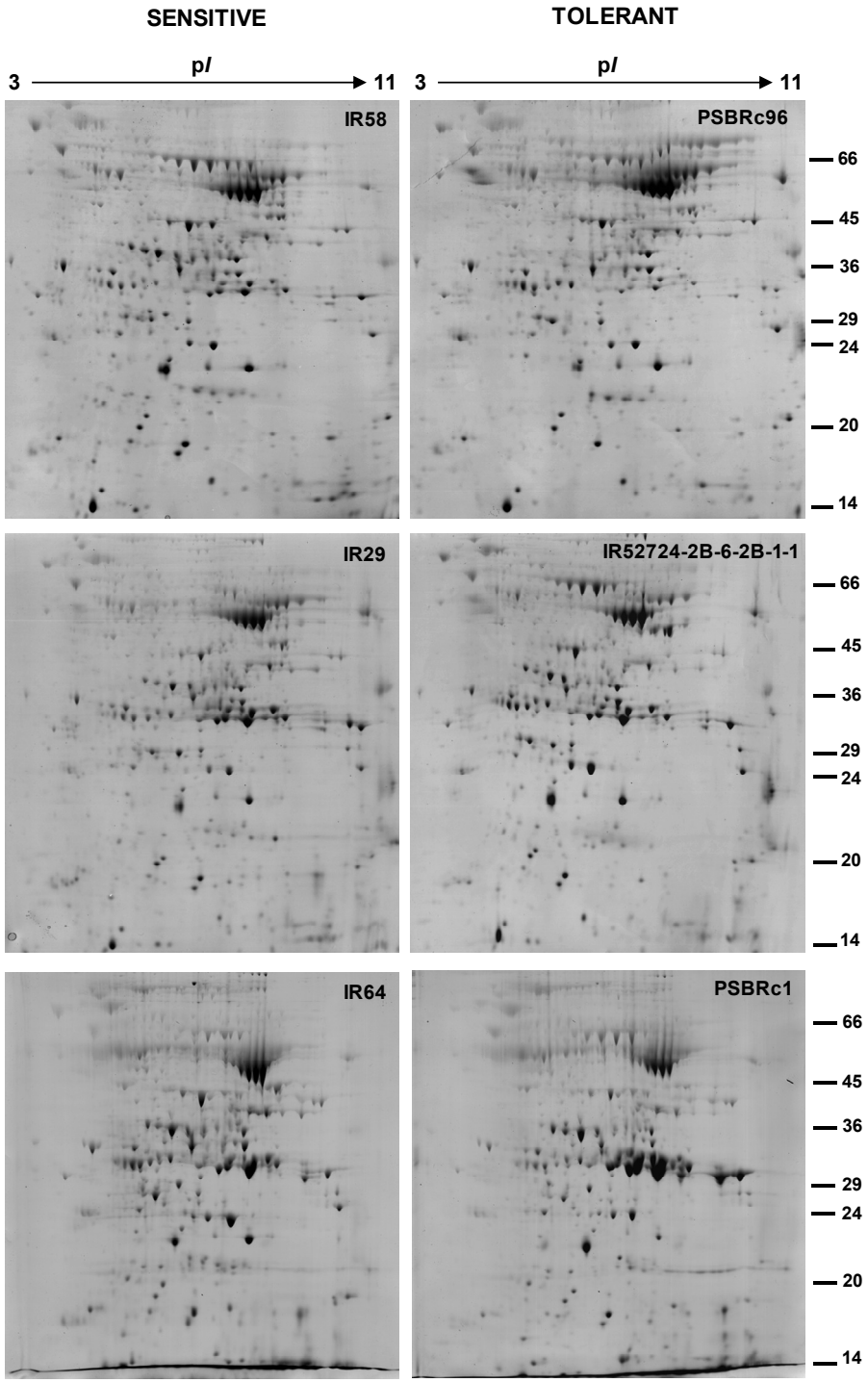


Figure 1. **2-D representative gel images of embryo proteins in distinct rice varieties**. Molecular weight (MW) markers are shown on the right (kD).

The mean number of spots detected after Coomassie staining depended on the genotype, varying between 356 and 602 spots (Fig. 2). Despite the differences in the mean number of detected spots, the percentages of matching between the different sets of sensitive and tolerant varieties were quite close (Fig. 2). The percentage of matching between 'PSBRc96'- the only *japonica* variety included in this study- and 'IR58' (an *indica* rice) was 78%, the highest registered (Fig. 2). After comparing the six embryo proteomes taking 'PSBRc96' as reference, only less than 10% of all spots were genotype-specific.

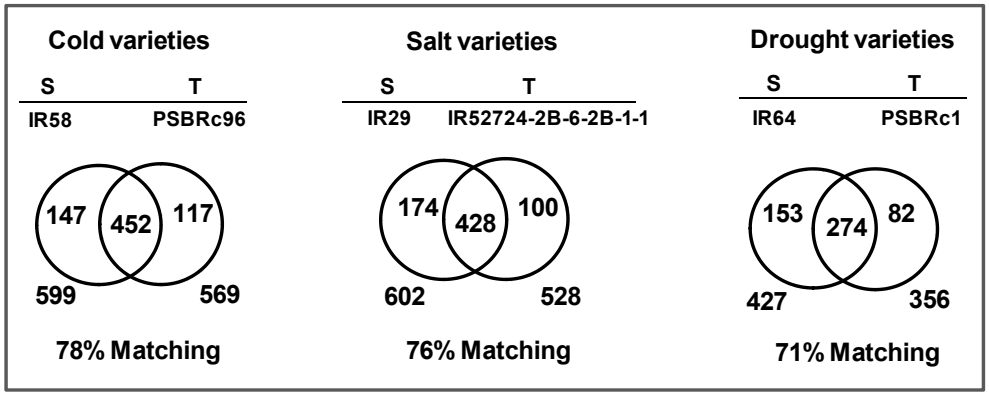


Figure 2. **Venn diagrams showing the number of matching spots between rice varieties with contrasting abiotic stress tolerance.** Abbreviations: S, Sensitive; T, Tolerant.

The assessment of technical and biological variation is essential to find relevant and meaningful changes in the comparison of different samples (Hunt *et al*., 2005; Karp *et al*., 2005). The degree of variation among rice embryo protein samples was first estimated by determining the linear relationship between normalised spot volumes either from the same protein sample, or from different protein extracts (Table I). Detailed information about the experimental design and normalisation of spot volumes is described in 'Materials and Methods'. Correlation among technical gel replicates was strong, registering values equal to, or higher than 0.96, whereas biological gel replicates exhibited slightly lower correlation

coefficients, ranging from 0.67 to 0.89 (Table I). In both cases the correlation found was significant (Student's *t*-test, $P < 0.01$). An example of the scatter plots generated by the ImageMaster 2D Platinum 5.0 software is shown in Figure 3. Correlation between most abundant proteins was lower as compared to that among less abundant proteins (Fig. 3). Coefficients of variation (CVs) were also estimated among rice protein samples. The average CV was higher between biological replicates (0.35 to 0.44) than across technical ones (0.19 to 0.24) (Table I). Protein samples from the 'PSBRc1' variety registered the highest biological variation, while samples from 'PSBRc96', the lowest (Table I). Taking all the data together, the biological variation found among the embryo protein samples was higher than the analytical variation.

Table I. ***Comparison of the analytical and biological variation across rice embryo proteins separated by 2-DE***

Average values of correlation coefficients (*r*) and coefficients of variation (CV) obtained from pairwise comparison between normalised spot volumes of distinct gel replicates.

	IR58	PSBRc96	IR29	IR52724-2B-6-2B-1-1	IR64	PSBRc1
Technical Replicates	CV= 0.21 *r*= 0.97	CV=0.23 *r*=0.98	CV=0.21 *r*=0.97	CV=0.24 *r*=0.97	CV=0.22 *r*=0.96	CV=0.19 *r*=0.98
Biological Replicates	CV=0.37 *r*=0.82	CV=0.35 *r*=0.89	CV=0.39 *r*=0.83	CV=0.41 *r*=0.68	CV=0.43 *r*=0.72	CV=0.44 *r*=0.67

3.2. Identification of rice embryo proteins

Protein spots revealing qualitative differences (presence/absence) or important changes in protein abundance across the different varieties were selected for protein identification. Selected protein spots covered diverse p*I* and M*r* ranges to broaden the representation of the rice embryo proteome as much as possible. Proteins were identified through MALDI-TOF MS, liquid chromatography tandem mass spectrometry (LC-MS/MS), or *de novo* sequencing, in the particular case of the Rab21 protein.

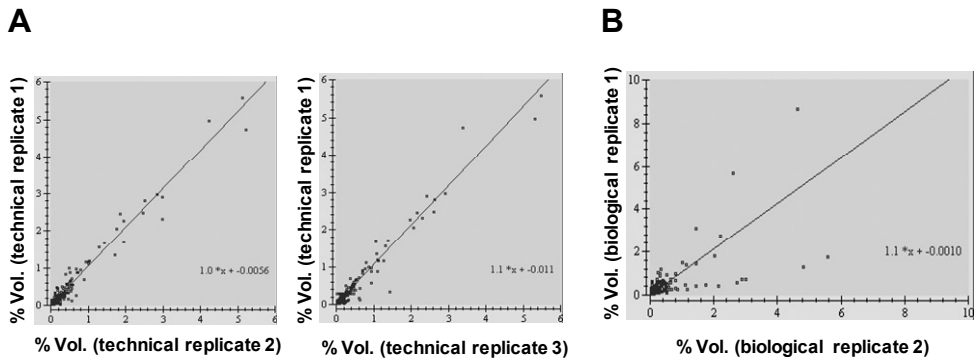


Figure 3. **Linear regression of protein amounts between 2-DE gel replicates from the 'PSBRc1' variety**. **A**, The linear relationship between normalized spot volumes (% Vol.) is shown for triplicate gels running the same protein extract. **B**, Linear regression of % Vol. between gels running different protein extracts.

From the 34 spots selected among the 'PSBRc96', 'IR52724-2B-6-2B-1-1' and 'PSBRc1' varieties, 30 could be successfully identified as rice proteins (Table II). Detailed information about the sequence of the matched peptides is provided in Supplemental Table S1. High quality mass spectra with intense peptide peaks were also obtained for the remaining four protein spots, though mass signals did not match significantly to any peptide in protein databases (data not shown). When using MASCOT for protein identification, individual ions scores > 49 indicated identity or extensive homology ($P < 0.05$) (Table II). However, in the identification of spot 79, a MASCOT score lower than 49 was accepted, since the observed M*r* and p*I* were close to the predicted, no peptide modifications were allowed in databases search and the peptide matched to a rice protein covering 13% of the sequence (Table II).

The largest proportion of identified proteins was constituted by proteins involved in nutrient reservoir activity (Table II). The next most representative class included proteins related to cellular protection against abiotic stress and involved in redox state control e.g., LEAs and a Cu-Zn superoxide dismutase (SOD) chloroplast precursor.

Table II. ***Identification of rice embryo proteins separated by 2-DE***

Protein spots with quantitative (QTV) or qualitative variations (QLV) were identified by LC-MS/MS, MALDI-TOF MS, or *de novo* sequencing. Unless indicated by letters 'a' and 'b', spot numbers correspond to numeration in cv. 'PSBRc96'. S, MASCOT score (MOWSE score is indicated with 'c'); Cov, protein sequence coverage; M*r*, molecular mass; p*I*, isoelectric point; ND, not determined.

Spot no.	Accession No.	Protein Name/Species	Identification Method	S	Cov. (%)	M*r* (kD)/p*I* observed	M*r* (kD)/p*I* predicted
13 (QLV)	P46520	Embryonic abundant protein 1 (EMP1)/ *O. sativa* (ssp. *japonica*)	LC- MS/MS	83	27	14.00/4.44	10.16/5.57
34[a] (QLV)	AAO37499	Expressed protein (calcium-binding EF-hand family protein)/ *O. sativa* (ssp. *japonica*)	LC- MS/MS	116	19	15.53/3.12	16.53/4.26
37[a](QLV)	BAC19997	Allergen RA5B precursor/ *O. sativa* (ssp. *japonica*)	LC- MS/MS	132	31	15.69/9.20	17.29/8.36
40 (QLV/QTV)	CAA32565	Preprolamine (AA-19 to 137)/ *O. sativa* (ssp. *japonica*)	LC- MS/MS	78	21	15.92/8.63	17.89/8.91
ST37[a]	CAA37850	Prolamin (precursor)/ *O. sativa* (ssp. *japonica*)	LC- MS/MS	155	21	14.97/8.00	16.93/9.17
DT32[b]	CAA32565	Preprolamine (AA-19 to 137)/ *O. sativa* (ssp. *japonica*)	LC- MS/MS	49	21	15.14/8.43	17.89/8.91
66 (QTV)	P93407	Superoxide dismutase [Cu-Zn], chloroplastic (precursor)/ *O. sativa* (ssp. *japonica*)	LC- MS/MS	315	42	18.06/5.20	21.30/5.79
79[b] (QLV)	BAB32715	Putative late embryogenesis abundant protein/ *O. sativa* (ssp. *japonica*)	LC- MS/MS	43	13	18.56/3.85	16.24/5.00
108 (QLV)	ABF95523	Mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein, putative, expressed/ O. *sativa* (ssp. *japonica*)	LC- MS/MS	321	35	20.68/5.78	18.37/6.42

(Table continues on following page)

Table II *(Continued from previous page)*

Spot no.	Accession No.	Protein Name/Species	Identification Method	S	Cov. (%)	M*r* (kD)/p*I* observed	M*r* (kD)/p*I* predicted
116 (QTV)	A2ZDX9	Water stress-inducible protein Rab21 / *O. sativa* (ssp. *indica*)	*de novo* sequencing	ND	ND	21.29/6.58	17.32/9.19
119 (QTV)	A2ZDX9	Water stress-inducible protein Rab21/ *O. sativa* (ssp. *indica)*	*de novo* sequencing	ND	ND	21.40/5.80	17.32/9.19
123 (QLV)	A2ZDX9	Water stress-inducible protein Rab21/ *O. sativa* (ssp. *indica*)	*de novo* sequencing	ND	ND	21.65/5.58	17.32/9.19
129[b] (QLV)	P29421	Alpha-amylase/subtilisin inhibitor (precursor)/ *O. sativa* (ssp. *japonica*)	LC- MS/MS	138	19	22.16/8.79	21.42/8.66
156[b] (QTV)	P0C5C8	Protein Rab24 (i.e. Thioredoxin peroxidase A)/ *O. sativa* (ssp. *indica*)	MALDI-TOF MS	50[c]	41	24.00/5.92	24.07/5.97
DT162[b] (QLV)	Q02897	Glutelin type-B 2 (precursor)/ *O. sativa* (ssp. *japonica*)	LC-MS/MS	155	7	31.67/7.68	56.04/9.11
182 (QLV)	AAS07324	Putative globulin (with alternative splicing)/ *O. sativa* (ssp. *japonica*)	LC- MS/MS	222	11	29.06/9.22	63.43/8.35
193 (QLV)	BAD81113	Putative embryonic abundant protein (group 3)/ *O. sativa* (ssp. *japonica*)	LC- MS/MS	236	27	29.20/5.94	24.48/6.07
207 (QTV)	BAD19796	Glutelin C precursor / *O. sativa* (ssp. *japonica*)	MALDI-TOF MS	174[c]	27	30.75/8.16	54.86/9.34
231[b] (QTV)	Q09151	Glutelin type-A 3 (precursor)/ *O. sativa* (ssp. *japonica*)	MALDI-TOF MS	45[c]	24	32.54/5.78	56.01/8.81
255 (QLV/QTV)	Q02897	Glutelin type-B 2 (precursor)/ *O. sativa* (ssp. *japonica*)	LC- MS/MS	347	14	33.70/8.55	56.04/9.11

(Table continues on following page)

Table II *(Continued from previous page)*

Spot no.	Accession No.	Protein Name/Species	Identification Method	S	Cov. (%)	M*r* (kD)/p*I* observed	M*r* (kD)/p*I* predicted
263 (QTV)	Q02897	Glutelin type-B 2 (precursor)/ *O. sativa (*ssp. *japonica)*	MALDI-TOF	121	15	34.16/8.20	56.04/9.11
266[b] (QTV)	P14614	Glutelin type-B 4 (precursor)*/* *O. sativa* (ssp. *japonica*)	MALDI-TOF MS	123[c]	24	34.26/7.72	56.82/9.00
284[a] (QLV)	AAS07324	Putative globulin (with alternative splicing)*/* *O. sativa* (ssp. *japonica*)	MALDI-TOF MS	101[c]	24	35.41/9.29	63.43/8.35
427[b] (QLV)	AAS07324	Putative globulin (with alternative splicing)/ *O. sativa* (ssp. *japonica*)	LC- MS/MS	183	11	51.19/6.71	63.43/8.35
434 (QLV)	BAD10058	Putative aminoacylase/ *O. sativa* (ssp. *japonica*)	LC- MS/MS	141	10	52.20/5.61	49.82/5.88
436[a] (QLV)	AAS07324	Putative globulin (with alternative splicing)/ *O. sativa* (ssp. *japonica*)	MALDI-TOF MS	80[c]	35	52.82/6.99	63.43/8.35
456[b] (QTV)	AAO37963	Putative globulin/ *O. sativa* (ssp. *japonica*)	MALDI-TOF MS	147[c]	33	52.00/6.10	52.13/6.79
468 (QLT)	P45960	Tubuline beta-4 chain/ *O. sativa* (ssp. *japonica*)	MALDI-TOF MS	143	46	57.56/4.26	50.29/4.74
496 (QTV)	Q0DEV5	Granule-bound starch synthase I, chloroplastic/ amyloplastic / *O. sativa* (ssp. *japonica*)	MALDI TOF MS	51[c]	36	62.73/5.67	66.48/8.34
534 (QLV)	AAS07324	Putative globulin (with alternative splicing)/ *O. sativa* (ssp. *japonica*)	LC- MS/MS	219	13	69.44/7.92	63.43/8.35

[a] Spot protein picked from the salt tolerant variety 'IR52724-2B-6-2B-1-1' (ST). [b] Spot protein picked from the drought tolerant variety 'PSBRc1' (DT).
[c] MOWSE score obtained with Protein Prospector software. MASCOT score S > 47 indicates identity or extensive protein homology (P< 0.05).
MASCOT score (S) is a probability-based MOWSE scoring defined as S= -10*Log (P); P is the probability that the observed match is a random event.
Spots 40, ST37 and DT32 matched in 'PSBRc96', 'IR52724-2B-6-2B-1-1' and 'PSBRc1', respectively.
ExPASy prediction tools were used to compute theoretical p*I*/MW.

The remaining proteins were implicated in diverse cellular functions, such as signal transduction (calcium-binding EF-hand family protein), protein degradation (putative aminoacylase), cytoskeleton assembly (tubuline beta-4 chain) and transporter activity (mitochondrial import inner membrane translocase subunit Tim17) (Table II). A seed allergenic protein belonging to the protease inhibitor I6 (cereal trypsin/ alpha-amylase inhibitor) family was also identified.

After comparing our 2-DE embryo reference map (Fig. 4) with that reported by Woo *et al*. (2002) and Fukuda *et al*. (2003), spots 16 and 154 matched to spots identified by these authors as the EMP1 and Rab24 proteins, respectively (Table III). Furthermore, we also identified EMP1 in spot 13, closely positioned to spot 16, and Rab24 in spot 156, closely positioned to spot 154 (Table II; Fig. 4). We therefore propose that spots 16 and 154 may constitute putative additional forms of the EMP1 and Rab24 proteins, correspondingly.

3.3. Classifying varieties according to their proteomic profiles

As logarithmic (log_{10}) transformation improves Gaussian distribution of data (Hunt *et al*., 2005; Karp *et al*., 2005), normalised spot volumes were log transformed before establishing any statistical analyses (Supplemental Fig. S1). We selected eighty three reproducible spots matching simultaneously across the six embryo proteomes for further statistical analyses. The distribution's range of the normalised volumes of the 83 selected spots was compared with that presented by all detected spots in each variety, to ensure that no bias occurred during the selection procedure. Taking as example the protein spots in 'IR58' and 'PSBRc96', it was observed that the distribution of protein abundance of the 83 selected spots was similar to that presented by all detected spots (Fig. 5). Identical results were obtained for all the other varieties (Supplemental Fig. S2).

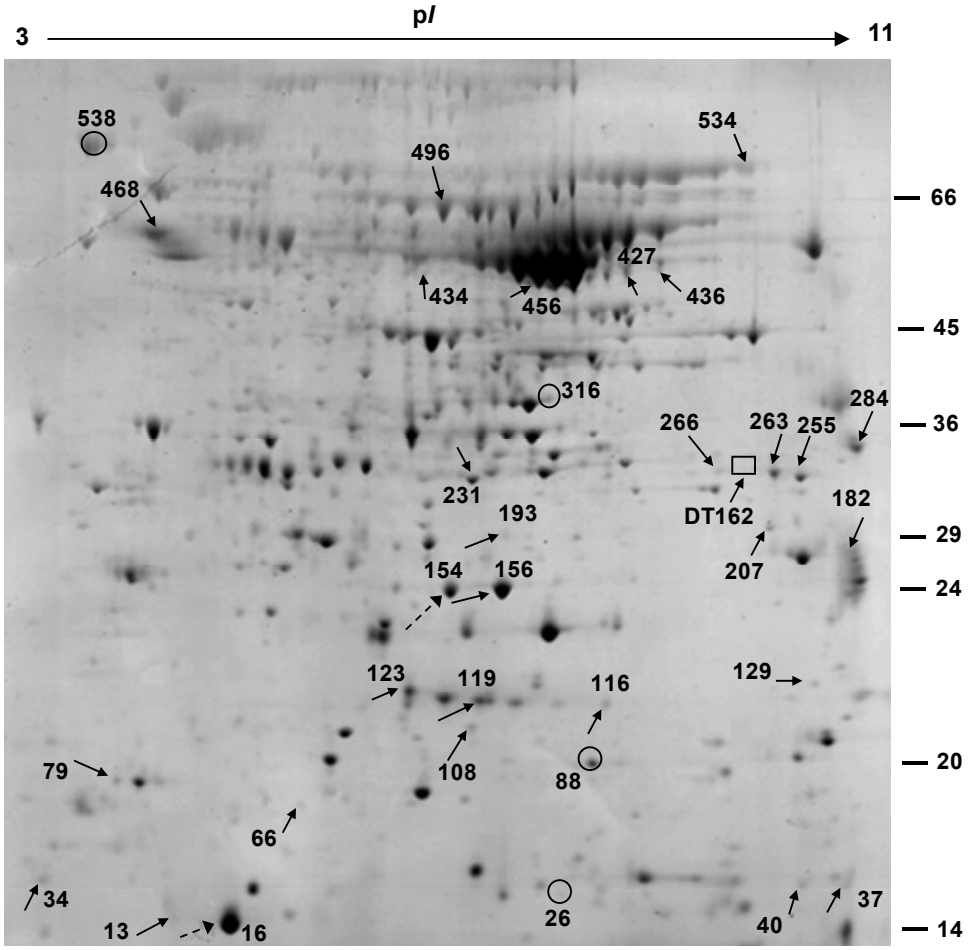


Figure 4. **2-DE reference map of rice embryo proteins (cv. 'PSBRc96')**. Spots successfully identified are indicated by arrows, whereas those that could not be identified are surrounded by circles. The squared box signs the position of a protein spot identified in cv. 'PSBRc1'. Arrows with open lines indicate proteins that were not identified in this work, but that match to spots identified by MS in other reported rice embryo maps (see Table III). MW markers are shown on the right (kD).

Table III. ***Protein spots matching across our 2-DE reference map and other reported for the rice embryo proteome***

Spots 16 and 154 in our reference map are proposed to match with the spots indicated below. The corresponding experimental (exp.) molecular mass (M*r*) and isoelectric point (p*I*) can be compared to those obtained by Woo *et al*. (2002) and Fukuda *et al*. (2003). The predicted M*r* and p*I* are also indicated.

Present Work		**Woo *et al*. (2002)**		**Fukuda *et al*. (2003)**		**Protein identified**	
Spot no.	M*r* (kD)/p*I* exp.	Spot no.	M*r* (kD)/p*I* exp.	Spot no.	M*r* (kD)/p*I* exp.	Protein identity	M*r* (kD)/p*I* predicted
16	14.0/4.88	46	13.0/5.26	107	13.0/5.2	EMP1 protein/ P46520	10.16/5.57
154	24.0/5.7	34	27.5/5.85	62	28.0/5.8	Protein Rab24/ P52573	24.07/5.97

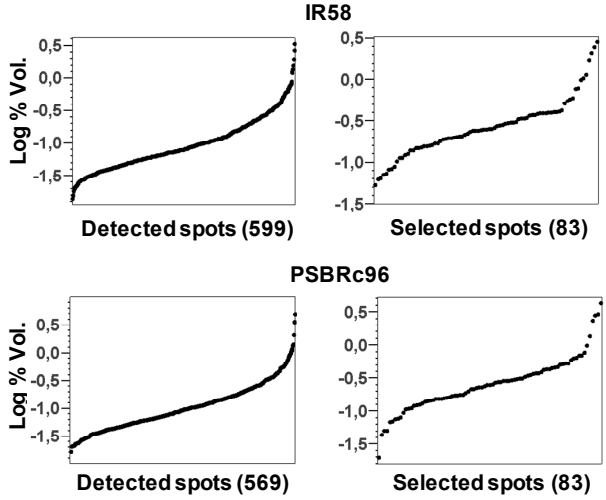


Figure 5. **Comparison of the distribution of protein abundance across all spots detected in 'IR58' and 'PSBRc96', and that exhibited by 83 proteins randomly selected in each variety**. Dot-plot graphs show protein abundance (log % Vol.) sorted in ascending order.

Hierarchical clustering and multidimensional scaling analysis were used to decipher the relationships between the distinct varieties, based on their embryo proteome profiles (Fig. 6). Cluster analysis showed that the

cold- and drought-sensitive varieties (‘IR58’ and ‘IR64’, respectively) were closely related, whereas their contrasting tolerant genotypes (‘PSBRc96’ and ‘PSBRc1’, accordingly) were more divergent, grouping into separate clusters (Fig. 6A). On the other hand, the salt-sensitive (‘IR29’) and the salt-tolerant (‘IR52724-2B-6-2B-1-1’) genotypes clustered together, being proximal to the cold- and drought-sensitive varieties (Fig. 6A). ‘PSBRc96’ was the most divergent genotype, clustering separately from all the other varieties (Fig. 6A).

Hierarchical clustering besides allowing the classification of varieties was useful in the clustering of proteins with similar expression profiles. The 83 embryo protein spots were distributed into four major clusters (Fig. 6A). The first cluster included proteins with high levels of expression, like seed storage proteins, though globulins and glutelins were then linked into distinct subgroups (Fig. 6A). The second major cluster grouped proteins with the lowest levels of abundance, while the third included again abundant proteins such as a putative Rab24 isoform (Fig. 6A). The fourth main cluster was then subdivided into many smaller groups, represented by proteins with more heterogeneous expression patterns across the six varieties e.g., two distinct forms of the Rab21 protein. A graphical representation of the proximities between the different varieties was obtained by multidimensional scaling analysis (Fig. 6B). This approach allowed a more detailed analysis of the pairwise relationships between the distinct varieties. Hence, the most distantly related varieties were ‘PSBRc96’ and ‘IR29’, whereas the closest were ‘IR58’ and ‘IR64’. Interestingly, it was also observed that the salt-sensitive ‘IR29’ and salt-tolerant ‘IR52724-2B-6-2B-1-1’ genotypes were not only closely related as indicated by HCA, but were also close to the drought-sensitive (‘IR64’) and drought-tolerant varieties (‘PSBRc1’), respectively (Fig. 6B). Moreover, a subtle separation between tolerant and sensitive genotypes could be observed in the second-dimension of the plot (Fig. 6B).

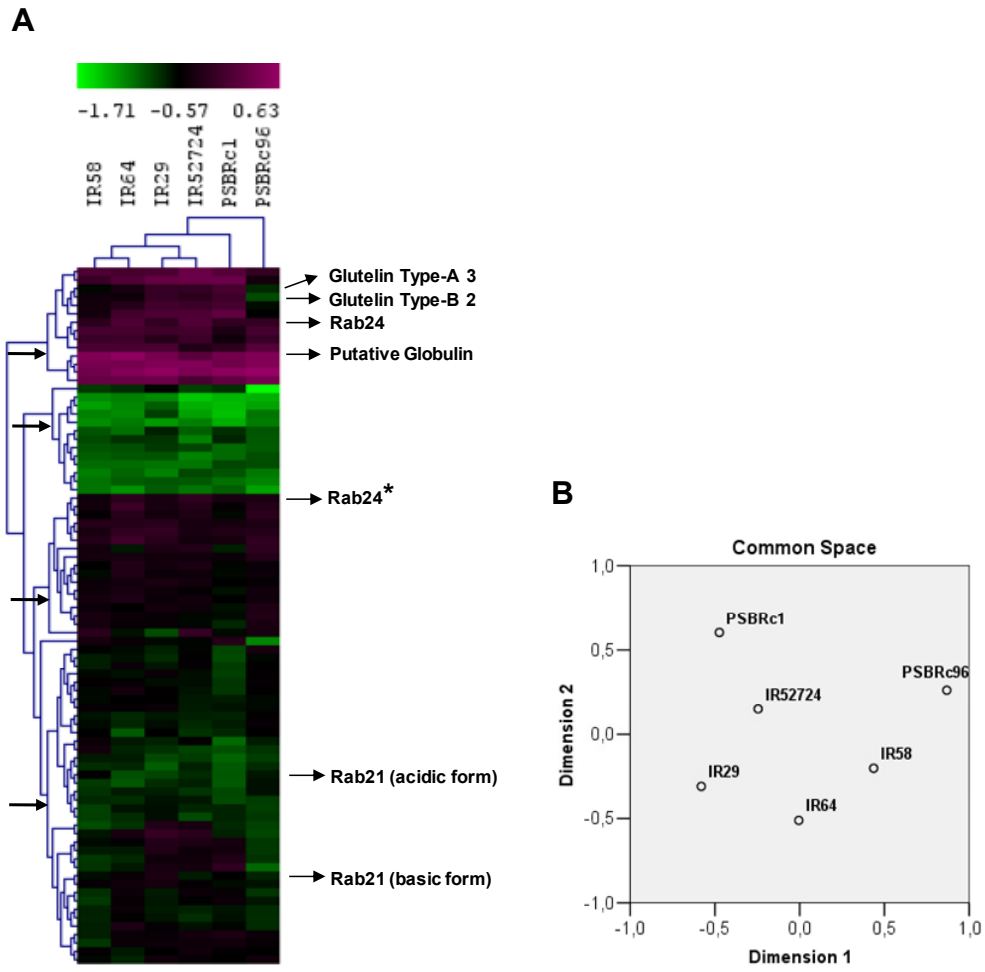


Figure 6. **Analysis of the relationship between distinct rice varieties with basis on their protein profiles**. **A**, Hierarchical clustering analysis based on 83 representative embryo protein spots commonly expressed across the six varieties. Node heights represent euclidean distances i.e. dissimilarity between objects (proteins or varieties). Vertical axis: clustering of individual proteins; horizontal axis: clustering of varieties. The higher the node height, lower the similarity between objects. Arrows on the left indicate 4 main clusters of proteins. Asterisk signs a protein that was not identified in this work but that may putatively correspond to a Rab24 isoform (see Table III). Magenta brighter colours represent high levels of protein expression; brighter green colours represent low levels. **B**, Multidimensional scaling analysis. Similar pairs of objects are represented by two points that are close in the two-dimensional plot.

3.4. Changes in protein abundance across the embryo proteome from varieties with contrasting stress tolerance

The differences in the means of protein abundance across the embryo proteomes was analysed for each abiotic stress set of varieties with contrasting stress tolerance (Student's *t*-test, $P < 0.05$). The proteomes of 'IR64' and 'PRSBRc1' - the varieties with contrasting response to drought - revealed a significant difference in protein abundance (Student's *t*-test, $P < 0.05$). Meanwhile, when comparing the proteomes of 'IR29' *vs.* 'IR52724-2B-6-2B-1-1' (salt set) or 'IR58' *vs.* 'PSBRc96' (cold set) no significant differences were found. The changes within each group of genotypes with contrasting abiotic stress tolerance were identical when analysing all the matching spots, or only the 83 proteins, despite that different *P* values were obtained (i.e 0.022 and 0.042, respectively, for the drought set; data not shown for the other varieties). Individual spots with significant fold-changes ratios ≥ 2.0 were also checked across the different sets of varieties with contrasting stress responses (Fig. 7). A few spots with more dramatic changes in protein accumulation e.g., spots 255 and 496 (Fig. 7), were further selected for protein identification, revealing to fall in the category of proteins related to nutrient reservoir activity, that will be next referred.

3.5. Differentially expressed proteins

3.5.1. Proteins related to nutrient reservoir activity

Granule-bound starch synthase I (GBSS-I) (spot 496) was definitely the identified protein with the most significant difference in accumulation not only between 'IR29' and 'IR52724-2B-6-2B-1-1' (Fig. 7), but also across the six varieties (Fig. 8). Glutelins were another class of proteins with most significant changes in the embryo proteome, exhibiting a complex pattern of accumulation across the distinct varieties (Fig. 8). Despite the diversity of glutelins observed in the rice embryos (type-A 3, -B 2, -B 4 and C-type) and of their accumulation profiles, they were consistently less abundant

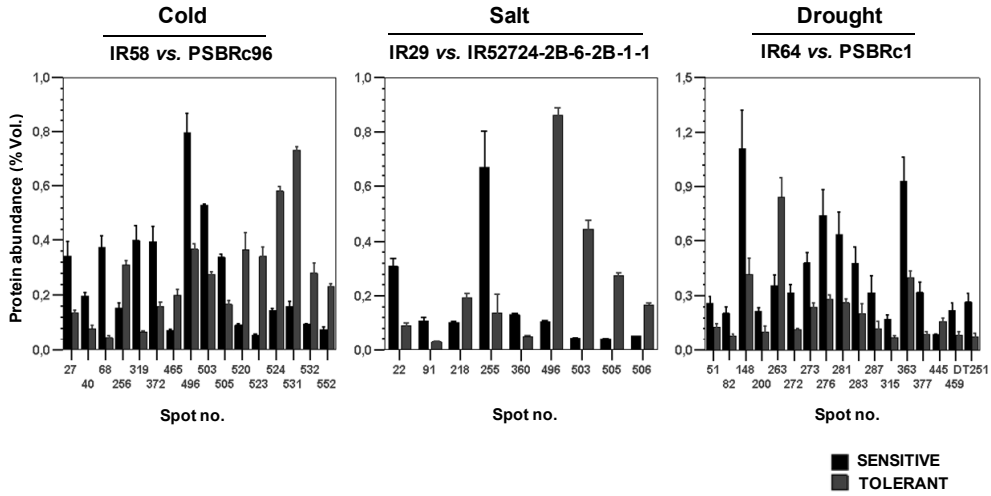


Figure 7. Analysis of protein spots with significant quantitative variation between sensitive and tolerant varieties. Spots with a significant (Student's *t*-test, $P < 0.05$) fold-change in protein abundance ≥ 2.0 are shown across each set of varieties with contrasting abiotic stress tolerance. Sixteen spots out of 452 spots presented a significant difference in abundance between 'IR58' and 'PSBRc96' (cold set), while 9 spots out of 428 matching spots between 'IR29' and 'IR52724-2B-6-2B-1-1' (salt set) had a significant variation. When comparing 'IR64' and 'PSBRc1' (drought set), 17 out of 274 matching spots displayed a significant difference in accumulation. See Fig. 2 to check the mean number of matching spots across the distinct varieties. Error bars show means of 3 replicates ± standard deviation. All spot numbers correspond to numeration in 'PSBRc96', except spot DT251 that corresponds to numeration in 'PSBRc1'.

in 'PSBRc96' than in any other variety (Fig. 8A; see also Fig. 6A). Glutelins showed similar levels of accumulation in the embryos of 'IR29' and 'IR52724-2B-6-2B-1-1', whereas they were in general less accumulated in 'IR64' than in 'PSBRc1' (Fig. 8A). The only glutelin with significant changes between 'IR64' and 'PSBRc1' was glutelin type-B 2, being more accumulated in 'PSBRc1' - the drought tolerant genotype - than in 'IR64', the sensitive one (spot 263; Fig. 8A). Glutelin type-B 2 was further identified in two more spots, i.e. 255 and DT162, with an identical M*r* and p*I* close to spot 263 (Table II; Fig. 8B). Spot 255 had a significant difference in accumulation between the embryos of 'IR29' and 'IR52724-2B-6-2B-1-1' (Fig. 7), besides exhibiting qualitative changes between 'IR58' and 'PSBRc96' (Fig. 8B), though it was not detected neither in 'IR64' nor 'PSBRc1'. Meanwhile, spot DT162 was detected only in 'PSBRc1' (Supplemental Table S2).

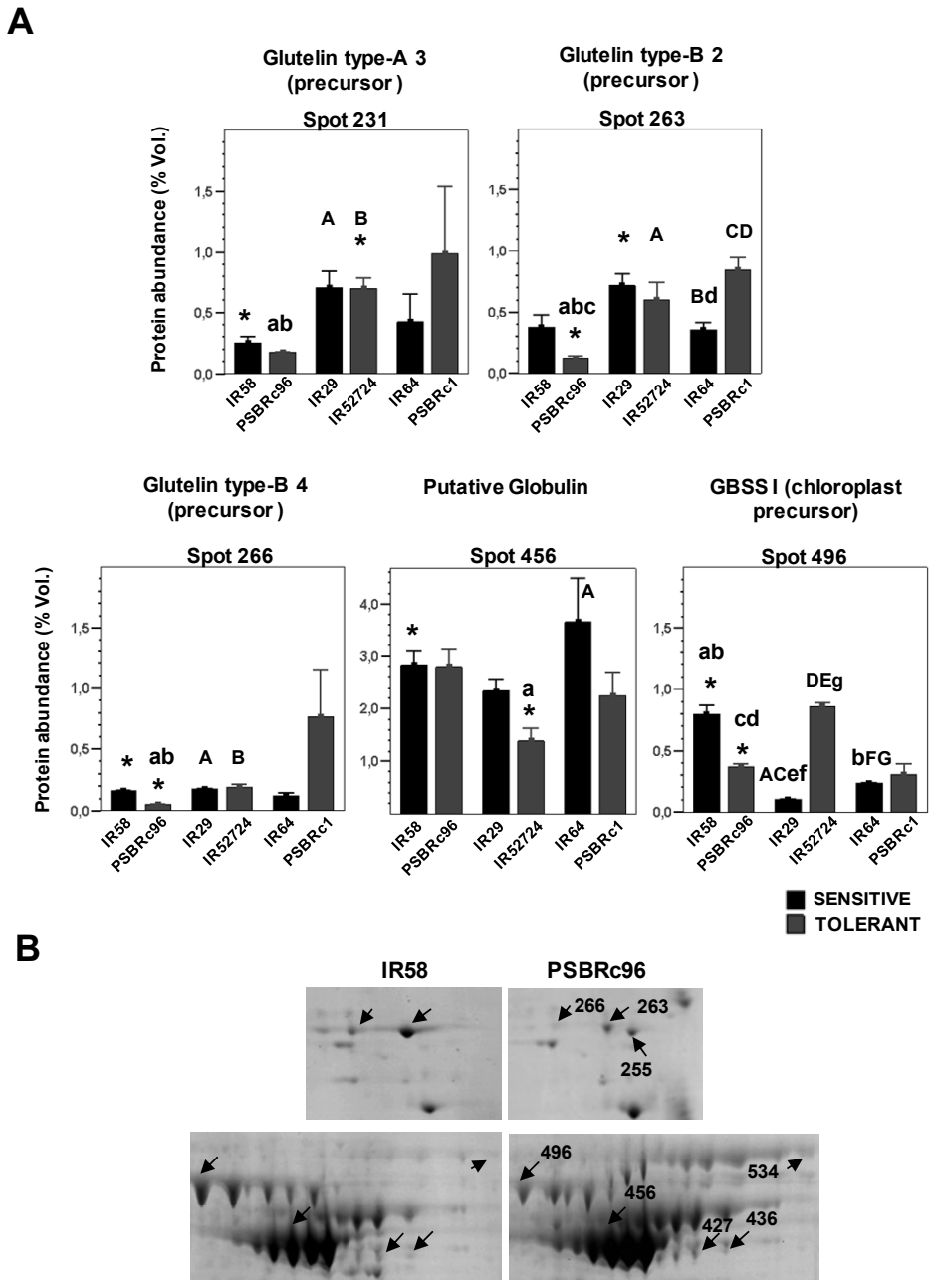


Figure 8. **Accumulation pattern of proteins related to nutrient reservoir activity in the seed embryo of distinct rice varieties**. **A**, Quantitative variations in the abundance of glutelins, a putative globulin and GBSS-I. Significant differences in the means of protein abundance between varieties are indicated by asterisks and uppercase/lowercase letters (Student's *t*-test, $P < 0.05$). Error bars show means of 3 replicates ± standard deviation. **B**, Expression pattern of glutelins (spots 263, 255 and 266), globulins (spots 427, 436, 456 and 534) and GBSS-I (spot 496) exemplified in 'IR58' and 'PSBRc96' varieties. See also Supplemental Table S2.

The abundance level of a putative globulin (spot 456) in the embryo of the different varieties was much higher than that presented by glutelins, being particularly abundant in 'IR64' (Fig. 8). The accumulation level of this putative globulin contrasted to that observed in other globulin forms with alternative splicing, that were much less abundant (spots 427, 436 and 534) (Fig. 8B). In fact, while spot 456 was detected in all varieties, displaying significant changes in protein abundance (Fig. 8A) the rest of globulin forms could not be detected in all genotypes (Supplemental Table S2).

3.5.2. Proteins involved in cellular protection against abiotic/biotic stress and in redox state control

LEA proteins may fall into nine groups based on their sequence similarity and conserved domains, according to a recent and revised classification (Bies-Etheve *et al*., 2008). Following this classification, we were able to identify LEAs included in four distinct groups, i.e. embryonic abundant protein 1 (EMP1) belonging to Group 1 (spot 13), a putative LEA with similarity to LEA14 (AtLEA7-1) from *Arabidopsis* (Group 7; spot 79), Rab21 belonging to Group 2 (also known as dehydrins) (spots 116, 119 and 123), and a putative embryonic abundant protein similar to AtLEA3-1 from *A. thaliana* (Group 3; spot 193). As mentioned in section 3.2, we propose that spot 16 with an identical M*r* to spot 13, but a slight more basic p*I*, could correspond to a putative additional form of the EMP1 protein (Table III, Fig. 9B).

The pattern of accumulation of the distinct LEAs in the rice mature embryo was quite diverse (Fig. 9). While spot 16 was most abundant in all rice varieties, spot 13 was barely detected in 'IR58' and 'PSBRc96' (Fig. 9). Rab21 was the most abundant identified LEA protein, although distinct forms of the protein (spots 116, 119 and 123) exhibited different levels of abundance (Fig. 9). The changes in protein abundance displayed

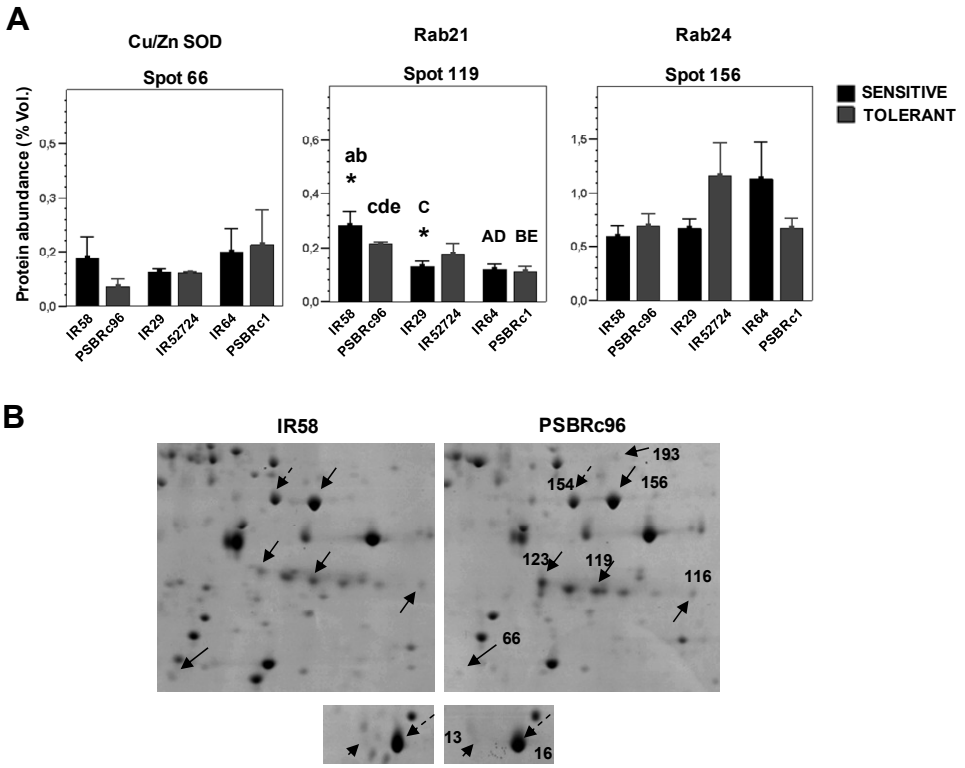


Figure 9. **Expression pattern of proteins involved in cellular stress protection**. **A**, Changes in protein abundance of LEA Rab21 and antioxidant scavenging proteins i.e. Cu-Zn SOD and Rab24. Significant differences in the means of protein abundance after pairwise comparisons across the 6 varieties are indicated by asterisks and uppercase/lowercase letters (*t*-test, $P < 0.05$). Error bars show means of 3 replicates ± standard deviation. **B**, Comparison of the expression pattern in 'IR58' and 'PSBRc96'. A putative LEA (spot 193) was detected only in 'PSBRc96' The presence of a more basic form of the EMP1 protein (spot 13) was almost undetectable in 'IR58' and 'PSBRc96', whereas a putative additional form (spot 16) was most abundant. The Rab24 (spot 156) and Cu-Zn SOD (spot 66) proteins displayed different levels of accumulation. See also Supplemental Table S2 for details of the qualitative differences of these proteins in the rest of varieties under study.

by spot 119 were significant across the embryos of most varieties (Fig. 9A), contrasting with those changes of spots 116 that were not significant (data not shown), while spot 123 was detected only in half of the genotypes (Supplemental Table S2). Spots 79 (putative LEA with similarity to LEA14-A) and 193 (putative embryonic abundant protein similar to AtLEA3-1) were

very low abundant, with similar levels of expression to spot 13, presenting only qualitative variations (Fig. 9B; Supplemental Table S2).

A bifunctional alpha-amylase/subtilisin inhibitor precursor (spot 129; see Fig. 4) involved in plant defense displayed qualitative changes (see Supplemental Table S2), and it was one of the proteins with a lower mean abundance (about 0.077±0.040).

Two antioxidant scavenging proteins were identified exhibiting a differential pattern of accumulation across the distinct rice embryos, namely the Cu-Zn SOD chloroplast precursor (spot 66) and the thioredoxin peroxidase A/Rab24 protein (spot 156) (Table II; Fig. 9). We suggested before in section 3.2 that spot 154 could represent a putative additional form of the Rab24 protein (see Table III). The Cu-Zn SOD protein showed similar levels of accumulation either between 'IR29' and 'IR52724-2B-6-2B-1-1', or between 'IR64' and 'PSBRc1', but it was more abundant in 'IR58' than in 'PSBRc96' with an approximate 2.5-fold change, though the difference was not significant (Fig. 9A). The levels of accumulation of the Rab24 protein (spot 156) were higher as compared with Cu-Zn SOD, and presented a quite different pattern (Fig. 9A).

3.5.3. Proteins implicated in other cellular processes

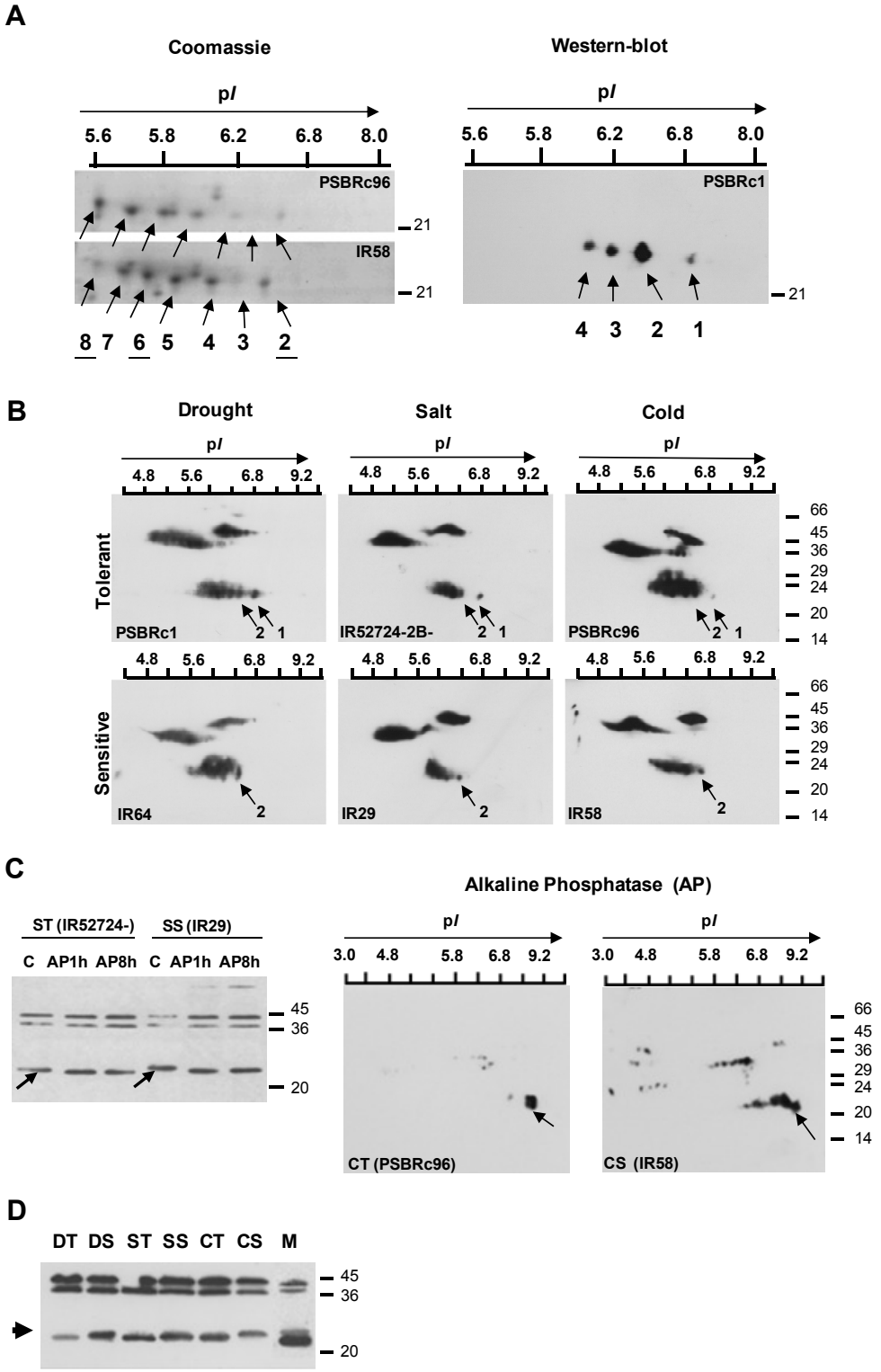
Tubulin beta-4 chain (spot 468; see Fig. 4) was considerably more abundant in 'PSBRc96' than in the other varieties (data not shown). It was among the proteins with a 3-fold change in abundance when comparing 'IR58' and 'PSBRc96', although this difference was not significant due to high variability in the expression (data not shown). Among the proteins with a lower level of abundance (i.e. with normalised spot volumes equal or lower than 0.1) and displaying qualitative variations across the six varieties, we could identify a calcium-binding EF-hand family protein (spot 34), a mitochondrial import inner membrane translocase subunit (spot 108) and a putative aminoacylase protein (spot 434). Their mean relative values of

accumulation were about 0.114 ± 0.015, 0.084 ± 0.036 and 0.032 ± 0.014, respectively. The qualitative variations presented by these proteins are summarised in Supplemental Table S2.

3.6. Differences in the phosphorylation pattern of the LEA protein Rab21 between tolerant and sensitive varieties

Three forms of Rab21 (spots 116, 119 and 123) with an approximate molecular mass of 21 kD and distinct isoelectric point were identified by MS (Fig. 4; Table II). Up to seven well resolved spots in the p*I* range 5.6 to 6.6 were observed in the 2-DE gels stained with CBB (Fig. 10A). This expression pattern of Rab21 was identical to that described for the Rab17 protein, the homologous protein in maize (Sánchez-Martínez *et al*., 1986; Goday *et al*., 1988; Vilardell *et al*., 1990). The protein spots of Rab17 found in the maize mature embryos with identical M*r* but different p*I* are due to post-translational phosphorylation occurring at the serine cluster region of the protein (Goday *et al*., 1988; Plana *et al*., 1991). To test the putative phosphorylation of Rab21 and compare its expression pattern across the distinct rice embryos, a polyclonal antibody raised against Rab17 (Goday *et al*., 1988) was used for cross-reaction. After 2-DE Western-blot, an

Figure 10. **Expression pattern of Rab21 in the embryo of rice varieties with contrasting abiotic stress tolerance**. **A**, **On the left**: seven distinct forms of Rab21 can be observed on 2-DE gels stained with CBB. Underlined numbers indicate spots identified by MS; **On the right**: an additional form (spot number 1) was detected by Western-blot on protein extracts separated by 2-DE, using 18-cm IPG strips (pH 3-11, NL). **B**, Immunodetection of Rab21 on rice embryo protein extracts separated by 2-DE, using 7-cm IPG strips (pH 3-11, NL). The additional form of Rab21 was detected only in the tolerant genotypes. **C**, Immunodetection of Rab21 on embryo protein extracts treated with alkaline phosphatase (AP); **On the left**: the band-shift of the 21-kD protein band (Rab21) after AP treatment was more pronounced in SS as compared with ST; **On the right**: a shift towards the basic end of the gel was observed on the 21-kD protein spots after AP, either in 'PSBR96' or 'IR58'. Compare with untreated embryos on B. Some less basic spots (p*I* close to 6.8) were still observed in 'PSBRc96' as compared with 'IR58', after AP treatment. **D**, Immunodetection of Rab21 in the embryo of distinct rice varieties. DT, Drought Tolerant; DS, Drought Sensitive; ST, Salt Tolerant; SS, Salt Sensitive; CT, Cold Tolerant; CS, Cold Sensitive. A maize embryo (M) extract was included as control.


A
Coomassie
Western-blot
pI
5.6 5.8 6.2 6.8 8.0
PSBRc96
IR58
PSBRc1
21
8 7 6 5 4 3 2
4 3 2 1
B
Drought
Salt
Cold
4.8 5.6 6.8 9.2
Tolerant
Sensitive
PSBRc1
IR52724-2B-
PSBRc96
IR64
IR29
IR58
66 45 36 29 24 20 14
C
Alkaline Phosphatase (AP)
ST (IR52724-)
SS (IR29)
C AP1h AP8h C AP1h AP8h
3.0 4.8 5.8 6.8 9.2
CT (PSBRc96)
CS (IR58)
45 36 20
D
DT DS ST SS CT CS M

additional and more basic form (spot 1; p*I* close to 6.8) was detected only in the tolerant varieties, suggesting some difference in the phosphorylation status of the protein across sensitive and tolerant genotypes (Fig. 10B; see also 10A). Total embryo protein extracts were treated with alkaline phosphatase (AP) to confirm on one hand that the several forms of Rab21 were due to post-translational phosphorylation as reported for Rab17; and on the other hand to check the differences in the phosphorylation status of the protein between tolerant and sensitive genotypes. Embryo proteins were separated either by 1-DE or 2-DE and analysed by Western-blot (Fig. 10C). Phosphorylation may result in protein migration at a higher M*r,* since phosphate residues modify the SDS coating of the proteins thus retarding their migration in the gel (Peck, 2006). Therefore, we analysed if the target protein, the 21-kD polypeptide, presented any band shift after AP treatment. A faster electrophoretic mobility of Rab21 was observed in different embryo extracts treated with AP, when compared with untreated extracts (Fig. 10C). The band shift was more accentuated in the embryos of the sensitive variety ('IR29') than in the embryos of the tolerant one ('IR52724-2B-6-2B-1-1') (Fig. 10C). However, the differences after AP treatment were more dramatic on the embryo extracts separated by 2-DE (Fig. 10C). There was an important change in the p*I* distribution of the Rab21 protein, with a shift towards the basic end of the gel and disappearance of the most acidic forms, either in the embryos of the tolerant ('PSBRc96') or the sensitive ('IR58') genotype. While some less acidic forms were still detected in the embryos of the sensitive genotype, such forms almost completely disappeared in the embryos of the tolerant one, after treatment with AP (Fig. 10C).

Band gel mobility differences in Rab21 were also observed in embryo protein extracts separated by 1-DE, across the different rice varieties (Fig. 10D). Rab21 presented a slightly faster migration rate in tolerant varieties as compared to sensitive genotypes (Fig. 10D). The difference was even more pronounced between 'PSBRc96' and 'IR58', the cold-related varieties (Fig. 10D). Taken together, the results indicate important differences in the

phosphorylation status of Rab21 across the rice embryos of varieties with contrasting responses to abiotic stress.

4. Discussion

The mechanisms underlying desiccation tolerance in seed embryos can elucidate about the components required for water-deficit tolerance in plants and contribute for the identification of candidate genes for further crop improvement (Bartels *et al*., 1996; Jensen *et al*., 1996; Campalans *et al*., 1999; Buitink *et al*., 2006; Sreenivasulu *et al*., 2007). Desiccation-tolerant tissues (or organisms) are also considered to be drought-tolerant, despite that the inverse is not true, probably because desiccation tolerance requires additional protective factors as compared to those under milder drought stress conditions (Moore *et al*., 2008; Caramelo and Iusem, 2009). In this study, we compared the embryo proteome of rice varieties with contrasting abiotic stress adaptation, aiming to get further clues on the molecular mechanisms underlying water-stress tolerance.

To validate the differences found when comparing the proteomes of the distinct rice varieties, we estimated the variation among the distinct rice embryo samples and the variation associated to the experimental procedures. The variation between 2-D gel replicates running rice proteins from different extracts was higher than that across running the same protein sample. A higher variation at the biological, than at the analytical level, was registered in other plant proteomic studies e.g., in legume *Medicago truncatula* (Asirvatham *et al*., 2002), holm oak (Jorge *et al*., 2005; 2006) and *Pinus radiata* (Valledor *et al*., 2008). Parker and collaborators (2006) reported that 93% to 95% of the stressed rice proteins from leaf lamina separated by 2-DE were within a coefficient of biological variation of about 0.7. These values were higher than those found among the embryo protein samples (0.35 to 0.44) (Table I). It is not surprising if we consider that biological variability among rice seedlings exposed to salt treatment (Parker *et al*., 2006) should be higher than across non-treated samples, as in the

case of the embryos from rice mature seeds. On the other hand, the biological variation was not the same across the distinct rice genotypes, this being highest in 'PSBRc1' and lowest in 'PSBRc96'. The higher variability registered among some of the most abundant proteins, could account for the differences in the biological variation across the distinct varieties. For instance, in 'PSBRc1', glutelins type A-3 and B-4 - two most abundant proteins - exhibited high levels of variation, besides accumulating to a higher extent in the embryos of this variety as compared to the rest of genotypes. A higher degree of variation among more abundant proteins was previously reported by other authors in maize, holm oak and pine (de Vienne *et al*., 2001; Jorge *et al*., 2005; Valledor *et al*., 2008). The analytical CVs were lower than biological CVs, and their values ranged between 0.19 and 0.24 across the different genotypes. Parker *et al*. (2006) obtained similar analytical CVs (0.26) for rice leaf lamina proteins separated by 2-DE. In addition, the correlation found between the 2-D gel replicates running the same embryo protein extract was high and significant (≥ 0.96; $P < 0.01$). Hence, the strong reproducibility exhibited by the different types of 2-D gel replicates proved to be appropriate for further comparison analyses across the embryo proteome of the distinct varieties.

In the comparison of *indica vs. japonica* full-length cDNAs, 45.8% of the homologous pairs presented important differences at protein level due to single nucleotide polymorphisms (SNPs) and insertions/deletions (Indels), among other variations (Liu *et al*., 2007). However, we found a high similarity in the embryo proteome between the *japonic*a 'PSBRc96' and *indica* 'IR58' rice varieties. The number of proteins expressed in common by these two genotypes with contrasting cold-response was even the highest in pairwaise comparisons between varieties with differential stress tolerance (i.e. 452 spots corresponding to 78% of the proteins detected in both varieties). Very similar percentages of matching were obtained in the comparison analyses across the rest of varieties, all belonging to *indica subspecies,* i.e. 76% between salt-tolerant *vs.* -sensitive, and 71% between drought-tolerant *vs.* -

sensitive. Wang *et al*. (2008) when comparing the mature embryo proteome of an elite rice hybrid (*LYP9*) to its parental lines *9311 (indica* ssp*.)* and *P64S* (mixed genetic background), observed not only similar expression profiles across the hybrid and its parents as expected, but also between the unrelated parental lines. Furthermore, the mean number of embryo proteins detected by Wang *et al*. (2008) varied between 427 and 478, a similar range to that found across the varieties under study, i.e. 352 to 602 embryo proteins. Therefore, in our study, as reported by Wang and collaborators (2008), the mean number of detected proteins depended on the genotype and the percentages of matching were quite similar among genotypes with very different genetic backgrounds.

Chevalier *et al*. (2004) verified that the classification of eight *Arabidopsis* ecotypes based on hierarchical clustering analysis was almost identical when using the whole proteome dataset, or a subset of 25 most abundant (major) proteins. This subset defined a total of 49 differentially matching spots across the different *Arabidopsis* ecotypes. In our work, we selected 83 representative spots expressed in all varieties irrespective of their abundance, for further statistical purposes. But first, it was confirmed that no bias occurred during the spot selection procedure. The pattern of distribution of protein abundance exhibited by all detected spots in each variety was compared to that presented by the 83 selected spots. The dot-plots obtained showed that the patterns of distribution of spot abundance were identical, indicating the absence of any bias during spot selection (Fig. 5 and Supplemental Fig. S2). The 83 selected spots could therefore be considered as representative of the proteomes under study.

Hierarchical clustering analysis (HCA) is a powerful data mining tool that allows to group biological samples and/or proteins blindly according to their expression patterns (Chevalier *et al*., 2004; Gallardo *et al*., 2007; Méchin *et al*., 2007; Meunier *et al*., 2007; Witzel *et al*., 2009). We performed HCA to check how the rice varieties adapted to different environments could be related on the basis of their embryo proteome fingerprints. The classification

of the varieties was based uniquely on the embryo protein quantitative variability, rather than on both quantitative and qualitative changes (protein presence or absence), hence selecting only protein spots that were expressed in all rice embryo proteomes. The 2-DE patterns of embryo proteins have already demonstrated to be useful in pedigree analysis of rice cultivars (Xie *et al*., 2006; Wang *et al*., 2008). In barley, eighteen cultivars with different grain characteristics were discriminated on the basis of their seed protein variations, with each cultivar exhibiting a unique combination of polymorphic spots (Finnie *et al*., 2009). But most interesting, the clustering analysis of barley seed proteomes grouped more closely the cultivars with best malting quality, as compared to SSR (simple sequence repeat) markers (Finnie *et al*., 2009), indicating the power of seed proteomes to disclose important physiological traits.

Cluster analysis demonstrated that the proteome of 'PSBRc96' was the most distant in relation to the rest of varieties. It has already been mentioned that this variety despite belonging to the *japonica* subspecies, shared a high similarity with an *indica* variety at the embryo proteome level. However, the genetic differences between 'PSBRc96' and the other varieties came to light with HCA, evidenced by important quantitative changes across commonly expressed proteins e.g., glutelins (Fig. 6A). The differentiation between *japonica* and *indica* rice cultivars based on the differences of 2-DE expression patterns of glutelins was reported by Abe *et al*. (1996). On the other hand, multidimensional scaling analysis showed that 'IR29' and 'PSBRc96' were the most distantly related pair of varieties. This could be due to the different type of traits (e.g., pests and disease resistance) that were incorporated along past and more recent breeding programes. In fact, 'IR29' was released in the Philippines in 1974, belonging to the first-generation of modern varieties (MVs1) with higher yielding than traditional varieties, while 'PSBRc96' was released in 2000, integrating the fourth-generation MVs (IRRI, 2000; Estudillo and Otsuka, 2002; Khush and Virk, 2005). However, the incorporation of novel traits within distinct breeding

programes did not seem to interfere in the relationships across the remaining genotypes. Indeed, ‘IR58’ released in 1983 as a second-generation MV (MV2) was close to ‘PSBRc96’, whereas ‘IR64’ and ‘PSBRc1’, both belonging to third-generation of MVs (release in 1985 and 1990, respectively; IRRI, 1991; Khush and Virk, 2005) appeared distantly related (Fig. 6B).

Despite the limited number of genotypes analysed in this work, we propose that physiological features related to stress adaptation could be revealed at the embryo proteome level, thus influencing the classification of the varieties. This way, the closer relationship between the varieties would reflect more similar strategies to cope with environmental conditions. Indeed, clustering analysis showed that the cold- and drought-tolerant varieties clustered separately from their corresponding sensitive genotypes, although the salt-tolerant and -sensitive genotypes grouped in the same cluster (Fig. 6A). Nevertheless, in the multidimensional scaling analysis, the salt-tolerant variety showed its proximity to the drought-tolerant genotype, whereas the salt-sensitive was closely related to the drought-sensitive (Fig. 6B). In the second dimension of the plot, it was even observed that the tolerant varieties separated from the sensitive ones. This suggests that stress-sensitive genotypes are closely related, sharing proteomic features distinct from those displayed by tolerant ones. Apparently, the tolerant varieties were more distantly related than the sensitive ones (Fig. 6). It is not surprising that the sensitive genotypes under study may share more similar physiological features than tolerant ones (and consequently more similar proteome profiles), given that these three rice varieties were all released for irrigated lands. By contrast, the higher distance that separated the three tolerant genotypes probably reflected their adaptation to more diverse environmental conditions, conferring to these varieties a higher plasticity to face stressful challenges, as compared to sensitive ones.

On the other hand, if the closer relationship between the drought- and salt-tolerant varieties, compared to the higher distance that separates the cold- and the drought-tolerant, or the cold- and salt-tolerant genotypes, reflects more similar mechanisms involved in water-stress adaptation, is purely speculative. The comparison of the proteomic features in vegetative tissues during water deficit would be necessary to evaluate the similarities/differences across genotypes adapted to different abiotic stresses (drought, salinity and cold), but all them evoking a common component of water deficit. Moreover, such proteomic comparisons should be extended to a higher number of genotypes to be more conclusive. In addition, we cannot exclude that the classification of the varieties could be somehow changed if also including qualitative differences of protein expression. Damerval *et al*. (1987) demonstrated that the pattern of relationships across five maize lines based on the variations of protein amounts was different from that based on the qualitative variability of proteins. The relationship between the two closest lines was the same when using either quantitative or qualitative variability, but the relationships between the more distant lines was altered by the type of variability (Damerval *et al*., 1987).

We analysed in more detail the differences across sensitive *vs.* tolerant proteomes for each abiotic stress set of varieties, i.e. drought-, salt- and cold-associated varieties (Student's *t*-test, $P < 0.05$). The difference in protein accumulation between the drought-sensitive and the drought-tolerant genotype was significant, either when taking for analysis the 83 representative spots ($0.042 < 0.05$) or all the matching spots ($0.022 < 0.05$). But after comparing the protein abundance across the proteomes of the cold-sensitive *vs.* cold-tolerant, and salt-sensitive *vs*. salt-tolerant varieties, the differences found were not significant. We further analysed protein spots with significant 2-fold (or higher) changes across the different sets of varieties (Fig. 7). The 2-fold threshold was adequate for the level of variation determined in our samples, besides that a more stringent threshold reduces the number of identified spots with 'false-positive' changes (Parker *et al*.,

2006). ‘IR64’ and ‘PSBRc1’ registered the largest proportion of spots with significant change, i.e. 17 out of 274 matching spots displayed significant differences in protein abundance, representing about 6% of their commonly expressed proteins, while the salt and cold sets of contrasting varieties registered only about 3%. This was consistent with the relationship found among the varieties, since ‘IR64’ and ‘PSBRc1’ were more distantly related than ‘IR29’ and ‘IR52724-2B-6-2B-1-1’, or ‘IR58’ and ‘PSBRc96’ (Fig. 6), at the same time that protein abundance was significantly different between the proteomes of ‘IR64’ and ‘PSBRc1’.

Among the spots with most dramatic changes when comparing sensitive *versus* tolerant varieties were proteins involved in nutrient reservoir activity, like the granule-bound starch synthase I (GBSS-I). This protein not only exhibited significant changes across the cold- and salt-set of varieties (Fig. 7), but it was also the identified protein with the most significant difference in accumulation among the six varieties (Fig. 8). The GBSS-I enzyme (or waxy protein) is encoded by the *Waxy* gene, being involved in amylose biosynthesis (Tsai, 1974; Sano 1984). In higher plants, starch is composed of 20% to 30% of amylose and 70% to 80% of amylopectin - two homopolymers of α-D-glucose units - the former essentially linear, and the last, highly branched (Fujita *et al*., 2007). ‘IR58’ and ‘IR64’ are non-glutinous varieties, containing high and intermediate amounts of amylose, respectively, whereas ‘IR29’ is a well-known waxy (glutinous) variety with an amylose content of only 1% (Khush and Virk, 2005). In these three varieties, the abundance of GBSS-I was directly associated with amylose content. Hence, the expression of GBSS-I was higher in ‘IR58’ than in ‘IR64’, being lowest in ‘IR29’ (Fig. 8). The level of accumulation of GBSS-I in the rest of the varieties, suggests they are non-glutinous, with ‘IR52724-2B-6-2B-1-1’ probably constituting a high-amylose content variety, and ‘PSBRc96’ and ‘PSBRc1’ genotypes presumably with intermediate levels of amylose.

The presence of GBSS-I - an isoform of starch synthase - in the embryo is intriguing, since in cereal crops starch biosynthesis and accumulation

occurs in the endosperm (James *et al*., 2003). Moreover, histochemical studies performed on rice caryopsis revealed the absence of starch in the ungerminated embryo (Krishnan *et al*., 2001). Nevertheless, it was observed that after 12 hours of water imbibition, starch deposition begins in the rice embryo and continues for few days during germination, though in small amounts (Krishnan *et al*., 2001). An ADP (UDP)-glucose starch glycosyl transferase was among the differentially expressed proteins in the rice embryo proteome from a hybrid rice cultivar and its parental lines (Wang *et al*., 2008), reinforcing the presence of starch enzymes in the ungerminated embryo, besides the endosperm. Recently, a proteomic study conducted by Kim *et al*. (2009) involving the rice embryo proteome, pointed to the essential role of embryonic proteins in the regulation of seed germination. Therefore, the presence of GBSS-I in the rice mature embryo may suggest a role during germination, though the involvement of GBSS in the amylose biosynthesis in the ungerminated cereal embryos cannot be ruled out (Fujita and Taira, 1998). Surprisingly, Yang *et al*. (2007) observed that some enzymes involved in carbohydrate biosynthesis (e.g. pyruvate orthophosphate dikinase and GBSS) were also upregulated during rice seed germination, despite the major role of proteins involved in carbohydrate degradation (e.g., α-amylase and fructokinase) to fulfil the energy needs of the germinating embryo. All these data seems to indicate that although carbohydrate degradation is essential for the success of seed germination, the biosynthesis of sugar/starch carbohydrates still occurs at very low levels during germination. Seedling growth depends not only on both reserve mobilization, but also on CO_2 assimilation, and their rates change with environmental conditions (Caton, 2002). It can be speculated that the presence of GBSS-I in the germinating embryo could be related to the biosynthesis of starch, either anticipating further seedling growth coinciding with autotrophy, and/or as a ‘safety’ mechanism ensuring that some basal levels of reserves are also available, while seedling growth is still heterotrophic.

Following Osborne's classification based on plant protein solubility, we identified two main classes of storage proteins, namely glutelins and globulins. Despite that rice glutelins belong to the 11-12S globulin gene family, they fall in the former category since they are not readily soluble in dilute salt solutions, inversely to typical globulins (7S) (Shewry and Halford, 2002). In the present work, distinct types of glutelin protein precursors were identified (i.e., type-A 3, -B 2, -B 4 and C-type), being in general more strongly accumulated in 'PSBRc1' - the drought tolerant genotype - than in 'IR64', the sensitive one (Fig. 8A). However, this difference in the accumulation of glutelins across the drought-set of compared varieties was statistically significant just in the case of glutelin type-B 2 precursor. The abundance of the other glutelins in 'PSBRc1' was highly variable, therefore not allowing the finding of meaningful changes between 'PSBRc1' and 'IR64', above the biological noise. The glutelin type-B 2 precursor (spot 263) was among the spots with significant fold-changes ≥ 2.0, and curiously it was one of the two spots with stronger accumulation in the drought-tolerant genotype, by contrast to the other 15 spots that accumulated to a higher extension in the drought sensitive variety (Fig. 7). The higher accumulation of glutelin type-B 2 in the embryos of 'PSBRc1' could thus be presumably related to stress tolerance.

A comprehensive functional genomics study of the gene network modulated by both stress and development conditions in rice, revealed that disease resistance proteins interacted with different seed proteins, among them a glutelin (Cooper *et al*., 2003). The authors of this work hypothesized that seed storage proteins could actually serve as stabilisers for proteins involved in stress responses (Cooper *et al*., 2003). On the other hand, a proteomic comparison between several aflatoxin-resistant and -tolerant maize genotypes showed that seed storage proteins (e.g., globulins) constituted part of the proteins with high levels of expression in the tolerant genotypes (Chen *et al*., 2002). Chen and co-workers (2002) suggested that resistance mechanisms not only require the accumulation of antifungal

proteins in maize kernels, but also other proteins such as hydrophilic storage proteins. Although these reports point to a relationship between seed storage and stress resistance, the precise function of seed storage proteins in the protection against biotic stress is unknown. A link between abiotic stress tolerance and seed storage proteins seems to be less obvious and data supporting some evidence is even scarcer. Interestingly, a glutelin protein was among those proteins strongly induced by cold in rice leaves under mild stress conditions, maintaining a constant level of expression even when cold stress was more severe (Cui *et al*., 2005). In our work, clustering analysis showed that Rab24, a protein related to redox state control, grouped in the same main cluster of a putative globulin and glutelins type A-3 and B-2 (Fig. 6A). Although the number of identified proteins included in the HCA was reduced, due to the random selection procedure of the 83 spots, it was noteworthy that one Rab24 isoform grouped together with distinct storage proteins. This interesting finding, together with the pattern of accumulation exhibited by glutelins (e.g., glutelin type B-2) across the drought-set of varieties, may suggest a possible role for storage proteins in the protection against water deficit. In addition, glutelins and globulins presented quite different patterns of accumulation across the embryos of the distinct varieties. Spot 456, corresponding to a putative globulin was much more abundant than glutelins, and accumulated to a higher level in sensitive genotypes than in tolerant ones (Figs 6A and 8A). Several other forms of a globulin with alternative splicing, much less abundant, were also detected, exhibiting mainly qualitative differences (Fig. 8). These complex expression patterns of distinct types and forms of glutelins, and globulins across the rice embryo of the different genotypes, reflect a high genetic variability likely involving different strategies related to the accumulation of nutrient reserves. However, it is possible that storage proteins exert other functions such as stabilisers of other proteins, as proposed by Cooper *et al*. (2003). It would be interesting to perform further studies to uncover the putative role of seed storage proteins in drought/desiccation tolerance, and to check if the

differences in the accumulation of glutelins and globulins could also be related to different roles contributing for desiccation tolerance in the rice embryo.

The expression patterns of the stress-related proteins were quite diverse among the embryos of the distinct rice varieties. For instance, LEAs such as EMP1 and the putative LEA similar to LEA14-A/AtLEA7-1 showed a clear genetic variation of expression, displaying essentially qualitative variations. Other proteins with lower levels of accumulation in the rice embryo and with a possible role in stress-tolerance also exhibited qualitative variations across the six varieties e.g., a calcium-binding EF-hand family protein, or a mitochondrial import inner membrane translocase subunit. Meanwhile, Rab24 was apparently more abundant than Cu-Zn SOD, though these differences may be relative, because we cannot exclude the existence of other forms of Cu-Zn SOD more abundant in the rice embryo. The large genetic variability (evidenced by quantitative and qualitative variations) of these proteins across the distinct genotypes, is most probably in the basis of different mechanisms that may confer desiccation tolerance to the embryo during seed maturation.

Distinct forms of LEA Rab21 with a molecular mass close to 21 kD and p*I* ranging between 5.6 and 6.6 were observed on Coomassie stained 2-D gels and further identified by MS (Table I; Fig. 10). We confirmed that the distinct forms of Rab21 were due to post-translational modification by phosphorylation, after submitting different embryo protein extracts to alkaline phosphatase treatments and separating them by 1-DE and 2-DE (Fig. 10C). The analysis of the rice embryo phosphoproteome using mass spectrometry (MS), revealed that the peptides derived from Rab21 were among the most densely phosphorylated, containing up to seven phosphoryl groups (Zhang *et al*., 2007). Our results together with those obtained by Zhang and collaborators (2007) show that Rab21 is extensively phosphorylated *in vivo* in the rice seed, similarly to what was found for its homologous proteins Rab17 and Rab18, in maize and *Arabidopsis*, respectively (Goday *et al*.,

1988; Irar *et al*., 2006). In the particular case of Rab17, it was demonstrated that phosphorylation takes place in the serine cluster region (SGSSSSSSSE), with CK2 playing a major role in the phosphorylation of the protein (Vilardell *et al*., 1990; Plana *et al*., 1991; Riera *et al*., 2004). A detailed analysis of the phosphorylation sites in Rab17 through ß-elimination coupled to tandem MS, showed that each of the seven consecutive serine residues (Ser78-Ser84) could be phosphorylated (Jiang and Wang, 2004). Meanwhile, in Rab21 that contains a serine cluster identical to Rab17, it was further demonstrated that phosphorylation could also take place in the serine residue that is not contiguous to the others (SGSSSSSSSE), though seven was the maximum number of phosphoryl groups observed in the serine tract (Zhang *et al*., 2007). The seven well-resolved spots observed in the 2-D gels stained with Coomassie (Fig. 10A) seem to be consistent with the seven possible phosphorylation sites observed by Zhang *et al*. (2007) in the serine tract. Nevertheless, an additional most basic protein spot with a p*I* close to 6.8 was detected by Western-blot in the tolerant genotypes (spot 1 in Fig. 10B). Most probably it was not detected by Coomassie staining due its low abundance. Consistent with this idea, this most basic protein spot would be more abundant in the tolerant genotypes than in the sensitive ones. The Rab17 protein exhibited a similar pattern of eight isoforms in maize mature embryos labelled with [^{35}S] methionine (Sánchez-Martínez *et al*., 1986). The rice most basic protein spot could thus represent an additional less phosphorylated form of Rab21. In the rice embryos, the treatment with alkaline phosphatase (AP) caused an important protein shift towards the basic end of the gel to a p*I* close to 9.2, with the most acidic forms disappearing, either in the embryos of the tolerant, or the sensitive genotype (Fig. 10C). Interestingly, some less acidic forms with p*I* close 6.8 were still detected in the embryos of the sensitive genotype, whereas such forms almost completely disappeared in the embryos of the tolerant, after AP treatment (Fig. 10C). The results suggest that the unphosphorylated form of Rab21 should be highly basic with a p*I* close to 9.2, hence supporting that

the most basic Rab21 spot may represent an additional phosphorylated form. Interestingly, in the data obtained by Zhang *et al*. (2007) for the Rab21 phosphopeptides, we checked that in the case of the presence of three phosphoryl groups, tiny differences in the M*r* of the same phosphophopeptide could occur (i.e., 1828.47, 1828.48 and 1828.49) depending on the position of the phosphorylated (p) serine (S) residue in the cluster, thus influencing the mass/charge (m/z) ratios (see Supplemental Table S2 on Zhang *et al*., 2007). Therefore, most probably the different isoforms of Rab21 observed in the mature embryos, including the most basic one, are not only influenced by the number of phosphoryl groups present in the serine cluster region, but also by the position of the phosphorylation site even when the total charge of the protein is the same (e.g., pSpSpSSE, pSSpSpSE and SpSpSpSE; Zhang *et al*., 2007).

In addition, it was not only possible to observe some less acidic spots in the sensitive genotype after treatment with AP, but the band shift of Rab21 was also more pronounced in the sensitive than in the tolerant genotype (Fig. 10C). This seemed to indicate that Rab21 was apparently more strongly phosphorylated in the sensitive varieties than in the tolerant ones. Furthermore, the 21-kD band corresponding to Rab21 also evidenced mobility differences across the embryo protein extracts of different rice genotypes, showing a faster migration in all three tolerant genotypes than in the corresponding sensitive ones (Fig. 10D). These band gel mobility differences were consistent with a stronger phosphorylation in the sensitive varieties, as phosphate residues modify the SDS protein coating, thus retarding their migration in the gel (Peck, 2006). Taking the results together, this is the first time that it is reported a clear difference in the phosphorylation status of Rab21 in the seed embryos between stress-sensitive and -tolerant genotypes adapted to diverse environmental conditions (Fig. 10).

Reversible protein phosphorylation is crucial in the regulation of many cellular processes, influencing protein activity, subcellular localization, protein-protein interactions and protein turnover (de la Fuente van Bentem *et*

al., 2006; Vlad *et al*., 2008). The differences found in the phosphorylation status of Rab21 between stress-sensitive and -tolerant genotypes (Fig. 10), supports the hypothesis that phosphorylation plays an essential role in the acquisition of water stress tolerance. The analyses of the proteome of desiccated vegetative tissues from the resurrection plant *Craterostigma plantagineum* provided considerable evidence that phosphorylation is involved in desiccation tolerance (Röhring *et al*., 2006; 2008). Not less interesting was the observation that both LEAs and storage proteins made part of the heat-stable fraction of the *Arabidopsis* seed phosphoproteome (Irar *et al*. 2006; Oliveira *et al*., 2007). In rice seeds, it was also demonstrated that glutelins and waxy proteins were strongly phosphorylated (Lin *et al*., 2005). Furthermore, the analysis of the embryo phosphoproteome revealed that up to 125 proteins were phosphorylated (Zhang *et al*., 2007). Hence, phosphorylation seems not only to be fundamental for the function of stress-related proteins, but also important for the function of proteins in the desiccated seeds, related or not, to stress tolerance.

So many protein forms of LEA Rab21 (or Rab17 in maize) with different degrees of phosphorylation - up to seven phosphoryl groups – suggests different physiological roles, rather than a redundancy of forms. In the hierarchical clustering analysis it was observed that two distinct forms of Rab21 - one more basic, and another more acidic - clustered separately (Fig. 6A), giving support to our hypothesis that the different isoforms may play different roles. Boudet *et al*. (2006) proposed that LEAs associated with desiccation tolerance could exert different protective functions depending on the hydration level reached by plant tissues during drying. The authors suggested that LEAs linked to desiccation tolerance would thus protect at both high and low hydration levels. Curiously, among the distinct isoforms of a group 3 LEA (PM18) and a group 2 LEA (DHN3) some were related to desiccation tolerance, whereas others were clearly not. Riccardi *et al*. (2004) in a proteomic study of the response to water deficit in maize leaves, reported that two more basic forms of Rab17 were present after 8 days of

water stress, while most acidic forms appeared after 12 and 14 days. In addition, we observed that a most basic form of Rab21 corresponding putatively to a less phosphorylated form was only detected in the tolerant genotypes (Fig. 10). Taking the data altogether, it is tempting to speculate that some isoforms of LEA Rab21 could be more directly related to extreme conditions of water deficit (i.e., desiccation), whereas others could protect cells at milder stress conditions, with phosphorylation mediating their function.

Rab21 was apparently more strongly phosphorylated in the embryos of the sensitive varieties, likely due to the fact that most protein isoforms should be extensively phosphorylated, with only a very small proportion being less phosphorylated and/or unphosphorylated. By contrast, in the embryos of tolerant genotypes as the protein was not so heavily phosphorylated, there should have a much higher proportion of less phosphorylated and/or unphosphorylated isoforms as compared with sensitive genotypes. The detection of a most basic form of Rab21 only in the tolerant genotypes supports this hypothesis. This further suggests that less phosphorylated and/or unphosphorylated forms of Rab21 have an important role in stress tolerance, most probably because, if necessary, they are readily accessible for further phosphorylation. Kim *et al*. (2009) pointed to the essential role of embryonic proteins in the regulation of seed germination. They demonstrated that LEA proteins are even degraded first than storage proteins during germination. Furthermore, there is some evidence that phosphorylated forms of proteins are preferred substrates for degradation by the proteasome pathway (Perales *et al*., 2006). In this context, under normal germination conditions, more densely phosphorylated forms of Rab21 could be rapidly degraded; but in the case of environmental constraints during germination, the less phosphorylated and/or unphosphorylated forms would be already accessible to further phosphorylation, without ‘waiting’ for *de novo* synthesis of the protein. Protein phosphorylation could thus trigger the signal for retarding seedling growth in adverse environmental conditions.

Riera *et al*. (2004) showed that phosphorylation of Rab17 was crucial for seedling growth arrest in osmotic stress conditions. Thus, the expression pattern of Rab21 seems to indicate that tolerant genotypes possess a higher preparedness to face environmental constraints when compared with sensitive ones.

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6. Literature cited:

Abe T, Gusti RS, Ono M, Sasahara T (1996) Variations in glutelin and high molecular weight endosperm proteins among subspecies of rice (*Oryza sativa* L.) detected by two-dimensional gel electrophoresis. Genes Genet Syst 71: 63-68.

Agrawal GK, Rakwal R (2006) Rice proteomics: a cornerstone for cereal food crop proteomes. Mass Spec Rev 25: 1-53.

Asirvatham VS, Watson BS, Sumner LW (2002) Analytical and biological variances associated with proteomic studies of *Medicago truncatula* by two-dimensional polyacrylamide gel electrophoresis. Proteomics 2(8): 960-968.

Bartels D, Furini A, Ingram J, Salamini F (1996) Responses of plants to dehydration stress: a molecular analysis. Plant Growth Regul 20: 111-118.

Bartels D, Salamini F (2001). Desiccation tolerance in the resurrection plant *Craterostigma plantagineum*. A contribution to the study of drought tolerance at the molecular level. Plant Physiol 127: 1346-1353.

Boudet J, Buitink J, Hoekstra FA, Rogniaux H, Larré C, Satour P, Leprince O (2006) Comparative analysis of the heat stable proteome of radicles of *Medicago truncatula* seeds during germination identifies late embryogenesis abundant proteins associated with desiccation tolerance. Plant Physiol 140(4): 1418-1436.

Bies-Etheve, N, Gaubier-Comella P, Debures A, Lasserre E, Jobet E, Raynal M, Cooke R, Delseny M (2008) Inventory, evolution and expression profiling diversity of the LEA (late embryogenesis abundant) protein gene family in *Arabidopsis thaliana*. Plant Mol Biol 67: 107-124.

Bray EA (1997) Plant responses to water deficit. Trends Plant Sci 2 (2): 48-54.

Buitink J, Leger JJ, Guisle I, Vu BL, Wuillème S, Lamirault G, Le Bars A, Le Meur N, Becker A, Küster H, Leprince O (2006) Transcriptome profiling uncovers metabolic and regulatory processes occurring during the transition from desiccation-sensitive to desiccation-tolerant stages in *Medicago truncatula* seeds. Plant J 47(5): 735-750.

Campalans A, Messeguer R, Goday A, Pagès M (1999) Plant responses to drought, from ABA signal transduction events to the action of the induced proteins. Plant Physiol Biochem 37 (5): 327-340.

Caramelo JJ, Iusem ND (2009) When cells lose water: lessons from biophysics and molecular biology. Prog Biophys Mol Biol 99(1): 1-6.

Caton BP (2002) Simulating seed reserve mobilization and seedling growth of rice in DSRICE1. Field Crops Res 76: 55-69.

Chakrabortee S, Boschetti C, Walton LJ, Sarkar S, Rubinsztein DC, Tunnacliffe A (2007) Hydrophilic protein associated with desiccation tolerance exhibits broad protein stabilization function. Proc Natl Acad Sci 104 (46): 18073-18078.

Chen ZY, Brown RL, Damann KE, Cleveland TE (2002) Identification of unique or elevated levels of kernel proteins in aflatoxin-resistant maize genotypes through proteome analysis. Phytopathol 92:1084-1094.

Chevalier F, Martin O, Rofidal V, Devauchelle AD, Barteau S, Sommerer N, Rossignol M (2004). Proteomic investigation of natural variation between *Arabidopsis* ecotypes. Proteomics 4(5): 1372-1781.

Cooper B, Clarke JD, Budworth P, Kreps J, Hutchison D, Park S, Guimil S, Dunn M, Luginbühl P, Ellero C, Goff SA, Glazebrook J (2003) A network of rice genes associated with stress response and seed development. Proc Natl Acad Sci 100(8): 4945-4950.

Cui S, Huang F, Wang J, Ma X, Cheng Y, Liu J (2005) A proteomic analysis of cold stress responses in rice seedlings. Proteomics 5(12): 3162-3172.

Damerval C, Hébert Y, de Vienne D (1987) Is the polymorphism of protein amounts related to phenotypic variability? A comparison of two-dimensional electrophoresis data with morphological traits in maize. Theor Appl Genet 74: 194-202.

de la Fuente van Bentem S, Anrather D, Roitinger E, Djamei A, Hufnagl T, Barta A, Csaszar E, Dohnal I, Lecourieux D, Hirt H (2006) Phosphoproteomics reveals extensive *in vivo* phosphorylation of Arabidopsis proteins involved in RNA metabolism. Nucleic Acids Res 34(11): 3267-3278.

de Vienne D, Bost B, Fiévet J, Zivy M, Dillmann C (2001) Genetic variability of proteome expression and metabolic control. Plant Physiol Biochem 39: 271-283.

Estudillo JP, Otsuka K (2002) 'Lessons from three decades of green revolution in the Philippines'. Paper presented at the workshop on 'Green Revolution in Asia and its Transferability to Africa,' sponsored by FASID and held in Tokyo, December 8-10, 2002, pp 1-51.

FAO (2002) World agriculture: towards 2015/2030. Summary report, *on World Wide Web URL:* http://www.fao.org/DOCREP/004/Y3557E/y3557e06.htm#e

Finnie C, Bagge M, Steenholdt T, Østergaard O, Bak-Jensen KS, Backes G, Jensen A, Giese H, Larsen J, Roepstorff P, Svensson B (2009) Integration of the barley genetic and seed proteome maps for chromosome 1H, 2H, 3H, 5H and 7H. Funct Integr Genomics 9(1): 135-143.

Fujita N, Taira T (1998) A 56-kDa protein is a novel granule-bound starch synthase existing in the pericarps, aleurone layers, and embryos of immature seed in diploid wheat (*Triticum monococcum* L.). Planta 207: 125-132.

Fujita N, Yoshida M, Kondo T, Saito K, Utsumi Y, Tokunaga T, Nishi A, Satoh H, Park JH, Jane JL, Miyao A, Hirochika H, Nakamura Y (2007) Characterization of SSIIIa-deficient mutants of rice: the function of SSIIIa and pleiotropic effects by SSIIIa deficiency in the rice endosperm. Plant Physiol 144(4): 2009-2023.

Fukuda M, Islam N, Woo SH, Yamagishi A, Takaoka M, Hirano H (2003) Assessing matrix assisted laser desorption/ ionization-time of flight-mass spectrometry as a means of rapid embryo protein identification in rice. Electrophoresis 24: 1319-1329.

Galau GW, Hugues DW, Dure L III (1986) Abscisic acid induction of cloned cotton late embryogenesis abundant (LEA) messenger RNAs. Plant Mol Biol 7: 155-170.

Gallardo K, Firnhaber C, Zuber H, Héricher D, Belghazi M, Henry C, Küster H, Thompson R (2007) A combined proteome and transcriptome analysis of developing *Medicago truncatula* seeds: evidence for metabolic specialization of maternal and filial tissues. Mol Cell Proteomics 6(12): 2165-2179.

Goday A, Sánchez-Martinez D, Gómez J, Puigdomènech P, Pagès M (1988) Gene expression in developing *Zea mays* embryos: regulation by abscisic acid of a highly phosphorylated 23- to 25-kD group of proteins. Plant Physiol 88: 564-569.

Golovina EA, Hoekstra FA, Van Aelst AC (2001) The competence to acquire cellular desiccation tolerance is independent of seed morphological development. J Exp Bot 52(358): 1015-1027.

Gomez J, Sanchez-Martinez D, Stiefal V, Rigau J, Puigdomenech P, Pagès M (1988) A gene induced by the plant hormone abscisic acid in response to water stress encodes a glycine-rich protein. Nature 334 (6179): 262-264.

Gorantla M, Babu PR, Lachagari VBR, Reddy AMM, Wusirika R, Bennetzen JL, Reddy AR (2007) Identification of stress-responsive genes in an *indica* rice (*Oryza sativa* L.) using ESTs generated from drought-stressed seedlings. J Exp Bot 58: 253-265.

Goyal K, Walton LJ, Tunnacliffe A (2005a) LEA proteins prevent protein aggregation due to water stress. Biochem J 388: 151-157.

Goyal K, Pinelli C, Maslen SL, Rastogi RK, Stephens E, Tunnacliffe A (2005b) Dehydration-regulated processing of late embryogenesis abundant protein in a desiccation-tolerant nematode. FEBS Lett 579: 4093-4098.

Grelet J, Benamar A, Teyssier E, Avelange-Macherel MH, Grunwald D, Macherel D (2005) Identification in pea seed mitochondria of a late-embryogenesis abundant protein able to protect enzymes from drying. Plant Physiol 137:157-167.

Hajduch M, Ganapathy A, Stein JW, Thelen JJ (2005) A systematic proteomic study of seed filling in soybean. Establishment of high-resolution two-dimensional reference maps, expression profiles, and an interactive proteome database. Plant Physiol 37(4): 1397-1419.

Hajela RK, Horvath DP, Gilmour SJ, Thomashow MF (1990) Molecular cloning and expression of cor (cold-regulated) genes in *Arabidopsis thaliana*. Plant Physiol 93: 1246-1252.

Hoekstra FA, Golovina EA, Buitink J (2001) Mechanisms of plant desiccation tolerance. Trends Plant Sci 6 (9): 431-436.

Hunt SM, Thomas MR, Sebastian LT, Pedersen SK, Harcourt RL, Sloane AJ, Wilkins MR (2005) Optimal replication and the importance of experimental design for gel-based quantitative proteomics. J Prot Res 4(3): 809-819.

Irar S, Oliveira E, Pagès M, Goday A (2006) Towards the identification of late-embryogenic abundant phosphoproteome in *Arabidopsis* by 2-DE and MS. Proteomics 6 (Suppl 1): S175-185.

IRRI (1991) Program Report for 1990: Upland Rice Ecosystem Research Program, Los Baños, Philippines, pp 98-118.

IRRI (2000) Program Report for 2000: Irrigated Rice Ecosystem Research Program. Los Baños, Philippines, p 7, *on World Wide Web URL:* www.irri.org/publications/program/pdfs/00ProgramReport/Irrigated.pdf

James MG, Kay Denyer K, Myers AM (2003) Starch synthesis in the cereal endosperm. Curr Opin Plant Biol 6: 215-222.

Jensen AB, Busk PK, Figueras M, Albà MM, Peracchia G, Messeguer R, Goday A, Pagès M (1996) Drought signal transduction in plants. Plant Growth Reg 20: 105-110.

Jiang X, Wang Y (2004) *ß*-Elimination coupled with tandem mass spectrometry for the identification of *in vivo* and *in vitro* phosphorylation sites in maize dehydrin DHN1 protein. Biochem 43 (49): 15567-15576.

Jorge I, Navarro RM, Lenz C, Ariza D, Porras C, Jorrín J (2005) The holm oak leaf proteome: analytical and biological variability in the protein expression level assessed by 2-DE and protein identification tandem mass spectrometry de novo sequencing and sequence similarity searching. Proteomics 5(1): 222-234.

Jorge I, Navarro RM, Lenz C, Ariza D, Jorrín J (2006) Variation in the holm oak leaf proteome at different plant developmental stages, between provenances and in response to drought stress. Proteomics 6 Suppl 1: S207-214.

Karp NA, Spencer M, Lindsay H, O'Dell K, Lilley KS (2005) Impact of replicate types on proteomic expression analysis. J Prot Res 4: 1867-1871.

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

Khush GS, Virk PS (2005) IR varieties and their impact. International Rice Research Institute. Los Baños, Philippines, 163 p.

Kim ST, Wang Y, Kang SY, Kim SG, Rakwal R, Kim YC, Kang KY (2009) Developing rice embryo proteomics reveals essential role for embryonic proteins in regulation of seed germination. J Proteome Res 8(7): 3598-3605.

Krishnan S, Ebenezer GAI, Dayanandan P (2001) Histochemical localization of storage components in caryopsis of rice (*Oryza sativa* L.) Curr Sci 80(4): 567-571.

Lang V, Palva ET (1992) The expression of a rab-related gene, rab18, is induced by abscisic acid during the cold acclimation process of *Arabidopsis thaliana* (L.) Heynh. Plant Mol Biol 20(5): 951-962.

Langridge P, Paltridge N, Fincher G (2006) Functional genomics of abiotic stress tolerance in cereals. Brief Funct Genomic Proteomic 4: 343-354.

Leung J, Giraudat J (1998) Abscisic acid signal transduction. Ann Rev Plant Physiol Plant Mol Biol 49: 199–222.

Lin S-K, Chang M-C, Tsai Y-G, Lur H-S (2005) Proteomic analysis of the expression of proteins related to rice quality during caryopsis development and the effect of high temperature on expression. Proteomics 5: 2140-2156.

Liu X, Lu T, Yu S, Li Y, Huang Y, Huang T, Zhang L, Zhu J, Zhao Q, Fan D, Mu J, Shangguan Y, Feng Q, Guan J, Ying K, Zhang Y, Lin Z, Sun Z, Qian Q, Lu Y, Han B (2007) A collection of 10,096 *indica* rice full-length cDNAs reveals highly expressed sequence divergence between *Oryza sativa indica* and *japonica* subspecies. Plant Mol Biol 65(4): 403-415.

Méchin V, Thévenot C, Le Guilloux M, Prioul JL, Damerval C (2007) Developmental analysis of maize endosperm proteome suggests a pivotal role for pyruvate orthophosphate dikinase. Plant Physiol 143(3): 1203-1219.

Meunier B, Dumas E, Piec I, Béchet D, Hébraud M, Hocquette JF (2007) Assessment of hierarchical clustering methodologies for proteomic data mining. J Proteome Res 6(1): 358-366.

Moore JP, Vicré-Gibouin M, Farrant JM, Driouich A (2008) Adaptations of higher plant cell walls to water loss: drought vs desiccation. Physiol Plantarum 134: 237-245.

Mundy J, Chua NH (1988) Abscisic acid and water-stress induce the expression of a novel rice gene. EMBO J 7(8): 2279-2286.

Nguyen NV (2008) Sustainable Intensification of Rice Production for food security in the near future – A Summary Report. FAO International Rice Comission (IRC) - Plant Production and Protection Division (AGP), *on World Wide Web URL:* http://www.fao.org/fileadmin/templates/agphome/documents/Rice/sustintriceprod.pdf

Oliveira E, Amara I, Bellido D, Odena MA, Domínguez E, Pagès M, Goday A (2007) LC-MSMS identification of *Arabidopsis thaliana* heat-stable seed proteins: Enriching for LEA-type proteins by acid treatment. J Mass Spect 42: 1485-1495.

Pandey S, Bhandari H, Hardy B (2007). Economic costs of drought and rice farmers' coping mechanisms: a cross-country comparative analysis. Los Baños (Philippines): International Rice Research Institute. 203 p.

Parker R, Flowers TJ, Moore AL, Harpham NVJ (2006) An accurate and reproducible method for proteome profiling of the effects of salt stress in the rice leaf lamina. J Exp Bot 57: 1109-1118.

Peck SC (2006) Analysis of protein phosphorylation: methods and strategies for studying kinases and substrates. Plant J 45: 512-522.

Perales M, Portolés S, Más P (2006) The proteasome-dependent degradation of CKB4 is regulated by the Arabidopsis biological clock. Plant J 46(5): 849-860.

Plana M, Itarte E, Eritja R, Goday A, Pagès M, Martínez MC (1991) Phosphorylation of maize RAB-17 protein by casein kinase 2. J Biol Chem 266(33): 22510-22514.

Riccardi F, Gazeau P, Jacquemot MP, Vincent D, Zivy M (2004) Deciphering genetic variations of proteome responses to water deficit in maize leaves. Plant Physiol Biochem 42(12): 1003-1011.

Riera M, Figueras M, López C, Goday A, Pagès M (2004) Protein kinase CK2 modulates developmental functions of the abscisic acid responsive protein Rab17 from maize. Proc Natl Acad Sci 101(26): 9879–9884.

Roberts EH (1973) Predicting the storage life of seeds. Seed Sci Technol 1: 499-514.

Röhrig H, Schmidt J, Colby T, Bräutigam A, Hufnagel P, Bartels D (2006) Desiccation of the resurrection plant *Craterostigma plantagineum* induces dynamic changes in protein phosphorylation. Plant Cell Environ 29(8): 1606-1617.

Röhrig H, Colby T, Schmidt J, Harzen A, Facchinelli F, Bartels D (2008) Analysis of desiccation-induced candidate phosphoproteins from *Craterostigma plantagineum* isolated with a modified metal oxide affinity chromatography procedure. Proteomics 8(17): 3548-3560.

Saeed AI, Bhagabati NK, Braisted JC, Liang W, Sharov V, Howe EA, Li J, Thiagarajan M, White JA, Quackenbush J (2006) TM4 microarray software suite. Methods Enzymol 411: 134-193.

Sánchez-Martínez D, Puigdomènech P, Pagès M (1986) Regulation of gene expression in developing *Zea mays* embryos: protein synthesis during embryogenesis and early germination of maize. Plant Physiol 82(2): 543-549.

Sano Y (1984) Differential regulation of waxy gene expression in rice endosperm. Theor Appl Genet 68: 467-473.

Shewry PR, Halford NG (2002) Cereal seed storage proteins: structures, properties and role in grain utilization. J Exp Bot 53(370): 947-958.

Sreenivasulu N, Radchuk V, Strickert M, Miersch O, Weschke W, Wobus U (2006) Gene expression patterns reveal tissue-specific signaling networks

controlling programmed cell death and ABA-regulated maturation in developing barley seeds. Plant J 47: 310 - 327.

Sreenivasulu N, Sopory SK, Kavi Kishor PB (2007) Deciphering the regulatory mechanisms of abiotic stress tolerance in plants by genomic approaches. Gene 388(1-2): 1-13.

Tester M, Bacic A (2005) Abiotic stress tolerance in grasses. From model plants to crop plants. Plant Physiol 137: 791–793.

Thelen JJ, Peck SC (2007) Quantitative proteomics in plants: choices in abundance. Plant Cell 19: 3339-3346.

Tsai CY (1974) The function of waxy locus in starch synthesis in maize endosperm. Biochem Genet 11: 83-96.

Valledor L, Castillejo MA, Lenz C, Rodríguez R, Cañal MJ, Jorrín J (2008) Proteomic analysis of *Pinus radiat*a needles: 2-DE map and protein identification by LC/MS/MS and substitution-tolerant database searching. J Prot Res 7(7): 2616-31.

Vilardell J, Goday A, Freire MA, Torrent M, Martínez MC, Torné JM, Pagès M (1990) Gene sequence, developmental expression and protein phosphorylation of RAB-17 in maize. Plant Mol Biol 14: 423-432.

Vinocur B, Altman A (2005) Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. Curr Opin Biotechnol 16: 123-132.

Vlad F, Turk BE, Peynot P, Leung J, Merlot S (2008) A versatile strategy to define the phosphorylation preferences of plant protein kinases and screen for putative substrates. Plant J 55(1): 104-117.

Wang W, Meng B, Ge X, Song S, Yang Y, Yu X, Wang L, Hu S, Liu S, Yu J (2008) Proteomic profiling of rice embryos from a hybrid rice cultivar and its parental lines. Proteomics 8: 4808-4821.

Wasilewska A, Vlad F, Sirichandra C, Redko Y, Jammes F, Valon C, Frei dit Frey N, Leung J (2008) An update on abscisic acid signaling in plants and more. Mol Plant 1(2): 198-217.

Wehmeyer N, Vierling E (2000) The expression of small heat shock proteins in seeds responds to discrete developmental signals and suggests a general protective role in desiccation tolerance. Plant Physiol 122: 1099-1108.

Wise MJ, **Tunnacliffe A** (2004) POPP the question: what do LEA proteins do? Trends Plant Sci 9(1): 13-17.

Witzel K, Weidner A, Surabhi G-K, Borner A, Moc H-P (2009) Salt stress-induced alterations in the root proteome of barley genotypes with contrasting response towards salinity. J Exp Bot 60 (12): 3545-3557.

Woo SH, Fukuda M, Islam N, Takaoka M, Kawasaki H, Hirano H (2002) Efficient peptide mapping and its application to identify embryo proteins in rice proteome analysis. Electrophoresis 23(4): 647-654.

Xie Z, Wang J, Cao M, Zhao C, Zhao K, Shao J, Lei T, Xu N, Liu S (2006) Pedigree analysis of an elite rice hybrid using proteomic approach. Proteomics 6(2): 474-486.

Yang P, Li X, Wang X, Chen H, Chen F, Shen S (2007) Proteomic analysis of rice (*Oryza sativa*) seeds during germination. Proteomics 7: 3358-3368.

Zhang Q (2007) Strategies for developing Green Super Rice. Proc Natl Acad Sci 104: 16402-16409.

Zhang X, Ye J, Jensen ON, Roepstorff P (2007) Highly efficient phosphopeptide enrichment by calcium phosphate precipitation combined with subsequent IMAC enrichment. Mol Cell Proteomics 6: 2032-2042.

Supplemental data

Supplemental Table S1. Summary of the matched peptides obtained in protein identification by MS.

Supplemental Table S2. Proteins presenting qualitative variation across the six rice varieties.

Supplemental Figure S1. Effectiveness of logarithmic data transformation in the improvement of Gaussian distribution.

Supplemental Figure S2. Comparison of the distribution of protein abundance across all detected spots in ‘IR29’, ‘IR52724-2B-6-2B-1-1’, ‘IR64’ and ‘PSBRc1’, to that presented by 83 proteins randomly selected in each variety.

Supplemental Table S1. ***Summary of the matched peptides obtained in protein identification by MS***

Spot no.	Protein Name	No. Matched Peptides	Matched Peptides
13	Embryonic abundant protein 1	2	EGQTVVPGGTGGK GGLSTGDESGGER
34	Expressed protein (calcium-binding EF-hand family protein)	2	GFVADDDAFAR SVDARFEALDANGDGVLSR
37	Seed allergenic protein RA5 precursor	5	GTAAAAEQVR GTAAAAEQVRR QLAAVDDSWCR CEAISHMLGGIYR ELGAPDVGHPMSEVFR
40	Preprolamine (AA-19 to 137)	2	FDPLSQSYR QYQLQSHLLLQQQVLSPCSEFVR
ST37	Prolamin (precursor)	3	NLAQAQALLAFNVPSR NLAQAQALLAFNVPSR YYGAPSTITTLGGVL
DT32	Preprolamine (AA-19 to 137)	2	FDPLSQSYR QYQLQSHLLLQQQVLSPCSEFVR
66	Putative superoxide dismutase [Cu-Zn], chloroplast precursor	5	GTSQVEGVVTLTQDDQGPTTVNVR HAGDLGNIVANAEGVAEATIVDK QIPLSGPNSVVGR GGHELSLSTGNAGGR LACGVVGLTPL
79	Putative late embryogenesis abundant protein	1	VGLTVDLPILGNFTLPLTK
108	Mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein, putative, expressed	6	TFLDEVR NWMVDLGHPLLNR AAGIGAVQAVAR AAGIGAVQAVAR SFPDLNGGNSSK NSVVAGALTGAAVALTSDR
116	Water stress-inducible protein Rab21	——	EHGMEGQHGHVTSR/ m/z 521.15/3 (1560.4)
119	Water stress-inducible protein Rab21	——	QTGTGGGQFQDER / m/z 690.73/2 (1379.44)
123	Water stress-inducible protein Rab21	——	QTGTGGGQFQDER/ m/z 690.82/4 (1379.72)
129	Alpha-amylase/subtilisin inhibitor (precursor)	3	FTPWGGAAAPEDR VVTGPLIGPSPSGR DSCQDLGVSR

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Supplemental Table S1. *(Continued from previous page)*

156	Protein Rab24 (i.e. Thioredoxin peroxidase A)	8	LLGISCDDVQSHK DIEAYKPGNR VTYPIMADPSR QLNMVDPDEKDSNGGHLPSR ALHIVGPDKK LSFLYPSCVGR EKFPQGFDTADLPSGK FPQGFDTADLPSGK
DT162	Putative glutelin type-B 2 precursor	3	RVIQPQGLLVPR VIQPQGLLVPR QKEFLLAGNNNR
182	Putative globulin (with alternative splicing)	5	LLPPFHQASSLLR VAVLEANPR ILHTISVPGQIQFFFAPGGR NPESFLSSFSK NPESFLSSFSK
193	Putative embryonic abundant protein (group 3)	4	DKIPGPGSGGAGAGAAAGEGK SEPQPSSEEK SATENIYGSAASAAEAFR MGEEGTGMAAGDGGR
207	Glutelin C precursor	15	LQAFEPLR LQAFEPLRK CAGVFVIR VIEPQGLVVPR YSNTPALAYIIQGK GYVGLTFPGCPATHQQQFQLFEQ FRDEHQK SFANQLEPR QKEFLLAGNNQR EFLLAGNNQR LQSQNDQR RLQSQNDQR LQSQNDQRGDIIR HGLQLLKPTLTQR YQQVQYR
231	Glutelin type-A 3 [Precursor]	12	FDRLQAFEPIR LQAFEPIR GLLLPHYSN GATLVYVIQGR FRDEHQK HRDFFLAGNNK IGQQLYR NVFGGFSVELLSEALGISSGVAR QLQCQNDQR LQCQNDQRGEIVR LQCQNDQRGEIVR DYGQTQYQQK NIDNPNLADTYNPR

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255	Putative glutelin type-B 2 precursor	8	LQAFEPLR SEAGVTEYFDEK SEAGVTEYFDEKNELFQCTGTFVIR VIQPQGLLVPR YSNTPGLVYIIQGR YSNTPGLVYIIQGR QKEFLLAGNNNR EFLLAGNNNR
263	Putative glutelin type-B 2 precursor	10	FDRLQAFEPLR LQAFEPLR LQAFEPLRK NELFQCTGTFVIR RVIQPQGLLVPR VIQPQGLLVPR YSNTPGLVYIIQGR QKEFLLAGNNNR EFLLAGNNNR FPILNLIQMSATR
266	Glutelin type-B 4 [Precursor]	18	LQAFEPLR LQAFEPLRR SEAGVTEYFDEK SEAGVTEYFDEKNEQFQCTG NEQFQCTGTFVIR RVIEPQGLLVPR VIEPQGLLVPR YSNTPGMVYIIQG YSNTPGMVYIIQG FRDEHQK QKEFLLAGNNNR FLLAGNNNR EQQMYGR RLQGQNDQR LQGQNDQRGEIIR LQGQNDQR ITRLNSQK AMPVDVIANAYRISR
284	Putative globulin (with alternative splicing)	11	CEQDRPPYER CVQECKDQQQQQQER EHGGHDDDRR RPYVFGR RPYVFGRR LLPPFHQASSLLR VAVLEANPR WSYAIR QGDVFVAPAGTINYLANTDGR ILHTISVPGQIQFFFAPGGR NPESFLSSFSK
427	Putative globulin (with alternative splicing)	4	VAVLEANPR QGDVFVAPAGTINYLANTDGR ILHTISVPGQIQFFFAPGGR NPESFLSSFSK

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Supplemental Table S1. *(Continued from previous page)*

434	Putative aminoacylase	4	GQAGAAGLEAR TLELVAGKPLLLLR LYDGSAMENLMK GIGIYESIIR
436	Putative globulin (with alternative splicing)	14	ATILLLLAAVLFAAAAAASGEDR RPYVFGR LLPPFHQASSLLR VAVLEANPR WSYAIR QGDVFVAPAGTINYLANTDGR ILHTISVPGQIQFFFAPGGR NPESFLSSFSK ASEEQVRELR GPFNILEQRPR SFHDLAEHDIR GQEEEEEEQVGQGYETIR GTVFVVPSGHPIVVTSSR DSTLQIVCFDVHANNNE
456	Putative globulin	16	FSVLERFPDEQVVGAAVGGYR FPDEQVVGAAVGGYR VAVLEAAPR AFLQPSHYDADEVFYVK EGEGVIVLLR RESFCVR QREGGEITTAPEEQIR EGGEITTAPEEQIR GGGGGSGSEWEIKPSSLTGK SPYFSNNHGK LFELTGDECR GSMIAPNYNTR KLVFGGSAAR LVFGGSAAR EADRVLAAQPEQILLR VLAAQPEQILLR
468	Tubuline beta-4 chain	14	FWEVVCDEHGIDPTGR VNVYYNEASCGR VNVYYNEASCGRFVPR AVLMDLEPGTMDSVR TGPYGQIFRPDNFVFGQSGAGNN WAK GHYTEGAELIDSVLDVVR LTTPSFGDLNHLISATMSGVTCCLR FPGQLNSDLR LAVNLIPFPR LHFFMVGFAPLTSR ALTVPELTQQMWDAK YLTASAMFR NSSYFVEWIPNNVK GLSMASTFIGNSTSIQEMFR

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Supplemental Table S1. *(Continued from previous page)*

496	Granule-bound starch synthase I, chloroplastic/amyloplastic	19	QQRSVQR TGGLGDVLGGLPPAMAANGHR DAWDTSVVAEIK FFHCYK VFIDHPSFLEK FSLLCQAALEAPR ILNLNNNPYFK NNYQPNGIYR VAFCIHNISYQGR FAFEDYPELNLSER SSFDFIDGYDTPVEGR VLTVSPYYAEELISGIAR EALQAEAGLPVDR KIPLIAFIGR IPLIAFIGR FNAPLAHLIMAGADVLAVPSR FEPCGLIQLQGMR FEPCGLIQLQGMR VVGTPAYEEMVR
534	Putative globulin (with alternative splicing)	5	ILHTISVPGQIQFFFAPGGR NPESFLSSFSK GPFNILEQRPR GTVFVVPSGHPIVVTSSR MYLAGMNSVLK

(1) *de novo* sequencing. Note: m/z = mass/charge ratio

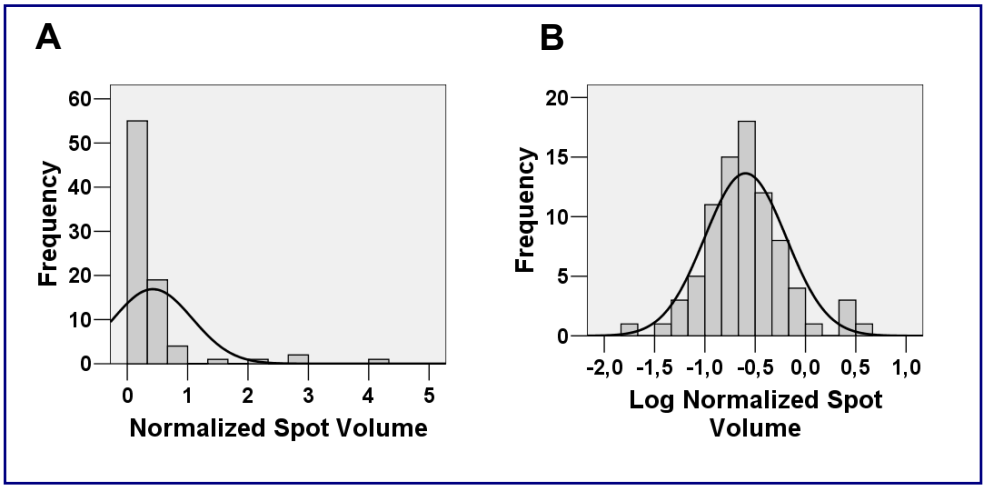
Supplemental Table S2. ***Proteins displaying qualitative variation across the six rice varieties***
A mark (√) indicates protein detection.

Spot no./Protein identity	IR58	PSBRc96	IR29	IR52724-2B-6-2B-1-1	IR64	PSBRc1
13 /Embryonic abundant protein 1	√	√	×	×	×	×
34/ Expressed protein (calcium-binding EF-hand family protein)	√	√	×	√	×	×
37/ Seed allergenic protein RA5 precursor	√	√	√	√	×	√
40/ Preprolamine (AA-19 to 137)	√	√	√	√	×	√
79/ Putative late embryogenesis abundant protein	√	√	×	×	×	√
108/ Mitochondrial import inner membrane translocase subunitTim17/Tim22/Tim23 family protein, putative, expressed	√	√	√	×	√	√
123/ Water stress-inducible protein Rab21	√	√	√	×	×	×
129/ Alpha-amylase/subtilisin inhibitor (precursor)	×	√	√	√	×	√
DT162/ Glutelin type-B 2 precursor	×	×	×	×	×	√
182/ Putative globulin (with alternative splicing)	×	√	√	√	√	√
193/ Putative embryonic abundant protein (group 3)	×	√	×	×	×	×

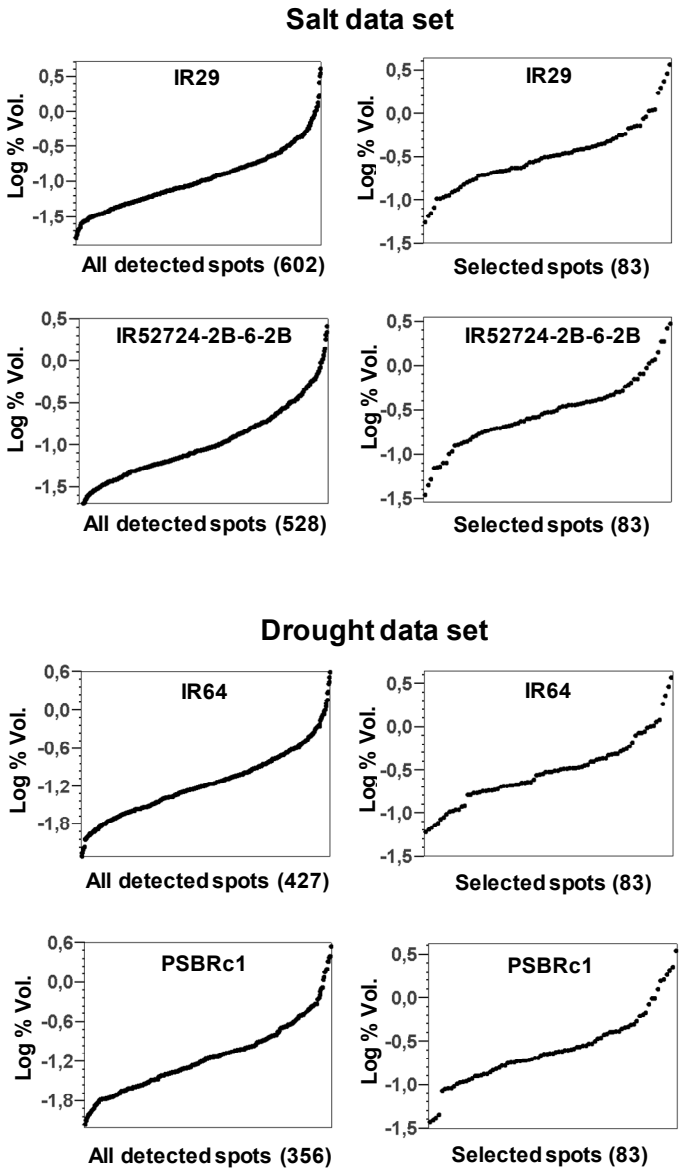
(Table continues on following page)

Supplemental Table S2. *(Continued from previous page)*

255/ Putative glutelin type-B 2 precursor	×	√	√	√	×	×
284/ Putative globulin (with alternative splicing)	×	√	√	√	√	×
427/ Putative globulin (with alternative splicing)	√	√	√	×	√	√
434/ Putative aminoacylase	√	√	√	√	×	×
436/Putative globulin	√	√	√	√	×	×
468/ Tubuline beta-4 chain	√	√	√	√	×	√
534/ Putative globulin (with alternative splicing)	√	√	√	√	×	×



Supplemental Figure S1. Effectiveness of logarithmic data transformation in the improvement of Gaussian distribution.



Supplemental Figure S2. Comparison of the distribution of protein abundance across all detected spots in 'IR29', 'IR52724-2B-6-2B-1-1', 'IR64' and 'PSBRc1', to that presented by 83 proteins randomly selected in each variety.

Chapter III

Characterisation of a putative rice DRE-binding protein responsive to water-deficit

Farinha AP [1,2], Oliveira MM [2] and Pagès M [1]. Characterisation of a putative rice DRE-binding protein responsive to water-deficit (under revision to be submitted)

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AP Farinha declares to have participated actively in this work, by performing the experimental design, all the laboratory work and manuscript writing.

Abstract

Transcription factors belonging to the AP2/ERF superfamily have important functions in the transcriptional regulation of both plant development and stress responses. While the DREB1- and DREB2-type proteins have been well characterised in *Arabidopsi*s and cereals in response to abiotic stress, much less is known about other members that evolved in a divergent way in relation to these proteins. The maize DBF1 transcription factor has been described as one of those members with little homology to DREB1- and DREB2-type proteins. We searched in GenBank for rice sequences with similarity to maize DBF1, in order to find potential homologous proteins. The best hit rice protein sequence with significant homology to DBF1 was designated as OsDBF1. In this study, we characterised a 20-kD rice protein that cross-hybridised with an antibody raised against the maize DBF1. The expression patterns exhibited by the 20-kD protein in rice were quite similar to those described for maize DBF1, both being induced by water-deficit, salinity and ABA. Furthermore, the 20-kD rice protein was also induced by cold, contrary to that found in maize. The anti-DBF1 antibody demonstrated to recognise proteins with identical molecular mass and p*I* in both maize and rice embryos. The whole data strongly suggests that the 20-kD rice protein may correspond to the putative OsDBF1 protein. In addition, high basal levels of the protein were detected in rice vegetative tissues of 'PSBRc1'- a drought-tolerant genotype - in pre-stress conditions, whereas it was strongly induced by water-deficit in the sensitive genotype. This expression pattern suggests that the protein may take part in 'stress-anticipating' mechanisms in the drought-tolerant variety.

Characterisation of a putative rice DRE-binding protein responsive to water-deficit

1. Introduction

Stress perception and signal transduction are essential in the transcriptional gene activation that leads to plant stress adaptation after exposure to adverse environmental conditions. Gene transcription is a complex process that requires coordinated regulatory machinery (Gómez-Porras *et al*., 2007; Nakashima *et al*., 2009). A single transcription factor (TF) may control the expression of many genes through specific binding to *cis*-acting elements in the core promoters of their target genes (Nakashima *et al*., 2009). Major 'regulons' (transcriptional regulatory systems) involved in the control of gene expression under abiotic stress, with or without the intervention of ABA, were initially identified in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki, 1994; Liu *et al*., 1998; Narusaka *et al*., 2003). The dehydration-responsive element binding protein 1 (DREB1)/C-repeat binding factor (CBF) and the DREB2 regulons participate in ABA-independent gene expression, among others like NAC and MYB/MYC (Nakashima *et al*., 2009). On the other hand, the ABA-responsive element (ABRE) binding protein (AREB)/ABRE binding factor (ABF) mediates an ABA-dependent signal transduction pathway. Interestingly, a single copy of ABRE is not sufficient for triggering ABA-dependent gene expression, but multiple copies of ABREs or a combination with a coupling element (CE) are required e.g., CE1, CE3 and DRE (Shen *et al*., 1996; Hobo *et al*., 1999; Narusaka *et al*., 2003). Moreover, there are some nuances in ABA regulatory mechanisms between rice and *Arabidopsis*. For instance, although ABRE is equally abundant in both species, CE3 is almost absent in *Arabidopsis* (Gómez-Porras *et al*., 2007). For a comprehensive comparison of the transcriptional regulatory networks involved in abiotic stress response between rice and *Arabidopsis,* see the review article by Nakashima *et al*. (2009).

The maize *rab17* gene encodes a LEA protein inducible by ABA and water deficit either in the embryo or vegetative tissues (Vilardell *et al*., 1990). Several *cis*-acting elements were found in the *rab17* promoter involved in gene induction by drought and ABA (Busk *et al*., 1997). Among them, a DRE2 *cis*-element with core sequence 'ACCGAC' (Busk *et al*., 1997) resembling the DRE/C-repeat (CRT) element from *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki, 1994). Two cDNAs coding for DNA-binding proteins - DBF1 and DBF2 - interacting with the DRE2 *cis*-element of the *rab17* promoter were isolated through yeast one-hybrid screening (Kizis and Pagès, 2002). The two maize DBF proteins belong to the AP2/EREBP superfamily of plant transcription factors (Riechmann and Meyerowitz, 1998) and phylogenetic analysis showed that DBF1 and DBF2 evolved in a divergent way to DREB1 and DREB2 proteins (Kizis and Pagès, 2002). The expression of *DBF1* is strongly induced by drought and ABA, whereas *DBF2* exhibits a constitutive expression pattern in most stress treatments, accumulating to a lower extent as compared to *DBF1* (Kizis and Pagès, 2002). On the other hand, DBF1 demonstrated to strongly enhance the activity of the *rab17* promoter in transiently transformed cells after ABA treatment, contrasting with DBF2 that decreased the promoter activity, either in the presence or absence of exogenous ABA (Kizis and Pagès, 2002). In *Arabidopsis,* DRE/CRT is involved in drought, osmotic stress and cold induction in an ABA-independent pathway. But in maize, the DRE2 cis-acting element participates in ABA-dependent gene expression, thus suggesting a different role of DRE in the two species (Kizis and Pagès, 2002). In addition, functional analysis of *DBF1* through transgenic approaches pointed for an involvement in drought and salinity stress tolerance (Saleh *et al*., 2006).

From a total of 2344 non-redundant TFs estimated in rice genomes, about 188 corresponded to members of the AP2/EREBP superfamily (Wu *et al*., 2006). Similar estimates (about 8%) can be obtained in the rice database of TFs (Gao *et al*., 2006). Several rice genes belonging to the AP2/EREBP

superfamily with significant homology to *DREB1/CBF* and *DREB2* from *Arabidopsis,* were reported in the last few years e.g., *OsDREB1A, OsDREB1B, OsDREB1C, OsDREB1D, OsDREB2A* (Dubouzet *et al*., 2003); *OsDREB1E, OsDREB1G, OsDREB2B* (Chen *et al*., 2008); and *OsDREB1F* (Wang *et al*., 2008). However, and most surprising, is the lack of any work reporting the characterisation a rice gene with significant homology to *DBF1* until date. Among cereals, the barley *HvDRF*1 gene has been reported to have some similarity to *DBF1* (Xue and Loveridge, 2004), and in *Arabidopsis,* the *RAP2.4* gene was described as homologous to *DBF1* (Lin *et al*., 2008). Both genes were involved in drought- and salt-responsive gene expression.

In this work, we report the characterisation of rice proteins that cross-hybridised with polyclonal antisera raised against the maize DBF1 transcription factor (Kizis and Pagès, 2002). Two proteins with a molecular mass of about 20 and 29 kD were immunoreactive in rice embryos. The 20-kD polypeptide was found to be involved in water-stress gene expression in vegetative tissues, besides exhibiting some induction by ABA. The comparison of the expression pattern of the 20-kD protein across rice genotypes with contrasting abiotic stress response further suggests an involvement in stress tolerance.

2. Materials and Methods

2.1. Preparation of total protein extracts and stress treatments

Seeds of cultivars ‘PSBRc1’, ‘IR64’, ‘IR29’, ‘PSBRc96’, ‘IR58’ and breeding line ‘IR52724-2B-6-2B-1-1’, were provided by Dr. Glenn B. Gregorio from IRRI (Los Baños, Philippines). Dried seeds were peeled and the mature embryos were separated from the endosperm with scalpel, immediately frozen in liquid nitrogen and kept at -80°C until protein extraction. Stress treatments were performed on fourteen days-old seedlings (with 3 leaves). Dehydration was performed by leaving plants over a dry filter paper at room temperature. Water deficit was induced by adjusting nutrient

solution with 15% PEG-6000 (Fluka), whereas salt stress was achieved with 200 mM NaCl, and cold treatment by regulating the temperature of the growth chamber to 4°C. Rice seedlings were also treated with 100 μM ABA (cis/trans isomer, Sigma-Aldrich) for 3 and 4 hours. All treatments were made by immersing germinated roots in fresh nutrient solution (MS diluted 1:4 v/v) adjusted to the mentioned concentrations of PEG, NaCl and ABA. Water-stress was induced for 3, 4, 24 and 72 hours, while salt and cold stresses were imposed for 3 hours. Control plants were immediately frozen before starting water-stress, salt or cold treatments, though in the case of the control of ABA, untreated plants were kept in nutrient solution for 3 hours. Stressful and non-stressfull treatments were performed under controlled conditions in a growth chamber, except for air drying. Rice tissues were quickly frozen in liquid nitrogen and stored at -80°C for protein extraction. Total protein extracts were prepared by grinding the frozen rice tissues with mortar and pestle in liquid nitrogen. For 1-DE, protein extracts were solubilized in 50 mM Tris-HCl pH 8.0, 10 mM NaCl, 1% (v/v) SDS, 5% (v/v) β-mercaptoethanol, with protease inhibitors (1mM PMSF, 50 μM leupeptin, 1 μM pepstatin, 10 μM E-64, 10 μg mL $^{-1}$ aprotinin). For 2-DE, proteins were solubilized in 8M Urea, 2M Thiourea, 4% (w/v) CHAPS, 40mM Tris-HCl pH 8.0 (Lysis Buffer) containing protease inhibitors in the same concentrations as previously mentioned, and a protease-free DNaseI-RNaseA mixture. Protein extracts were centrifuged at 10.000xg for 20 min at 4°C until supernatant was completely clear. Protein concentrations were determined using the Bio-Rad Protein Assay, and Bovine Serum Albumine (BSA) as standard. Equal protein loading was confirmed by staining proteins with CBB R-250 after SDS-polyacrylamide gel electrophoresis (PAGE).

2.2. Western-blot analysis

Rice embryo proteins were separated either by 1-DE or 2-DE for Western-blot analyses. Total protein extracts were separated by 1-DE on 15% (v/v) SDS-PAGE Laemmli gels (8x7.3x0.1cm) at 30mA (15mA/gel,

constant current) until the tracking dye reached the bottom of the gel. For protein identification by mass spectrometry, proteins were separated by 1-DE electrophoresis in Tris-tricine running buffer as described by Schagger and Von Jagow (1987), onto large format gels (26x20x0.1cm), using the Ettan DALT*six* System (GE Healthcare Amersham Biosciences).

For 2-DE Western-blot analysis, total protein extracts (100 µg) were diluted in 125 µl final volume containing rehydration solution: 8M Urea, 18 mM Tris-HCl pH 8.0, 4% (w/v) CHAPS, 0.5% (v/v) IPG (immobilized pH gradient) buffer in same range as the IPG strip (i.e. pH 3-11), 1.6% (v/v) DeStreak Reagent (GE Healthcare Amersham Biosciences) and 0.002% (w/v) Bromophenol Blue. Protein samples were separated using 7-cm IPG strips (NL pH 3-11; Immobiline DryStrips, GE Healthcare Amersham Biosciences) and strip rehydration was performed for 6 hours at room temperature, followed by 6.5h at 30V. Protein isoelectric focusing was performed at 500V for 1h, 1000V for 1h, followed by 8000V for 7h, using the Ettan^{TM} IPGphor^{TM} Isoelectric Focusing System (GE Healthcare Amersham Biosciences). Prior to second dimension, IPG strips were equilibrated with 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (v/v) SDS, a trace of Bromophenol Blue and 10 mg mL $^{-1}$ DTT for 15 min, followed by a second equilibration step with the same buffer containing 25 mg mL $^{-1}$ iodoacetamide for further 15 min, with gentle shaking. The protein samples were then run on 12% (v/v) SDS-PAGE gels (8x7.3x0.1cm) using the Mini-Protean 3 System (Bio-Rad), at 25V for 10 min and then 120 V until the dye front reached the bottom gel. The separated proteins were blotted onto nitrocellulose membranes (Schleicher and Schuell, 0.45 µm) using a Trans-blot Semidry Transfer Cell (Bio-Rad). Polyclonal rabbit antisera raised against rice Rab21 (Mundy and Chua, 1988), maize Rab17 (Goday *et al*., 1988) and maize DBF1 (Kizis and Pagès, 2002) proteins were used to cross-react with rice proteins. Primary Rab21 and Rab17 antibodies were diluted 1:2000, whereas anti-DBF1 was diluted 1:1000. The secondary antibody anti-rabbit IgG, Horseradish Peroxidase-linked (GE Healthcare Amersham

Biosciences) was diluted 1:15000. Signal detection was performed using the ECL Western blotting detection reagents and analysis system (GE Healthcare Amersham Biosciences) following the manufacturer's instructions.

3. Results

3.1. Sequence analysis of a putative rice DRE-binding protein with significant homology to maize DBF1

Rice proteins with significant homology to DBF1 were searched on GenBank databases using the BLAST sequence alignment program (Altschul *et al*., 1997). After BLASTP and BLASTX sequence alignment against databases, a best hit rice sequence (AAP56252) with 65% of identity with DBF1 was retrieved. This sequence corresponded to an AP2-domain DRE binding factor 1 (DBF1) protein. The full-length cDNA (AK105725) was searched on KOME database (http://cdna01.dna.affrc.go.jp/cDNA/) and it contained a longest open reading frame (ORF) of 280 amino acids encoding a putative protein with a predicted M*r* of 29.4 kD and p*I* of 7.07. Sequences from other cereals (e.g., *Sorghum bicolor* and *Triticum aestivum*) with significant homology to DBF1 were also retrieved by BLASTX and BLASTP (e.g., TaDBF). The nucleotide sequence alignment between the putative *OsDBF1* and *DBF1* is shown in Figure 1.

Protein sequence alignment of the putative OsDBF1 with DBF1 and a putative DBF-like protein from wheat (TaDBF) showed that they were not only quite conserved within the AP2/EREBP DNA-binding domain, but also at their N- terminal and especially at the C-terminal region (Fig. 2A). Furthermore, the rice protein contained a C-terminal region rich in serine (S)

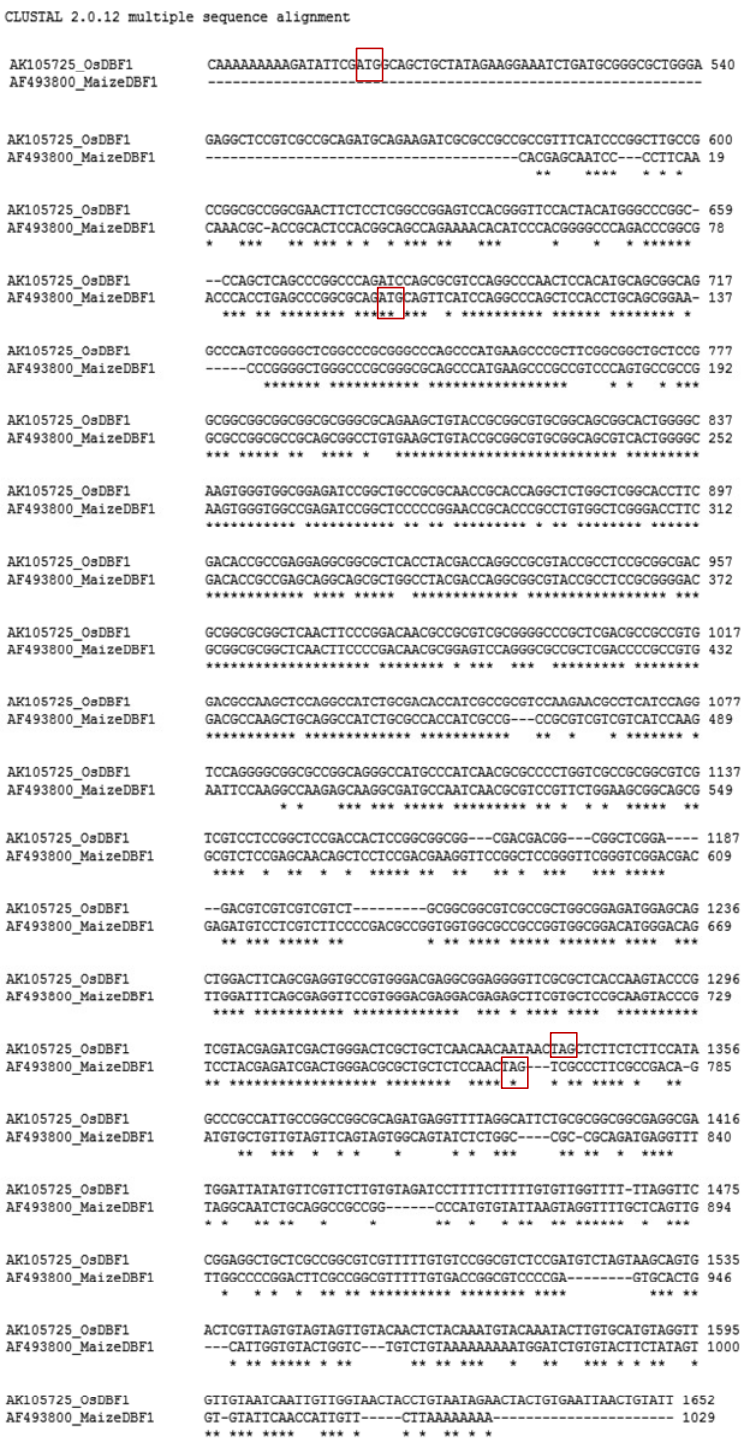

Figure 1. **Nucleotide sequence alignment between *DBF1* and a rice sequence putatively corresponding to *OsDBF1***. The translation initiation (ATG) and stop (TAG) codons are indicated by boxes.

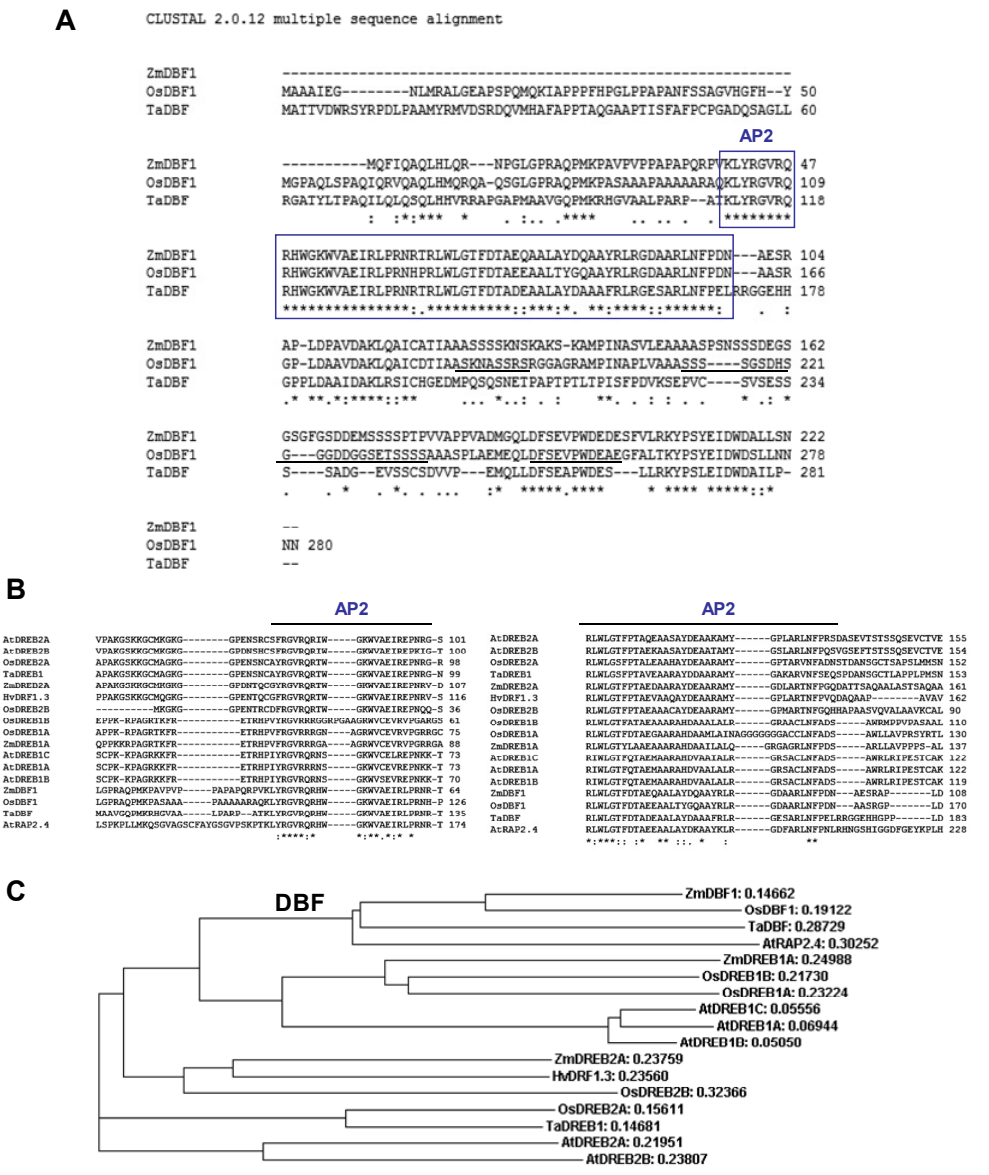

Figure 2. **Protein sequence alignment and phylogenetic analysis of DRE-binding proteins**. **A**. Amino acid sequence alignment between OsDBF1, (AAP56252) maize DBF1 (ZmDBF1; AAM80486) and a putative DBF protein from wheat (TaDBF; AAZ08560); underlined residues indicate acidic and Ser-rich regions. **B**. Sequence alignment of AP2/ERF proteins. **C**. Phylogram of DBF-type and DREB-type proteins. Alignment was performed using the CLUSTALW2 program. *Arabidopsis* AtDREB1A (Q9M0L0), AtDREB1B (P93835), AtDREB1C (BAA33436), AtDREB2A (AB007790), AtDREB2B (NM111939), AtRAP2.4 (AAN12993); Barley HvDRF1.3 (AAO27885). Maize ZmDREB1A (AF045481), ZmDREB2A (AB218832); Rice OsDREB1A (AAN02486), OsDREB1B (Q3T5N4), OsDREB2A (Q0JQF), OsDREB2B (AK099221); Wheat TaDREB1 (AAL01124). Distances are indicated by numbers.

and acidic amino acid residues (glutamic acid, E; aspartic acid D) that may serve as putative activation domain (Fig. 2A). The similarities of the putative OsDBF1 with DREB1- and DREB2-type proteins belonging to the AP2/EREBP TF superfamily (Riechmann and Meyerowitz, 1998) were analysed. The amino acid sequences of 17 proteins were aligned using the ClustalW2 multiple sequence alignment program available at the EMBL/EBI site (http://www.ebi.ac.uk/Tools/clustalw2/). The AP2 conserved domain is indicated by asterisks in Fig. 2B. Based on protein similarity, a phylogram was computed using the neighbour-joining (NJ) method (Fig. 2C). Phylogenetic analysis showed that the putative OsDBF1 shared a high similarity to DBF1, as well as to the putative TaDBF protein, besides to RAP2.4 protein from *Arabidopsis*. All these proteins clustered independently from DREB1- or DREB2-type proteins, in a distinct clade - here designated as the 'DBF' subgroup (Fig. 2C). On the other hand, all DREB1 proteins, except TaDREB1, clustered separately from DREB2 TFs. The members of monocots clustered together, and in distinct clades from the *Arabidopsis* members, either in the case of DREB1- or DREB2-type proteins (Fig. 2C).

3.2. Characterisation by 1-DE Western-blot of rice proteins cross-reacting with anti-DBF1

3.2.1. *Accumulation pattern in mature embryos*

The antibodies raised against maize proteins may be helpful in the recognition of rice homologous proteins. The antiserum raised against the maize Rab17 protein (Goday *et al*., 1988; Vilardell *et al*., 1990) was crucial for the characterisation of Rab21, the rice homologous protein, in the embryo of distinct rice varieties (see chapter II). In the upper panel of Figure 3, it is depicted an example of rice embryo proteins recognised by the anti-Rab17 and anti-Rab21 antisera (Goday *et al*., 1988; Mundy and Chua, 1988). The rice embryo proteins cross-reacting with both antibodies presented an identical electrophoretic mobility (Fig. 3). The band with lower

molecular mass corresponded to Rab21, indicating that either of the two antibodies recognised the protein. Two antibodies raised against the maize DBF1 transcription factor were hence used to cross-react with rice potential homologous proteins (Fig. 3). One of the polyclonal antibodies was raised against the entire sequence of the maize DBF1 protein, whereas the other was raised against the C-terminal region, thus not including the AP2 domain (Kizis and Pagès, 2002). DBF1 accumulates in maize mature embryos, being also induced by drought, salinity and ABA, in vegetative tissues (Kizis and Pagès, 2002). We started by analysing if the antibodies could cross-hybridise with rice proteins extracted from mature embryos of distinct varieties (Fig. 3).

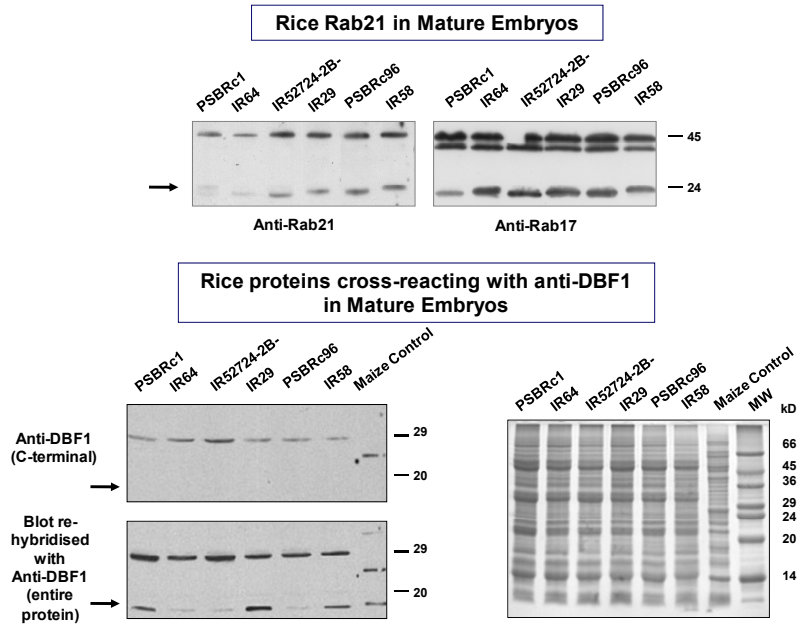

Figure 3. **Cross-reaction of rice mature embryo proteins with different polyclonal antibodies**. The **upper panel** shows the cross-reaction of rice proteins with anti-Rab17 and anti-Rab21 antibodies. Both antisera recognise the same type of rice proteins, with the lower band corresponding to Rab21. In the **lower panel**, the antibody raised against the entire sequence of the DBF1 protein cross-hybridizes with an additional protein of about 20 kD as compared with the antibody raised against the C-terminal region. On the right: total protein extracts from the embryos of different rice varieties and from maize maize embryos. Gels were stained with CBB R250.

Two rice proteins with a molecular mass close to 20 and 29 kD were immunoreactive in the embryos of varieties with contrasting abiotic stress tolerance (Fig. 3). The 29-kD protein was recognised by both antibodies in the embryo extracts, but the polypeptide of about 20 kD (herein simply referred as 20-kD) cross-reacted uniquely with the antibody raised against the entire DBF1 sequence (Fig. 3). For that reason, the antiserum raised against the entire sequence was selected to proceed with the analysis of protein expression in different rice tissues.

3.2.2. *Expression pattern in vegetative tissues in response to water-deficit and ABA*

A 20-kD polypeptide was detected in the rice shoots from different rice varieties submitted to abiotic stress treatments (Fig. 4). The protein was more strongly induced by dehydration than by salinity or cold stress (Fig. 4). As the response to dehydration was apparently the most accentuated, we further focused on the response to water-deficit. The water-stress response was then induced in rice seedlings treated with polyethylene glycol (PEG), at different times of exposure, and compared between a drought-tolerant and a -sensitive genotype (Fig. 5). The 20-kD protein accumulated in the roots and leaves of the tolerant variety even in the absence of stress, being induced in these same tissues in the sensitive genotype after 3 hours of water-stress (Fig. 5). Curiously, the accumulation pattern in roots and leaves seemed to occur in opposite directions, in both varieties (Fig. 5). Indeed, in ‘PSBRc1’, the protein was nearly undetected in roots after 24 hours of water-stress, whereas it attained a maximum of accumulation in the leaves. Meanwhile, in ‘IR64’ the protein was poorly accumulated in roots after 4 hours of stress, though it was strongly induced in leaves (Fig. 5). After 3 days of water-stress, the protein was still present in the leaves of ‘PSBRc1’, but by then it was no longer detected in ‘IR64’, neither in the aerial

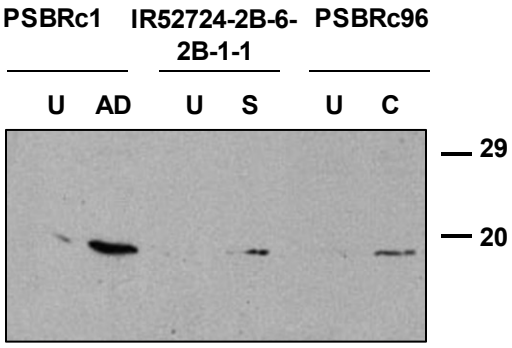

Figure 4. **Western-blot analysis of protein extracts from shoots of rice seedlings submitted to different abiotic stress treatments**. Abbreviations: Untreated control (**U**); air drying (**AD**; 3h); salt stress (**S**; 200 mM NaCl, 3h); cold stress (**C**; 4°C, 3h)

part of the plant, nor in the roots (Fig. 5). The accumulation of the 20-kD polypeptide in the two varieties also seemed to be influenced by the phytohormone ABA (Fig. 5). The protein was induced by ABA in the roots of both varieties, despite that it was observed some basal protein levels in untreated roots. In the leaves of 'PSBRc1' it was not possible to detect the accumulation of the protein in the presence of ABA, while in the leaves of 'IR64' the levels of the protein were slightly lower compared to those of control plants (Fig. 5). Again, basal levels of the protein were detected in control conditions.

3.2.3. *Protein identification by mass spectrometry*

To determine the identity of the 20- and 29-kD polypeptides, total embryo protein extracts were separated by 1-DE using the Tris-tricine buffer system (Shägger and Von Jagow, 1987), since it is suitable for the separation of low molecular weight proteins. Total embryo protein extracts were run on 1-D large-format gels (26x20x0.1cm) thus ensuring a greater separation of proteins (data not shown). After Western-blot analysis, the 20- and 29-kD bands were isolated from a preparative gel stained with CBB G250, to be identified by mass spectrometry (MS). The analysis by tandem MS (MS/MS)

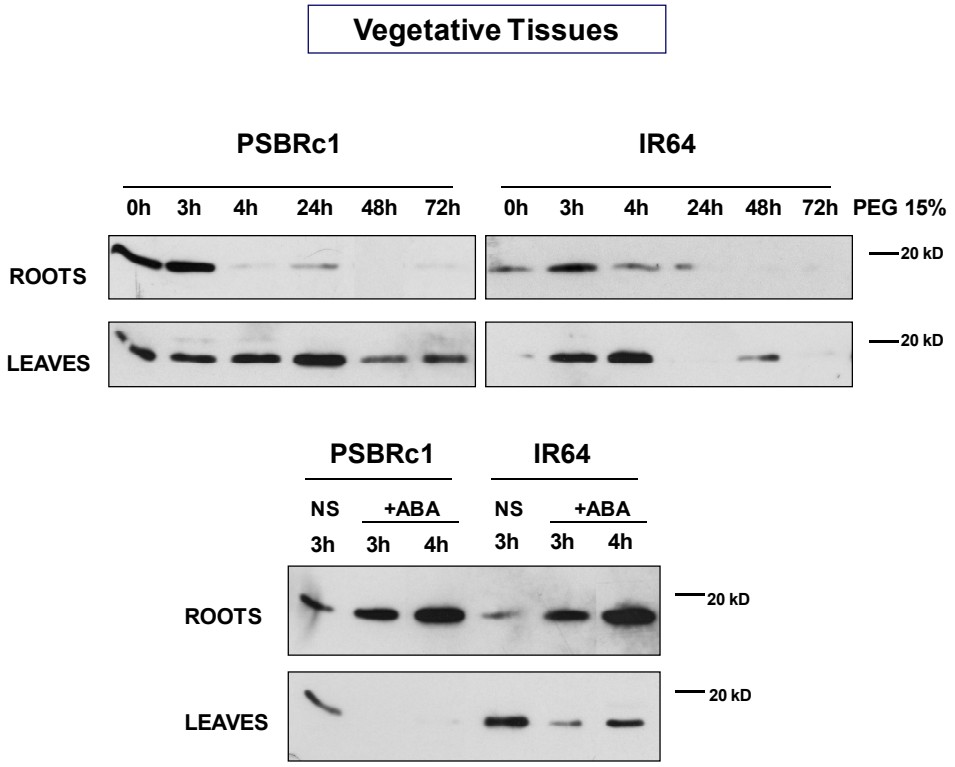

Figure 5. **Comparison of water-stress and ABA responses between a drought-tolerant ('PSBRc1') and a drought-sensitive genotype ('IR64')**. **Upper panel**: the anti-DBF1 antibody cross-reacted with a polypeptide of about 20-kD in the roots and leaves of seedlings submitted to water-stress (induced with 15% PEG). **Lower panel**: analysis of the expression pattern in seedlings treated with 100µM ABA; control plants were maintained in nutrient solution (NS) without ABA, for 3 hours.

revealed that the 29-kD protein band corresponded to a glutelin type-A 1 precursor from *O. sativa* (ssp. *japonica*) (Ac. P07728), being identified 6 peptides matching significantly the protein and covering 21% of the sequence (Fig. 6). However, the peptides digested from the 20-kD band did not allow the unequivocal identication of the protein by MS.

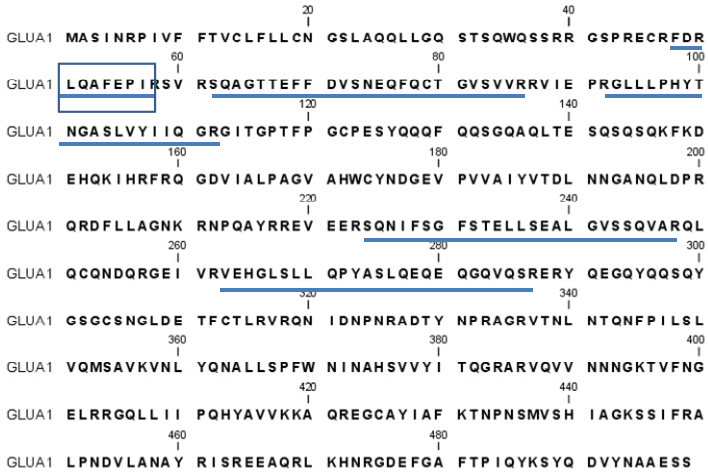

Figure 6. **Amino acid sequence of glutelin type-A 1 precursor**. The 29-kD rice embryo band was digested with trypsin and analysed by MS/MS. The resultant matched peptides that allowed protein identification are underlined; overlapping peptides are boxed.

3.3. 2-DE Western-blot analysis of rice mature embryo proteins cross-reacting with anti-DBF1

Embryo total proteins from six rice varieties were separated by 2-DE and analysed by Western-blot. The expression profile of the embryo proteins recognised by the anti-DBF1 antibody was compared across rice genotypes with contrasting responses to drought, high salinity and low temperature (Fig. 7A). Several basic embryo protein spots with molecular mass close to 20 kD (herein referred as 20-kD to simplify) and p*I* between 6.8 and 9.2 cross-reacted with the antibody (Fig. 7A). However, the less basic protein form with a p*I* close to 6.8 was only detected in the tolerant varieties, while most basic spots with p*I* close to 9.2 were detected in both tolerant and sensitive genotypes (Fig. 7A). Moreover, other rice protein spots with an electrophoretic mobility between 29 and 36 kD, and p*I* ranging from 5.6 to 9.2 also cross-reacted with the anti-DBF1 antibody (Fig. 7A).

We further compared the 2-D expression profile of rice and maize embryo proteins immunoreacting with anti-DBF1 (Fig. 7). The overall pattern

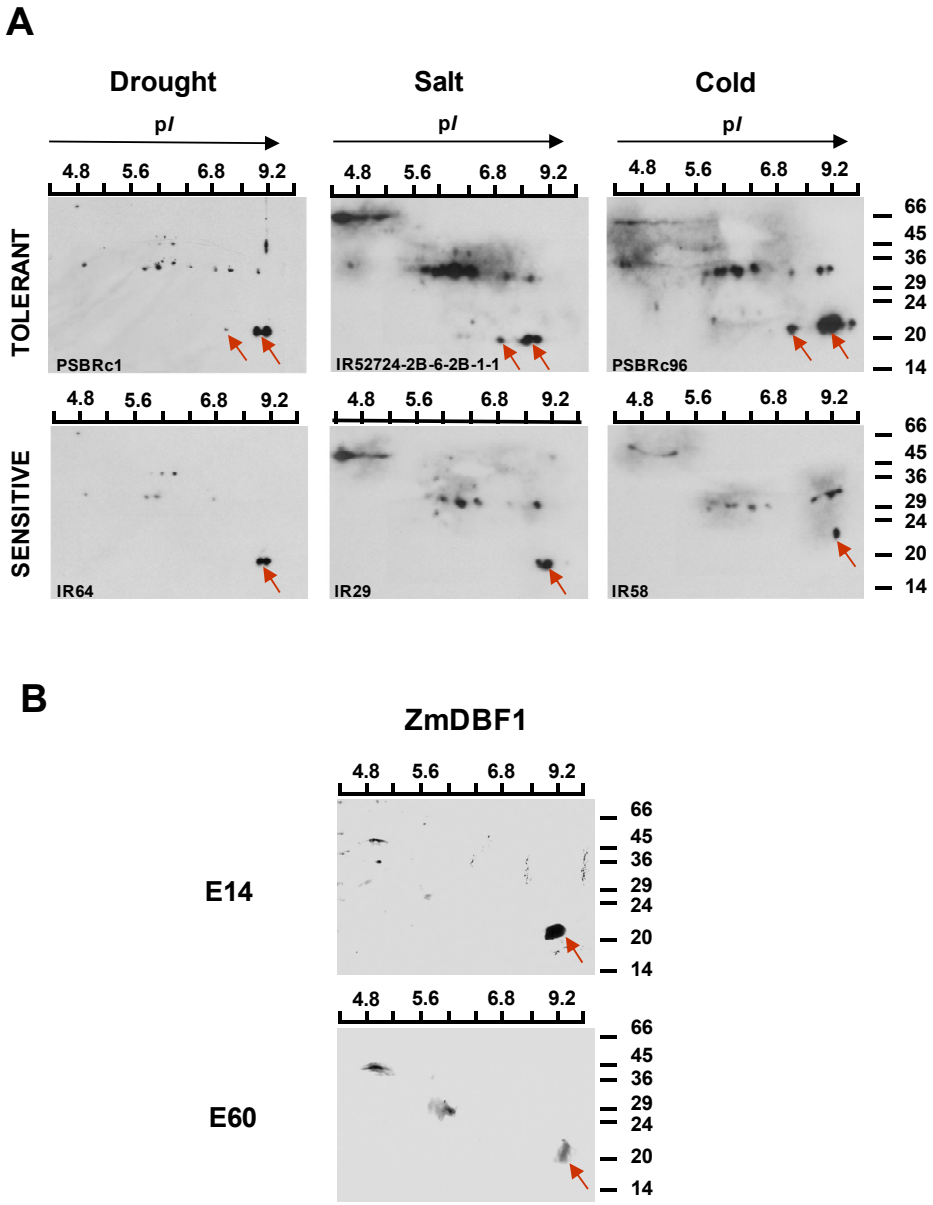

Figure 7. **Expression pattern of embryo proteins separated by 2-DE cross-reacting with anti-DBF1**. **A**. An additional spot of about 20 kD with an approximate p*I* of 6.8 was observed in rice tolerant genotypes when compared to sensitive ones. DBF1 antiserum cross-hybridized with other rice proteins with M*r* close to 29 and 36-kD. **B**. The anti-DBF1 antibody cross-reacted with a protein spot of about 20-KD in both immature and mature maize embryos. Other abundant protein spots with M*r* close to 29 and 45 kD were detected in mature embryos. E14, maize embryos 14 days after pollination (DAP); E60, maize embryos 60 DAP. Arrows indicate basic protein spots of about 20 kD.

of immunoreactive proteins was quite similar across the rice and maize embryos (Fig. 7). In maize embryos, similarly to rice, the anti-DBF1 antibody cross-hybridised with a 20-kD basic protein spot (Fig. 7B). The cross-reaction not only happened in mature embryos (60 DAP), but also in immature ones (14 DAP) (Fig. 7B). On the other hand, more acidic spot(s) close to 29 kD were detected in the maize mature embryos, as registered in rice (Fig. 7B). Interestingly, these 29-kD acidic spots failed to be detected in the immature maize embryos (Fig. 7B). In addition, a cross-reaction with other protein spots close to 45 kD was observed either in maize or rice embryo protein extracts (Fig. 7).

4. Discussion

After searching in Genbank for rice proteins with significant homology to DBF1, we found as the best hit a sequence (AAP56252) with 65% of identity, suggesting the existence of a putative rice homolog. A cDNA clone coding for this putative protein was found on the rice KOME database. Both nucleotide and amino acid sequence alignment indicated that the rice and maize sequences shared a high homology. The putative rice protein was thus named as 'OsDBF1'.

The maize DBF1 transcription factor belongs to the AP2/EREBP superfamily, being included in the DREB subfamily (Sakuma *et al*., 2002), or alternatively in the ERF family, according to a more recent classification (Nakano *et al*., 2006). Kizis and Pagès (2002) proposed that DBF1 evolved in a divergent way to DREB1-type and DREB2-type proteins, thus not considering DBF1 and DREBs as homologs. Previous phylogenetic analyses grouped DBF1 together with the *Arabidopsis* RAP2.4 protein in the A-6 subgroup of DREB proteins, separated from DREB1-type (subgroup A-1) and DREB2-type (subgroup A-2) transcription factors (Sakuma *et al*., 2002; Qin *et al*., 2004; Nakashima and Yamaguchi-Shinozaki., 2006). According to the new classification of Nakano *et al*. (2006), the A-6, A-1 and A-2

subgroups of the DREB subfamily are now included in Groups I, III and IV of the ERF family, respectively.

In order to clarify the phylogenetic relationship between the putative OsDBF1 and other members of the DREB subfamily, we performed a phylogenetic analysis by including other proteins from monocots (barley, maize, rice and wheat) and *Arabidopsis*, restrincting the analysis to members of A-1 (DREB1), A-2 (DREB2) and A-6 subgroups. The putative OsDBF1 was the most closely related to the maize DBF1, followed by the putative wheat TaDBF1 and the RAP2.4 protein from *Arabidopsis*, hence indicating a strong relationship between OsDBF1 and DBF1. The comparison of the amino acid sequence of DBF1 with the putative OsDBF1 demonstrated that these two proteins not only shared a significant homology within the AP2 conserved domain, but also outside of it. However, when comparing OsDBF1 with other DREB proteins, the homology shared at the amino acid level was much lower even within the AP2 domain. The DBF1 and RAP2.4 TFs were found to cluster independently from DREB1- and DREB2-type proteins (that in turn also grouped in distinct clades), as previously reported (Sakuma *et al*., 2002; Qin *et al*., 2004; Nakano *et al*., 2006; Nakashima and Yamaguchi-Shinozaki., 2006). Since the putative OsDBF1 and TaDBF proteins clustered together with DBF1 and RAP2.4, they were classified as new members of the DREB A-6 subgroup/Group I of ERF proteins (Sakuma *et al*., 2002; Nakano *et al*., 2006). The proteins included in this group are here proposed to be designated as ‘DBF’-type proteins, distinguishable from the commonly used ‘DREB1’ and ‘DREB2’ denominations. Curiously, Xue and Loveridge (2004) reported that HvDRF1 shared a significant homology with DBF1, though it clustered together with other members of the DREB2 subgroup, instead with DBF proteins. While the function of ERF proteins belonging to Groups III and IV has been extensively studied, being involved in freezing, salt and drought tolerance, the function of TFs belonging to Group I has received much less attention (Nakano *et al*., 2006). Hence, the characterisation of rice proteins with

homology to DBF1 may contribute to further elucidate about the function of this type of TFs.

The full-length cDNA clone (AK105725) corresponding putatively to *OsDBF1* contains a longest ORF of 280 amino acids, encoding a protein with a predicted molecular mass of 29.4 kD. Two rice proteins from mature embryos - one of 29 kD and another of about 20 kD - were immunoreactive with the antisera raised against the maize DBF1 transcription factor. Nevertheless, the 29-kD polypeptide was not detected in vegetative tissues, neither in unstressed conditions nor after abiotic stress treatments. Further analyses by MS revealed that the 29-kD polypeptide corresponded to glutelin type-A 1 precursor, a seed storage protein abundant in rice embryos (see Chapter II). Therefore, the 20-kD polypeptide was the stronger candidate for putative OsDBF1 protein. On the other hand, this polypeptide migrated at approximately 20 kD, a slightly lower M*r* than that predicted for the putative OsDBF1 protein (i.e. 29-kD). In addition, 2-D Western blot analysis showed that the anti-DBF1 antibody recognised a basic protein spot with a M*r* of about 20 kD and p*I* close to 9.2 in both maize and rice embryos. This showed that the antibody recognised identical proteins in maize and in rice, supporting that the 20-kD rice polypeptide is homologous to DBF1. The data taken together, strongly suggests that this 20-kD protein is most probably the one predicted to be encoded by the putative *OsDBF1* gene with the accession number 'AK105725'. However, as we were not able to identify the 20-kD protein band by MS, we cannot exclude the hypothesis that it may correspond to another gene product sharing a high homology with *DBF1*, also belonging to the 'DBF'-type subgroup of proteins.

The expression pattern of the 20-kD protein recognised by the anti-DBF1 antiserum was further characterised regarding the response to abiotic stress. In maize seedlings, the *DBF1* gene and its protein accumulate in response to drought, salinity and ABA, but are not induced by cold (Kizis and Pagès, 2002). In rice seedlings, the immunoreactive 20-kD protein was induced by water-deficit, salinity, ABA and cold as well. The 20-kD rice protein was

hardly detected in the roots after 4 hours of water stress, whereas it was strongly accumulated in the leaves in the same period, pointing to tissue-specific expression. At prolonged stress periods, i.e. after 2 and 3 days of water-stress imposition, the protein was no longer detected in roots although it was still possible to observe it in the leaves. The more transient accumulation in roots as compared to more prolonged expression in leaves, suggests that this putative transcription factor is involved in a well-coordinated response to water stress at the whole-plant level. In maize seedlings submitted to water stress, a detailed time-course showed that the *DBF1* transcript was strongly accumulated after 3 hours of stress, with higher levels of accumulation in roots than in leaves, although some basal mRNA levels were observed in both tissues (Kizis and Pagès, 2002). The expression pattern of the 20-kD rice protein observed in vegetative tissues during the first 3 hours of water-stress seems to agree with that reported for *DBF1* in maize water-stressed seedlings. In addition, the expression of the 20-kD rice protein was apparently induced by ABA in the roots, but not in the leaves pointing once again to tissue-specific expression. This further indicates that both ABA-dependent and independent-pathways may be involved in the expression of the 20-kD rice protein in response to water-stress. Interestingly, the transcript of *DBF1* was detected in maize *viviparous* mutants deficient in ABA (*vp2)*, suggesting that despite being induced by ABA, the accumulation of the gene was not only dependent on ABA (Kizis and Pagès, 2002). It was also demonstrated that the ABA-inducible genes *rab17* and *rab28* accumulated during the late embryogenesis in maize *viviparous* mutants impaired in ABA responses (Pla *et al*., 1989; Pla *et al*., 1991). These data seem to indicate that more than one mechanism may influence the level of expression of ABA-responsive genes. In the particular case of the 20-kD rice protein, only the precise identification of the corresponding gene and the analysis of the *cis*-elements present in its promoter, can elucidate about the possible regulation of gene expression

through ABA-dependent and -independent pathways in response to water-stress.

We compared the expression of the putative rice TF between genotypes with contrasting response to abiotic stress aiming to get further clues on the molecular mechanisms involving stress tolerance. The 20-kD rice polypeptide was differentially expressed between 'PSBRc1' - a drought-tolerant genotype – and 'IR64' a high yielding rice cultivar, but drought-sensitive. The protein was induced in 'IR64' in the first 3 hours of water stress, whereas a high constitutive level of expression of the protein was observed in 'PSBRc1', either in the roots or in the leaves. The constitutive level expression of this stress-responsive protein in the tolerant genotype could be related to increased stress tolerance. In the comparison of expression profiles between *Arabidopsis thaliana* - considered as salt sensitive - and its close relative *Thellungiella halophila* extremely tolerant to salt, in normal and salt stress conditions it was revealed that many genes with a role in stress tolerance were stress-inducible in *Arabidopsis*, but were expressed at high constitutive levels in pre-stress conditions in *Thellungiella* (Taji *et al*., 2004). Further studies based on the comparison of metabolite profiling between *Arabidopsis* and *Thellungiella,* reinforced the preparedness of *Thellungiella* towards salt stress, by disposing 'stress-anticipating' mechanisms, evidenced by high constitutive levels of metabolites acting as signalling molecules or osmolytes (Gong *et al*., 2005). Particular attention was given to the higher basal level of ascorbate peroxidase in 'Pokkali' than in 'IR29' (salt tolerant and sensitive rice genotypes, respectively), suggesting that constitutive levels of antioxidant capacity could play an important role in stress tolerance (Salekdeh *et al*., 2002). These data supports that the constitutive expression of the 20-kD protein in the drough-tolerant genotype may be related to stress tolerance. High constitutive levels of this putative rice DRE-binding protein could make part of the preparedness of 'PSBRc1' to face adverse environmental conditions. In addition, in the shoots of 'PSBRc1' the accumulation of the protein was maintained at prolonged

periods of water stress i.e after 24h and 72h, whereas in 'IR64' the protein was not detect at these time periods. But curiously it was detected in water-stressed shoots after at 48 h. This may indicate that at more extended periods of stress (after 24 hours), the protein accumulates more slowly and/or to a less extent in the sensitive *vs.* the tolerant variety. Nonetheless, the protein seemed not to accumulate in the roots at longer periods of water stress, either in the tolerant or sensitive genotype, indicating a clear difference in the regulation of the protein between the two organs.

When comparing the 2-D patterns of embryo proteins from genotypes with contrasting stress tolerance, the anti-DBF1 antibody recognised an additional basic 20-kD protein spot (p*I* close to 6.8) in the extracts of the tolerant genotypes (Fig. 7A). The 20-kD polypeptide was assumed to have several isoforms with distinct p*I*, with most basic forms that were present in all genotypes (p*I* close to 9.2) and a less basic form (p*I* close to 6.8) that was detected only in the tolerant ones. However, it cannot be discarded that the less basic form could constitute an unrelated protein in respect to other 20-kD protein spots with p*I* close to 9.2. Due to its very low abundance, we were not able to detect these distinct spots on 2-D gels stained with Coomassie, and consequently we could not identify it by MS.

The sequence alignment of DBF1 with the putative OsDBF1 protein showed that both possess a serine-rich region following the AP2 domain that could act as possible phosphorylation site. Reversible phosphorylation can modulate the activity of TFs by influencing their subcellular localization, their transactivating capacity and/or DNA-binding activity (Agarwal *et al*., 2007). In the majority of the DREB proteins, the activation by phosphorylation does not seem to be necessary due to the transactivating capacity of specific domains rich in acidic residues. However, there is some evidence that in particular cases phosphorylation/dephosphorylation plays an important role in the binding to the DRE element. As the over-expression of *DREB2* in transgenic *Arabidopsis* plants did not improve stress tolerance, it was argued that DREB2 proteins needed post-translational modification e.g,

phosphorylation to become activated (Liu *et al*., 1998; Sakuma *et al*., 2006). On the other hand, and most surprising, it was found that phosphorylation regulated negatively the DNA-binding activity of a DREB2A protein isolated from *Pennisetum glaucum* (a stress tolerant food grain crop) (Agarwal *et al*., 2007). Although we do not know the exact nature of the distinct forms of the 20-kD in the rice embryo, we can speculate that the less basic (more acidic) protein with a p*I* close to 6.8, could be due to post-translational modification, such as phosphorylation. Most interesting is the presence of this form in the embryos of the tolerant genotypes, which points for a possible role in stress tolerance. Future work would be necessary to elucidate if the nature of the less basic 20-kD rice protein could be related to post-translational modification by phosphorylation or with other type of modification. But first, it would be important to check its presence in vegetative tissues and the possible accumulation differences in response to abiotic stress.

Briefly, though we could not acertain the precise identity of the 20-kD rice protein, we suggest that it corresponds to a 'DBF'-type protein, due to its cross-reaction with anti-DBF1 and induction pattern by abiotic stress. DREB1-type proteins are mainly involved in cold response, whereas DREB2-type proteins are essentially responsive to osmotic stress, typically in ABA-independent pathways (despite few exceptions) (Nakashima and Yamaguchi-Shinozaki, 2006). Interestingly, the 20-kD rice protein was responsive to water-deficit, salinity and cold, and apparently to ABA, thus revealing unique features among the DREB proteins and reinforcing the importance of the isolation and characterisation of DBF-type proteins in rice. Once clearly identified, this rice protein may constitute a good candidate for further cereal crop improvement.

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6. Literature cited:

Agarwal P, Agarwal PK, Nair S, Sopory SK, Reddy MK (2007) Stress-inducible *DREB2A* transcription factor from *Pennisetum glaucum* is a phosphoprotein and its phosphorylation negatively regulates its DNA-binding activity. Mol Genet Genomics 277(2): 189-198.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389-3402.

Busk PK, Jensen AB, Pagès M (1997) Regulatory elements in vivo in the promoter of the abscisic acid responsive gene *rab17* from maize. Plant J 11(6): 1285-1295.

Chen JQ, Meng XP, Zhang Y, Xia M, Wang XP (2008) Over-expression of *OsDREB* genes lead to enhanced drought tolerance in rice. Biotechnol Lett 30 (12): 2191-2198.

Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. Plant J 33:751-763

Gao G, Zhong Y, Guo A, Zhu Q, Tang W, Zheng W, Gu X, Wei L, Luo J (2006) DRTF: a database of rice transcription factors. Bioinformatics 2006, 22(10):1286-1287.

Goday A, Sánchez-Martinez D, Gómez J, Puigdomènech P, Pagès M (1988) Gene expression in developing *Zea mays* embryos: regulation by abscisic acid of a highly phosphorylated 23- to 25-kD group of proteins. Plant Physiol 88: 564-569.

Gómez-Porras JL, Riaño-Pachón DM, Dreyer Y, Mayer JE, Mueller-Roeber B (2007) Genome-wide analysis of ABA-responsive elements ABRE and CE3 reveals divergent patterns in *Arabidopsis* and rice. BMC Genomics 8: 260.

Gong Q, Li P, Ma S, Rupassara SI, Bohnert HJ (2005) Salinity stress adaptation competence in the extremophile *Thellungiella halophila* in comparison with its relative *Arabidopsis thaliana*. Plant J 44: 826-839.

Hobo T, Asada M, Kowyama Y, Hattori T (1999) ACGT-containing abscisic acid response element (ABRE) and coupling element 3 (CE3) are functionally equivalent. Plant J 19 (6): 679-689.

Kizis D and Pagès M (2002) Maize DRE-binding proteins DBF1 and DBF2 are involved in *rab17* regulation through the drought-responsive element in an ABA-dependent pathway. Plant J 30(6): 679-689.

Lin RC, Park HJ, Wang HY (2008) Role of *Arabidopsis* RAP2.4 in regulating light- and ethylene-mediated developmental processes and drought stress tolerance. Mol Plant 1(1): 42-57.

Liu X, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, **Shinozaki K** (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 binding domain separate two cellular signal transduction pathways in drought- and low temperature responsive gene expression, respectively, in *Arabidopsis*. Plant Cell 10: 1391-1406.

Mundy J, Chua NH (1988) Abscisic acid and water-stress induce the expression of a novel rice gene. EMBO J 7(8): 2279-2286.

Nakano T, Suzuki K, Fujimura T, Shinshi H (2006) Genome-wide analysis of the ERF gene family in *Arabidopsis* and rice. Plant Physiol 140(2): 411-432.

Nakashima K, Yamaguchi-Shinozaki K (2006) Regulons involved in osmotic stress-responsive and cold stress-responsive gene expression in plants. Physiol Plantarum 126: 62-71.

Nakashima K, Ito Y, Yamaguchi-Shinozaki K (2009) Transcriptional regulatory networks in response to abiotic stresses in *Arabidopsis* and grasses. Plant Physiol 149: 88-95.

Narusaka Y, Nakashima K, Shinwari ZK, Sakuma Y, Furihata T, Abe H, Narusaka M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Interaction between two *cis*-acting elements, ABRE and DRE, in ABA-dependent expression of *Arabidopsis rd29A* gene in response to dehydration and high salinity stresses. Plant J 34(2): 137-148.

Pla M, Goday A, Vilardell J, Gómez J, Pagès M (1989) Differential regulation of ABA-induced 23-25 kDa proteins in embryo and vegetative tissues of the *viviparous* mutants of maize. Plant Mol Biol 13(4): 385-394.

Pla M, Gómez J, Goday A, Pagès M (1991) Regulation of the abscisic acid-responsive gene *rab28* in maize viviparous mutants. Mol Gen Genet 230(3): 394-400.

Qin F, Sakuma Y, Li J, Liu Q, Li YQ, Shinozaki K, Yamaguchi-Shinozaki K (2004) Cloning and functional analysis of a novel DREB1/CBF transcription factor involved in cold-responsive gene expression in *Zea mays* L. Plant Cell Physiol 45(8): 1042-1052.

Riechmann JL, Meyerowitz EM (1998) The AP2/EREBP family of plant transcription factors. Biol Chem 379(6): 633-646.

Sakuma Y, Liu Q, Dubouzet JG, Abe H, Shinozaki K, Yamaguchi-Shinozaki K (2002) DNA-binding specificity of the ERF/AP2 domain of *Arabidopsis* DREBs, transcription factors involved in dehydration- and cold inducible gene expression. Biochem Biophys Res Commun 290: 998-1009.

Sakuma Y, Maruyama K, Osakabe K, Qin F, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006) Functional analysis of an *Arabidopsis* transcription factor, *DREB2A*, involved in drought-responsive gene expression. Plant Cell 18: 1292-1309.

Saleh A, Lumbreras V, Lopez C, Dominguez-Puigjaner E, Kizis D, Pagès M (2006) Maize DBF1-interactor protein 1 containing an R3H domain is a potential regulator of DBF1 activity in stress responses. Plant J 46: 747-757.

Salekdeh GH, Siopongco J, Wade LJ, Ghareyazie B, Bennett J (2002) A proteomic approach to analyzing drought- and salt-responsiveness in rice. Field Crops Res 76: 199-219.

Schägger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range of 1 to 100 kDa. Anal Biochem 166: 368-37.

Shen QJ, Zhang P, Ho TH (1996) Modular nature of abscisic acid (ABA) response complexes: composite promoter units that are necessary and sufficient for ABA induction of gene expression in barley. Plant Cell 8 (7): 1107-1119.

Taji T, Seki M, Satou M, Sakurai T, Kobayash Mi, Ishiyama K, Narusaka Y, Narusaka M, Zhu JK, Shinozaki K (2004) Comparative genomics in salt tolerance between *Arabidopsis* and *Arabidopsis*-related halophyte salt cress using *Arabidopsis* microarray. Plant Physiol 135: 1679-1709.

Vilardell J, Goday A, Freire MA, Torrent M, Martínez MC, Torné JM, Pagès M (1990) Gene sequence, developmental expression and protein phosphorylation of RAB-17 in maize. Plant Mol Biol 14: 423-432.

Wang Q, Guan Y, Wu Y, Chen H, Chen F, Chu C (2008) Overexpression of a rice *OsDREB1F* gene increases salt, drought, and low temperature tolerance in both *Arabidopsis* and rice. Plant Mol Biol 67: 589-602.

Wu C-Q, Hu H-H, Zeng Y, Liang D-C, Xie K-B, Zhang J-W, Chu Z-H, Xiong L-Z (2006) Identification of novel stress-responsive transcription factor genes in rice by cDNA array analysis. J Integr Plant Biol 48 (10): 1216-1224.

Xue G-P, Loveridge CW (2004) *HvDRF1* is involved in abscisic acid-mediated gene regulation in barley and produces two forms of AP2 transcriptional activators, interacting preferably with a CT-rich element. Plant J 37: 26-339.

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel *cis*-acting element in *Arabidopsis* gene is involved in responsiveness to drought, low temperature, or high-salt stress. Plant cell 6: 251-264.

Chapter IV

Identification of water-stress responsive genes by cDNA-AFLP and comparison of their expression patterns in rice varieties with contrasting abiotic stress tolerance

Farinha AP [1,2], Lumbreras V [2], Pagès M [2] and Oliveira MM [1].
Identification of water-stress responsive genes by cDNA-AFLP and comparison of their expression patterns in rice varieties with contrasting abiotic stress tolerance (under revision to be submitted)

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AP Farinha declares to have participated actively in this work, by performing the experimental design, all the laboratory work (including the whole cDNA-AFLP experiments, gene cloning, data analysis, Northern and RT-PCR analysis) and manuscript writing.

Abstract

We used cDNA-AFLP, as a most powerful and robust transcript profiling methodology, to identify genes differentially expressed under water-deficit conditions. The cDNA-AFLP expression patterns were validated by reverse Northern and/or RT-PCR analysis. The expression profiles of six water-deficit responsive genes were further analysed after seedling exposure to salinity and cold stress treatments, across genotypes with contrasting responses to abiotic stress. The overall gene expression analysis indicated that the water-deficit responsive genes also responded to salinity, cold and ABA, in at least one of the compared genotypes. All cDNA clones presented significant homology to rice genes e.g., a calcium-binding EF hand family protein, a C-type cyclin and SC35-like splicing factor. Most of the identified genes seemed to be implicated in cellular signal transduction. This is not surprising since we focused on the early events of the stress response. In addition, the results evidenced both specific and common abiotic stress responses, pointing to gene expression patterns in tolerant varieties that may be related to more successful stress adaptation. The overall approach allowed the identification of critical genes with a potential role in abiotic stress tolerance.

Identification of water-stress responsive genes by cDNA-AFLP and comparison of their expression patterns in rice varieties with contrasting abiotic stress tolerance

1. Introduction

The management of water resources in the next decades will be challenging, given the need to meet both food security and environmental sustainability (CAWMA, 2007). A fifth of the world's population lives in areas with reduced water availability (CAWMA, 2007). But water scarcity can also occur where water is apparently abundant, when the water resources are overcommited to many competing uses, thus lacking enough water to keep pace with both human demands and environmental flow needs (CAWMA, 2007). A better water management is definitely an essential issue on the debate how to mitigate the negative consequences of water scarcity.

The lack of water is thus a threat to the production of food for millions of people. Rice is particularly susceptible to drought stress and high yielding production can only be achieved when water supply is adequate (Nguyen, 2008). In the near future it is expected that rice varieties still provide high yielding rates though with less water due to the increasing vulnerability of the water resources. Therefore, new irrigation systems for rice must be developed, together with varieties possessing increased drought tolerance. Genes involved in drought response and associated with stress tolerance must be available for crop improvement, either through genetic engineering or marker-assisted selection. In this sense, the identification of drought-responsive genes is far from being fulfilled, and understanding the way that plants in general, and rice in particular cope with water scarcity, is primordial for food production.

In the last decade, numerous rice genes have been identified in response to drought through genome-wide expression analysis (Cooper *et al*., 2003; Rabbani *et al*., 2003; Wang *et al*., 2007; Zhou *et al*., 2007;

Degenkolbe *et al*., 2009), most of them being microarray-based. Alternative approaches based on DNA sequencing or cDNA fragment analysis, though less commonly used, have also demonstrated its usefulness in rice transcript profiling under drought stress (Yang *et al*. 2004; Rodriguez *et al*., 2006; Gorantla *et al*., 2007). The cDNA-AFLP (amplified fragment length polymorphism) is a gel-based transcript profiling method that does not require prior sequence information, and therefore it can also be employed in gene discovery (Bachem *et al*., 1996; 1998). One major advantage of cDNA-AFLP over oligonucleotide or cDNA microarrays is the relatively low start-up costs (Breyne and Zabeau, 2001; Donson *et al*., 2002; Vuylsteke *et al*., 2007). The cDNA-AFLP technique is highly reproducible, allows the detection of rare transcripts, besides that needs small amounts of mRNA (Breyne and Zabeau, 2001; Donson *et al*., 2002; Vuylsteke *et al*., 2006; 2007). Nevertheless, the whole technical procedure is time consuming and labour intensive, requiring fragment recovery from the gel, re-amplification, subsequent cloning and sequencing (Breyne and Zabeau, 2001).

Here we used cDNA-AFLP, as a most powerful and robust transcript profiling methodology to identify genes differentially expressed under water stress. Six water-deficit responsive genes whose differential expression was validated by Northern and/or RT-PCR analysis, proved to be also responsive to salinity and low temperature, besides to ABA. The expression patterns of the identified genes were compared among six rice genotypes with contrasting stress tolerance either to drought, salinity or cold. The differences found in gene expression suggest an involvement in abiotic stress tolerance. The rice genes identified in this work, could therefore constitute good candidates for further assays aiming for crop improvement.

2. Materials and Methods

2.1. Plant material, seed germination and stress treatments

Seeds of rice (*Oryza sativa* L.) varieties with differential response to abiotic stress were used in this study, namely ‘PSBRc1’ (drought tolerant;

ssp. *indica*), 'IR64' (drought sensitive; ssp. *indica*), 'IR52724-2B-6-2B-1-1' (salt tolerant; ssp. *indica*), 'IR29' (salt sensitive; ssp. *indica*), 'PSBRc96' (cold tolerant; ssp. *japonica)* and 'IR58' (cold sensitive; ssp. *indica*). Seed material was obtained from the IRRI institute (Los Baños, Philippines), following the Material Transfer Agreement for IRRI-developed seeds and the Philippine phytosanitary rules: seeds fumigation with Phostoxin ($2g/m^3$) for 72 hours at 28° C, followed by seed dressing with Benlate 50WP and Dithane M-45 (0.3% by seed weight).

Seeds were surface-sterilised with 70% EtOH for 1 min, washed three times with bidestilled water (10 min each), treated with 1% sodium hipocloride for further 5 min, rinsed again and then left for one hour in sterilised bidestilled water. All the processes were carried out with shaking. After sterilisation, rice seeds were kept on moistened filter paper in Petri dishes for 2 days, in darkness. Pre-germinated seeds were then transferred to autoclaved filter paper towels partially submerged in 1:4 (v/v) Murashige and Skoog medium (1962) (Supplemental Fig. S1). The pH of the solution was adjusted every two days to 5.2 and nutrient solution was changed every seven days. Seeds were germinated in a growth chamber under 16 hours light (150 μmol m^{-2} s^{-1}) and 8 hours dark, at 28° C and 26° C, respectively.

Stress treatments were performed by immersing the roots of fourteen days-old seedlings (with 3 leaves) in fresh nutrient solution. Water-deficit was induced with 15% PEG-6000 (Fluka), whereas salt stress was induced with 200 mM NaCl. Cold treatment was performed by adjusting the temperature of the growth chamber to 4°C. Water-stress was induced for 3, 4 and 72 hours, while salt stress was applied for 3 and 72 hours, and cold stress imposed for 3 and 24 hours. Rice seedlings were also treated with 50 μM ABA (cis/trans isomer, Sigma-Aldrich) for 6 hours. Rice tissues were quickly frozen in liquid nitrogen and stored at -80°C for RNA extraction.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from the shoots of 14 days-old seedlings using the RNeasy Plant Mini Kit (Qiagen). Poly(A) RNA was purified from total RNA using the PolyATract® mRNA Isolation Systems III, IV (Promega), following the manufacturer's instructions. Poly(A^+) RNA (0.75 μg) was used for first-strand cDNA synthesis by reverse transcription with an oligo $(dT)_{12-18}$ primer, M-MLV reverse transcriptase (Invitrogen) and RNaseOUT™ (Invitrogen). Second-strand synthesis was performed by strand displacement with *E. coli* DNA ligase, *E. coli* DNA polymerase I and RNAse H. All reagents were supplied from Invitrogen. The double-stranded cDNA was purified using the QIAquick spin columns (QIAquick PCR Purification Kit, QIAGEN), being resuspended in 30 μL. A second and independent experiment was performed to ensure both biological and technical replicates.

2.3. cDNA-AFLP

The cDNA-AFLP procedure was adapted from Vos *et al*. (1995) and Bachem *et al*. (1996; 1998). Double-stranded cDNA samples obtained from untreated and 3 h water-stressed shoots of 'PSBRc1' and 'IR64' were digested with 10U *Eco*RI (Roche) and 10U *Taq*I (Fermentas) for 3 hours at 37°C. The *Eco*RI (E) (5 μM) and *Taq*I (T) (50 μM) adaptors were ligated onto cDNA digested ends by using T4 DNA ligase (Roche) for 3 hours at 37°C. The reaction volume containing the digested and annealed cDNA was diluted 1:5. An aliquot (5 μL) was used for pre-amplification with the *Eco*RI and *Taq*I primers, in 20 μL reaction volume. Pre-amplification reactions were carried out with non-selective primers and were initiated at 94°C for 3 min, followed by 25 cycles at 94°C for 30 s, 56°C for 1 min, 72°C for 1 min, plus 5 min at 72°C. The amplification products were checked on 1% agarose gels and a DNA smear was observed from 1000 to 100 bp (data not shown).

The pre-amplification reaction volume was diluted 1:20 and a 5 μL aliquot was used for selective amplification in 20 μL reaction volume. After initial

denaturation (94°C for 3 min), 11 cycles were performed with touchdown annealing temperature, reducing from 65°C to 56°C in 0.7°C steps for 30 s, followed by other 25 cycles (94°C for 30 s, 56 °C for 30 s and 72°C for 1 min) and a final elongation step of 5 min at 72°C. Eight primer combinations were used for selective amplification using two or three selective nucleotides. The combinations were: E-AC/T-CG, E-GC/T-AC, E-TG/T-AT, E-AG/T-TCT, E-AG/T-TC, E-CA/T-GC, E-CAA/T-GC and E-CG/T-AC. The sequences of adaptors and primers (Invitrogen) used are listed in Table I. All DNA amplification reactions were performed in a MJ Research PTC 200 Thermal Cycler (MJ Research, Inc, USA) with Takara *Ex Taq* ™ DNA polymerase (TAKARA Bio Inc, Japan).

2.3.1. Polyacrylamide gel electrophoresis

The amplified fragments were separated on a DNA sequencing gel (6% acrylamide gel (v/v) of acrylamide:bis-acrylamide 19:1 (Bio-Rad), 7M urea in 1x TBE), using the Sequi-Gen GT Sequencing Cell (Bio-Rad, Hercules, CA, USA). Gels (0.4 mm thick x 21 cm large x 50 cm high) were run at a constant power of 50 W until the xylene cianol migrated ⅔ in the gel. The amplified fragments were visualised using a non-radioactive silver-staining method (Bassam *et al*., 1991; Bassam and Gresshoff, 2007).

2.3.2. Isolation and sequencing the transcript-derived fragments (TDFs)

The TDF fragments from the 'PSBRc1' variety were isolated by gently scratching over the silver-stained band, using a sterile disposable needle prewetted with PCR MasterMix (Stumm *et al*., 1997). After scratching the

Table I. ***Sequences of adaptors and primers used in the cDNA-AFLP experiments***

***Taq*I Adaptor** :	5'-GACGATGAGTCCTGAC-3'
	5'-TACTCAGGACTGGC-3'
***Eco*RI Adaptor**:	5'-CTCGTAGACTGCGTACC-3'
	5'-CATCTGACGCATGGTTAA-3'
***Taq*I pre-amplification primer**:	5'-GACGATGAGTCCTGACCGA-3'
***Taq*I amplification primers**:	**+TC**: 5'-GATGAGTCCTGACCGATC-3'
	+TCT: 5'-GATGAGTCCTGACCGATCT-3'
	+AT: 5'-GATGAGTCCTGACCGAAT-3'
	+AC: 5'-GATGAGTCCTGACCGAAC-3'
	+CG: 5'-GATGAGTCCTGACCGACG-3'
	+GC: 5'-GATGAGTCCTGACCGAGC-3'
***Eco*RI pre-amplification primer**:	5'-GACTGCGTACCAATTC-3'
***Eco*RI amplification primers**:	**+CA**: 5'-GACTGCGTACCAATTCCA-3'
	+CAA: 5'-GACTGCGTACCAATTCCAA-3'
	+AG: 5'-GACTGCGTACCAATTCAG-3'
	+TG: 5'-GACTGCGTACCAATTCTG-3'
	+GC: 5'-GACTGCGTACCAATTCGC-3'
	+AC: 5'-GACTGCGTACCAATTCAC-3'
	+CG: 5'-GACTGCGTACCAATTCCG-3'

band in the gel, the needle was placed into 20 µL standard PCR mix for 1 min and then discarded. The cDNA was re-amplified using the corresponding selective primers and following the PCR profile described for pre-amplification, except that we performed 30 cycles and the elongation step was extended to 7 min. The PCR products were checked on 1% agarose gels and purified using the QIAquick Gel Extraction Kit (Qiagen) for further cloning. TDFs were cloned either into the pCR® 2.1-TOPO® vector, using the TOPO TA cloning ® Kit (Invitrogen), or into the pTZ57R cloning vector using the InsTAclone™ PCR cloning Kit (Fermentas) (see vector

maps in Supplemental Figures S2 and S3). After plasmid purification the insert size was confirmed by PCR amplification using M13 primers. The cloned inserts were sequenced on the Sequencing Service facilities of CRAG-CSIC (Barcelona-Spain) using an ABI PRISM ® 377 DNA sequencer (Applied Biosystems). The sequences obtained were compared with NCBI nucleotide non-redundant and refseq_rna databases, using the BLAST sequence alignment program (Altschul *et al*., 1997). All sequences were submitted to the dbEST NCBI (National Center for Biotechnology Information), Bethesda, MD, USA. The GenBank accession numbers are listed in Table II.

2.4. Non-radioactive reverse Northern

The cDNA clones were amplified by PCR in 50 μL reaction volume using 100 ng of plasmid DNA and 0.75 μM of forward and reverse universal M13 primers. The thermal cycling conditions were 94°C for 2 min, followed by 30 cycles at 94°C (30 s), 55°C (1 min) and 72°C (1 min), and a final extension step of 7 min at 72°C. Aliquots of this PCR product (12 μL) were run in duplicate in the same agarose gel to allow identical transfer of TDFs to nylon membranes (Hybond-N, Amersham Biosciences) for further hybridisation with distinct cDNA probes, i.e. from control and water-stressed leaves. Membranes were baked for 2 hours at 80°C. A cDNA clone with homology to an ubiquitin conjugating enzyme (UBC) i.e. TDF13, was also loaded as a control of constitutive expression to guarantee equal labelling of untreated and stressed cDNA probes. All DNA amplification reactions were performed in a MJ Research PTC 200 Thermal Cycler (MJ Research, Inc, USA) using Expand High Fidelity *Taq* DNA polymerase (Roche).

Total RNA was obtained from the shoots of both tolerant (‘PSBRC1’) and sensitive (‘IR64’) varieties. The RNA from untreated and 3 h water-stressed seedlings, as well as cDNA synthesis, were prepared as described in section 2.2. The cDNA probes were further labelled according to Campalans *et al*.

(2001). Briefly, cDNA probes were labelled by PCR with 70 μM Digoxigenin (DIG)-11-dUTP (Roche) using as template an aliquot of 4 μL of the double-stranded cDNA digested and annealed to EcoRI and TaqI adaptors, in a reaction volume of 20 μL. The primers and PCR parameters were the same as those used for the cDNA-AFLP pre-amplification step. Filters were pre-incubated in hybridisation solution (250mM Na_2HPO_4 pH 7.2, 1mM EDTA pH 8.0, 0.5% Blocking Reagent-Roche, 20% SDS) for 2 hours at 65°C. The labelled probes were then added to this solution (3μL probe/mL hybridisation solution) and the filters were incubated overnight at 65°C. The hybridised filters were washed 3 times for 20 min in 20mM Na_2HPO_4 pH 7.2, 1mM EDTA pH 8.0 and 1% SDS at 65°C.

Chemiluminescent detection of blots was made with the CSPD reagent (Roche) following the manufacturer's instructions with minor modifications. Kodak films were exposed between 5 and 45 min. The raw images were digitalized using the ImageScanner desktop instrument and LabScan software (GE Healthcare Amersham Biosciences). Band Optical density was measured using the Scion Image software (Scion Corporation, Frederick, MD, USA) and the expression of the UBC transcript was used as internal control to normalize the signals within and between blots. Gene fold induction was determined by the ratio of normalized gene expression between treated and control plants.

2.5. Semi-quantitive RT-PCR

Total RNA of 14 days-old seedlings submitted to water-stress, cold or salinity conditions (during 3, 24 or 72 hours), as well as of untreated plants, was extracted from the shoots of distinct rice varieties, using the RNeasy Plant Mini Kit (Qiagen). The RNA (1μg) from the distinct samples was reverse-transcribed with anchored oligo-dT primer, M-MLV reverse transcriptase (Invitrogen) in the presence of RNase inhibitor (RNaseOUT™, Invitrogen) for 2 hours at 37°C, in 20 μL. The reverse-transcribed cDNA was

diluted 1:10 and an aliquot of 5 μL was used for PCR amplification. PCR was carried out with 0.5 μM of each primer, in 50 μL reaction volume, using the following cycling parameters: preliminary denaturation at 94°C for 3 min, 27 cycles at 94°C (45 s), 50° to 55° C (45 s) (the annealing temperature depended on the amplified TDF) and 72°C (1 min). A final elongation step of 5 min at 72°C was performed. See Table III to check the sequences of the primers, the size of amplicons and the annealing temperatures used.

PCR products (10 μL) were separated on 1.5% agarose gels and images were captured using the Fluor-S MultiImager system (Bio-Rad). Two independent RT-PCR reactions were performed for all RNA samples. Band optical density was measured using the Scion Image software (Scion Corporation, Frederick, MD, USA). The constitutive expression of TDF13 (with homology to the UBC gene) was used to further normalize the gene expression of differential transcripts. Hence, relative values of gene expression were calculated and gene fold induction was determined by the ratio of normalized gene expression between treated and control plants. The rice *Osmyb*4 gene encoding the Myb4 transcription factor (Vannini *et al*., 2004) was included as a control of gene expression under abiotic stress.

3. Results

3.1. Identification of differentially expressed genes by cDNA-AFLP

The cDNA-AFLP analysis was performed on the shoots from rice seedlings submitted to water deficit with polyethylene glycol (PEG) for 3 hours, aiming to identify genes involved in the early events of water-stress response. The cDNA templates of a drought-tolerant ('PSBRc1') and a drought-sensitive variety ('IR64') were amplified using eight primer combinations and visualised by silver staining. The amplified transcript-derived fragments (TDFs) ranged in length between 100 and 800 base pairs (bp), and about 40-60 scorable bands were observed for each primer combination, generating a mean of 400 fragments. However, not all

combinations of primers provided differential gene expression (see panels II and III in Fig. 1). On the other hand, the overall expression pattern was quite similar between the tolerant and the sensitive genotype (Fig. 1). About 45 TDFs were water-deficit responsive in at least one of the genotypes, either by up- or down-regulation. Twelve out of twenty-five TDFs were water-stress inducible in both varieties (e.g., TDF1), whereas four of them were apparently inducible only in 'PSBRc1' (e.g., TDF2), and nine of them only in 'IR64' (e.g., TDF8) (Fig. 1). Considering the twenty down-regulated TDFs, ten seemed to be specifically repressed in 'PSBRc1' (e.g., TDF9; Fig. 1), seven in 'IR64', and three in both varieties.

Eight TDFs showing a clear variation in band intensity, as well as other two TDFs exhibiting constitutive expression (TDF C and TDF13) were recovered from the gel for further analysis. The expression patterns of the distinct TDFs obtained by cDNA-AFLP are summarized in Table II. The ten selected fragments were reamplified and cloned before sequencing in order to prevent problems related to direct sequencing of PCR products. The accuracy of the reamplification of each fragment was confirmed by the presence of the selective nucleotides in the sequence region corresponding to the 3´- and 5´-ends of primers. All ten TDF sequences have been referenced in GenBank (accession numbers from HO058537 to HO058546) (Table II). After comparing the ten TDF sequences with those available in the GenBank, all of them matched significantly to rice genes (Table II). Eight TDFs showed significant homology to genes encoding proteins with known functions e.g., protein kinase OsPK4 (TDF C), a C-type cyclin (TDF2) or a splicing factor (TDF8), indicating the implication in diverse cellular processes (Table II). Meanwhile, TDF1 and TDF3 were homologous to rice genes encoding hypothetical proteins (Table II).

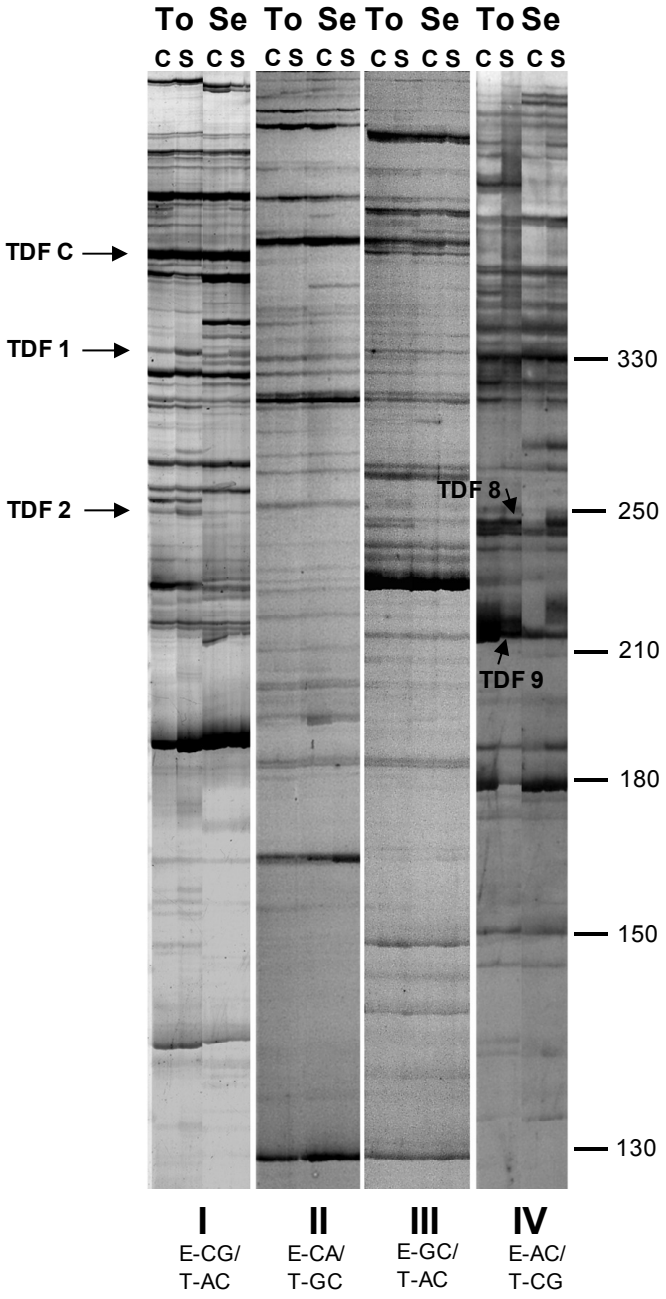

Figure 1. **Gel sections showing cDNA-AFLP profiles between 'PSBRc1' and 'IR64' rice varieties**. Examples of differentially expressed TDFs under water-stress, as well as a TDF with constitutive expression are indicated by arrows. Seedlings of stress-tolerant (**To**) and -sensitive (**Se**) rice genotypes were treated with 15% PEG-6000 for 3 hours. The cDNA templates were prepared from control (**C**) and stressed rice shoots (**S**). Panels I- IV indicate different primer combinations. MW markers are shown on the right (bp).

Table II. ***Putative functions of water-deficit responsive TDFs***

TDF/ GenBank Ac. no.	Primer combination	TDF size (bp)	Best Hit by BLASTN (score/ *E*-value/ Identities)	Definition	Homology	Functional Category	Expression 'PSBRc1'/ 'IR64' [(1)]
C/ HO058537	E-CG/ T-AC	390	NM_001062567/ (715/0.0/99%)	*O. sativa* ssp. *japonica,* Os05g0514200 mRNA, complete cds	Protein kinase OsPK4	Cellular communication/ Signal transduction mechanism	=/=
1/ HO058538	E-CG/ T-AC	332	NM_001189606/ (260/4e-69/81%)	*O. sativa* ssp. *japonica*, Os11g0471300, mRNA, partial cds	Hypothetical protein	Unclassified protein	+/+
2/ HO058539	E-CG/ T-AC	250	NM_001189000/ (331/2e-89/98%)	*O. sativa* ssp. *japonica,* Os09g0504400 mRNA, complete cds	C-type cyclin	Cell cycle and DNA processing	+/=
3/ HO058540	E-AG/ T-TC	230	NM_001072792/ (370/4e-101/ 99%)	*O. sativa* ssp. *japonica*, Os12g0169100 mRNA, complete cds	Hypothetical protein	Unclassified protein	+/=
5/ HO058541	E-AG/ T-TCT	130	NM_001058255/ (187/2e-46/ 100%)	*O. sativa* ssp. *japonica*, Os03g0820500, mRNA, complete cds	Actin-depolymerizing factor 3	Biogenesis of cellular component	–/0

(Table continues on following page)

Table II. *(continued from previous page)*

6/ HO058542	E-TG/ T-AT	168	NM_001063582/ (246/4e-64/97%)	*O. sativa* spp. *japonica,* Os06g0194900 mRNA, complete cds	Sucrose synthase	Metabolism	=/+
8/ HO058543	E-AC/ T-CG	244	NM_001066907/ (370/4e-101/96%)	*O. sativa* ssp. *japonica,* Os07g0633200 mRNA, complete cds	SC35-like splicing factor	Transcription	=/+
9/ HO058544	E-AC/ T-CG	216	NM_001189636 / (355/1e-96/100%)	*O. sativa* ssp. *japonica*, Os11g0522900 mRNA, complete cds	Serine carboxypeptidase	Protein fate	–/=
12/ HO058545	E-AG/ T-TC	100	NM_001186421/ (141/9e-33/100%)	*O. sativa* ssp. *japonica,* Os03g0250000 mRNA, complete cds	Calcium-binding EF hand family protein	Cellular communication/ Signal transduction mechanism	+/0
13/ HO058546	E-AG/ T-TC	180	NM_001051176/ (283/4e-75/99%)	*O. sativa* ssp. *japonica,* Os01g0819500 mRNA, partial cds	Ubiquitin conjugating enzyme E2-17 kDa	Protein fate	=/=

Gene functional categories follow MIPS classification (Mewes *et al*., 2002).
(1) Symbols refer to water-stress expression: +, induction; -, repression; =, no change in expression; 0, not detected.

3.2. Expression analysis of water-deficit responsive genes by reverse Northern and semi-quantitative RT-PCR

The eight selected TDFs differentially expressed were analysed by reverse Northern to confirm the expression patterns observed by cDNA-AFLP (Fig. 2). A cDNA clone with constitutive expression and homology to an ubiquitin conjugating enzyme (UBC) i.e. TDF13, was used as an internal control to further normalize band signal intensities. The Northern analysis confirmed that TDFs 1, 2, 3 and 12 were up-regulated by water-stress in 'PSBRc1', as well as TDFs 1 and 8, in 'IR64' (Fig. 2). On the other hand, the expression patterns of TDF5 and TDF9 observed in the reverse Northern could not confirm the results obtained in the cDNA-AFLP analysis. Hence, TDF5 was down-regulated in 'PSBRc1' whereas it was not detected in 'IR64', by cDNA-AFLP (data not shown), though blot analysis showed an induction by water-stress in 'PSBRc1' and in 'IR64' (Fig. 2). The cDNA-AFLP indicated that TDF9 was down-regulated by water-deficit in 'PSBRc1' (Fig. 1), but a constitutive expression was observed in the reverse Northern (Fig. 2).

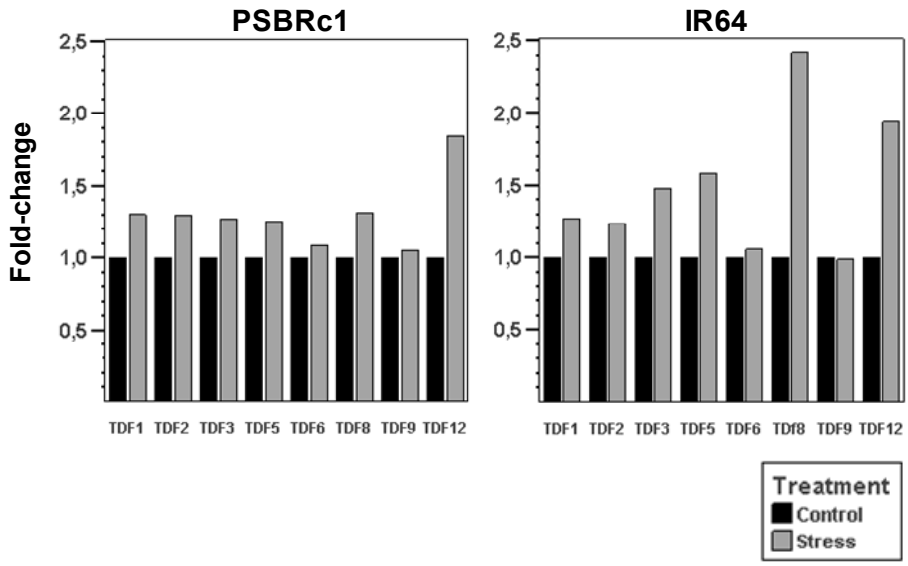

Figure 2. Reverse Northern-blot analysis to validate cDNA-AFLP patterns of distinct TDFs under water-stress conditions.

In ‘IR64’, the constitutive expression of TDF9 was observed either by cDNA-AFLP or reverse Northern (Figs. 1 and 2). As TDFs 5 and 9 were apparently down-regulated genes but its expression could not be fully validated by reverse Northern, they were not selected for the analysis by RT-PCR. Despite that the induction pattern of TDF6 in ‘IR64’ could not be confirmed by reverse Northern (Fig. 2), we selected it for further analysis. In fact, the induction of this transcript in ‘IR64’ after 3 hours of water deficit was confirmed by RT-PCR analysis (Fig. 3B), validating the cDNA-AFLP profiling.

The expression profiles of six TDFs were compared by semi-quantitative RT-PCR between ‘PSBRc1’ and ‘IR64’ (Fig. 3). The primers designed to amplify cDNA fragments of the putative water-stress responsive genes are listed in Table III. The constitutive expression of the UBC rice gene was used to normalize the expression levels of the stress-responsive genes. The profiles between ‘PSBRc1’ and ‘IR64’ were compared not only after water-stress injuries (3 and 72 hours stress), but also after ABA treatment to find a possible involvement of the hormone in the stress response (Fig. 3). The transcripts of TDF1 and TDF12 with homology to genes encoding a hypothetical protein and a calcium-binding EF-hand family protein, respectively, were strongly induced in the drought tolerant variety (Table II; Fig. 3B). In ‘IR64’, the drought-sensitive genotype, the levels of these transcripts were lower or not altered by water-stress (Fig. 3B).

By RT-PCR, the drought induction of TDF2 - with homology to a C-type cyclin - and TDF3, encoding a hypothetical rice protein, was observed after 72 hours of stress (Table II; Fig. 3B), though by cDNA-AFLP it was detected at an earlier time (3 h). Nevertheless, the up-regulation of TDF2 and TDF3 in the drought-tolerant variety, and its unchanged transcript levels in the drought-sensitive, confirmed the expression pattern observed in the cDNA-AFLP (Figs. 1 and 3B; Table II).

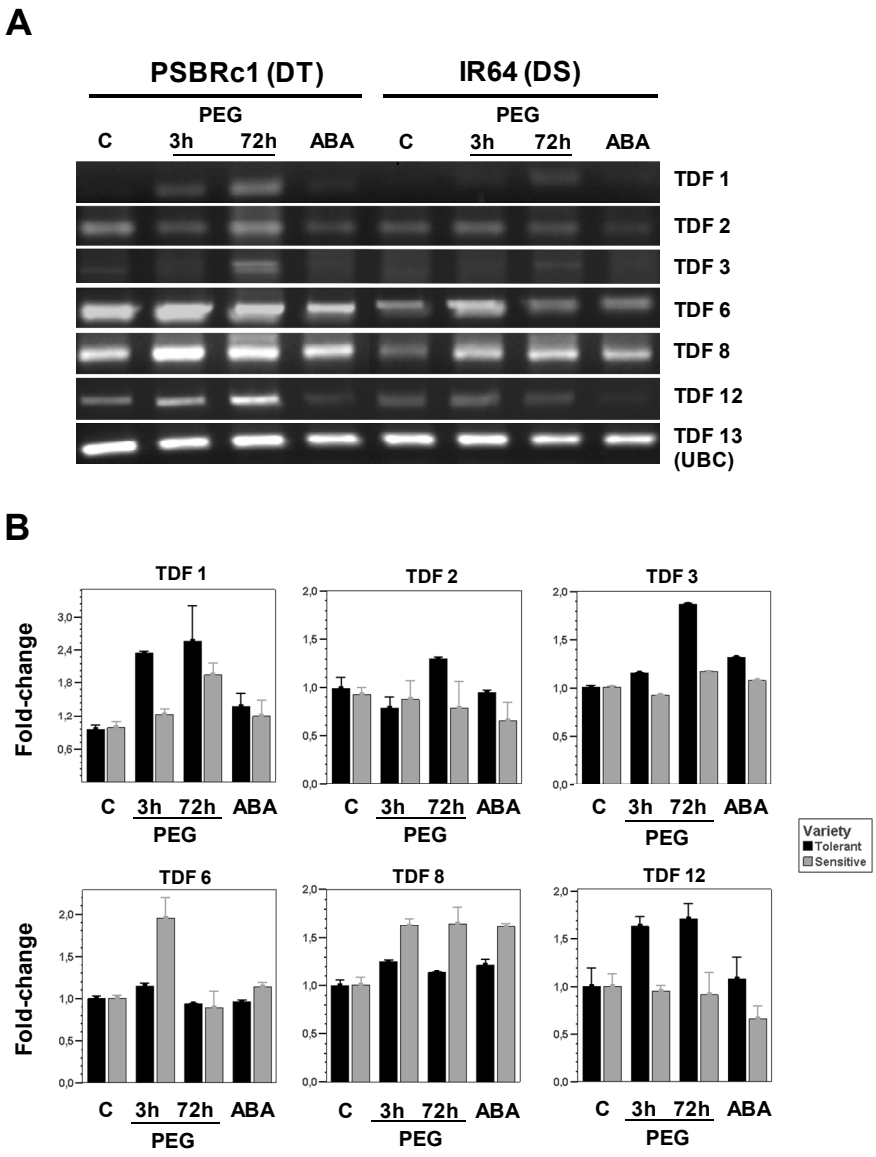

Figure 3. **Comparison of the expression pattern of water-stress responsive rice genes between varieties with contrasting response to drought**. **(A)** RT-PCR amplification profiles of six different TDFs in the shoots of untreated (control, **C**) and treated rice seedlings. Treatments were performed with 15% PEG-6000 for 3 and 72 hours; and with 50μM ABA for 6 hours. **(B)** Band optical density was determined and fold-change ratios were calculated on the basis of normalised values of gene expression. The UBC gene (TDF13) was used as a control of constitutive expression to normalise the mRNA levels of the different TDFs. Drought-tolerant (**DT**) genotype; drought-sensitive (**DS**) genotype. Error bars show means of 2 independent replicates ± standard deviation.

Table III. ***Primer sequences and annealing temperature (Tm) used in the RT-PCR reaction to assess the expression of different TDFs***

TDF	Amplified fragment size (bp)	Forward primer (5´-3´)/ Reverse primer (5´-3´)	Tm (°C)
TDF1	291	GAGTTATCGGTAATCCTCTCCTTA/ GTTCACAACTTACCAAAT	50
TDF2	959	GCCCTGCCCACGATGGCCGCCAA/ CGGAATTCTGAATTAAATCG	50
TDF3	1027	GAAGATCGAAGCAGAGAGAACTTC/ GAGCTTGCTGGAACGGAATTCA	56
TDF6	1002	GAATCACACATTGCATTCAC/ GAATGCAGCTGACGGCTTCT	50
TDF8	506	GCAGGTTATTCTGGGCAGGGAA/ GAGCAGAATTCACCGGCGTTGT	56
TDF12	1064	GTTCTCAGTAATCAGTTCAAAC/ GCATTGCATGGTAAAGTCTCTT	50
TDF13	342	GCTTTCGATCTGCTCGCTGCTCA/ GCAAGCATATGTTATCTCATTATC	56
Osmyb4	425	GGGAAGGAGCAAGCACAAT/ TCGGCTTCTTGTGCTTCTTG	56

The mRNA levels of TDF6, a putative member of the sucrose synthase family, and TDF8 - similar to a SC35-like splicing factor – were higher in the sensitive variety as compared with the tolerant (Table II; Fig. 3B). TDF6 was strongly induced by water-stress within the first hours of stress, not being detected after 72 hours, contrasting to TDF8 that was still up-regulated at this time (Fig. 3B).

TDFs 1, 3, and 12 besides being induced by water-deficit in ‘PSBRc1’, were apparently also slightly inducible by ABA (Fig. 3B). In a similar manner,

TDF8 was induced by both water-stress and ABA, but in 'IR64', the drought-sensitive variety. On the other hand, in 'IR64' the transcript levels of TDF2 and TDF12 seemed to diminish in the presence of ABA (Fig. 3B).

3.3. Comparison of the expression pattern of water-deficit responsive genes between tolerant and sensitive varieties in salinity and cold conditions

We were interested in verifying if the water-stress responsive genes could be also regulated by salinity and low temperature. Therefore, the expression patterns of the TDFs were checked after seedling exposure to salinity (Fig. 4) and cold stress treatments (Fig. 5). Furthermore, the expression profiles of the TDFs were compared between varieties with contrasting stress tolerance, i.e. salt- and cold-tolerant *vs.* salt- and cold-sensitive, respectively, to evaluate their possible involvement in stress tolerance. The periods of stress imposition were the same as those used in the induction of water deficit (Fig. 3), except in the case of the cold stress that instead of 72 h the stress was restricted to 24 hours.

The overall gene expression comparison analysis suggested that the water-deficit responsive cDNA clones also responded to salinity and cold, in at least one of the compared genotypes (Figs. 4B and 5B). Only TDF8 was induced by water-stress and salinity (Figs. 3B and 4B), but not by cold (Fig. 5B). On the other hand, the accumulation pattern of the distinct cDNA clones depended on the type of abiotic stress, as well as on the rice genotype and on the time of stress exposure. For instance, TDF1 was strongly induced by water-stress (Fig. 3B) and low temperature (Fig. 5B) and to a lower extent by salinity (Fig. 4B), with higher transcript levels in the tolerant varieties as compared to sensitive ones, particularly under water- and cold-stress conditions. Whereas TDF12 was strongly up-regulated by PEG in the drought-tolerant genotype after 3 hours of stress, there was no change in the accumulation of the transcript in the sensitive variety (Fig. 3B).

Nevertheless, TDF12 was induced to higher extent in the sensitive varieties than in the corresponding tolerant ones, after salt- and cold-stress treatments, in the same time period (Figs. 4B and 5B). Curiously, the differences in the expression of TDF1 and TDF12 between tolerant and sensitive genotypes became in general less accentuated after a more prolonged period of stress (24 or 72h) (Figs. 3B, 4B and 5B). In the case of TDF12, after longer periods of salt- and cold-stress, the levels between tolerant and sensitive genotypes were quite similar, being even slightly higher in the tolerant varieties (Figs. 3B, 4B and 5B).

The influence of ABA in the expression of the distinct TDFs under salt- and cold-stress conditions was also analysed. The levels of expression of the distinct TDFs in the presence of ABA were similar between the salt-tolerant and -sensitive variety, coinciding most of the times a slight induction by ABA and salinity (Fig. 4B). On the other hand, TDF1 was strongly induced by cold and ABA in the cold-tolerant variety, with no considerable change in the transcript levels in the sensitive genotype (Fig. 5B). The induction of TDF2 and TDF12 by low temperature in both cold-tolerant and -sensitive varieties seemed to coincide with slightly increased levels of the transcripts in the presence of ABA (Fig. 5B). In the particular case of TDF6, the up-regulation by cold occurred either in 'PSBRc96' or in 'IR58', but the increase of the transcript levels in the presence of ABA was registered only in the tolerant variety (Fig. 5B). In addition, we analysed the expression of the *Osmyb4* gene, a transcription factor that is strongly induced in rice coleoptiles by cold, but not by drought or salinity, after 4 hours of stress treatments (Vannini *et al*., 2004). The *Osmyb4* gene was clearly induced by cold, being strongly accumulated in the cold-tolerant genotype, though some increase in the transcript level was also observed in the sensitive genotype after 24 hours of stress (Fig. 5B). Furthermore, the *Osmyb4* gene was strongly induced by ABA in 'PSBRc96', whereas the mRNA levels were very similar in the cold-sensitive genotype under control conditions and after treatment with ABA (Fig. 5B).

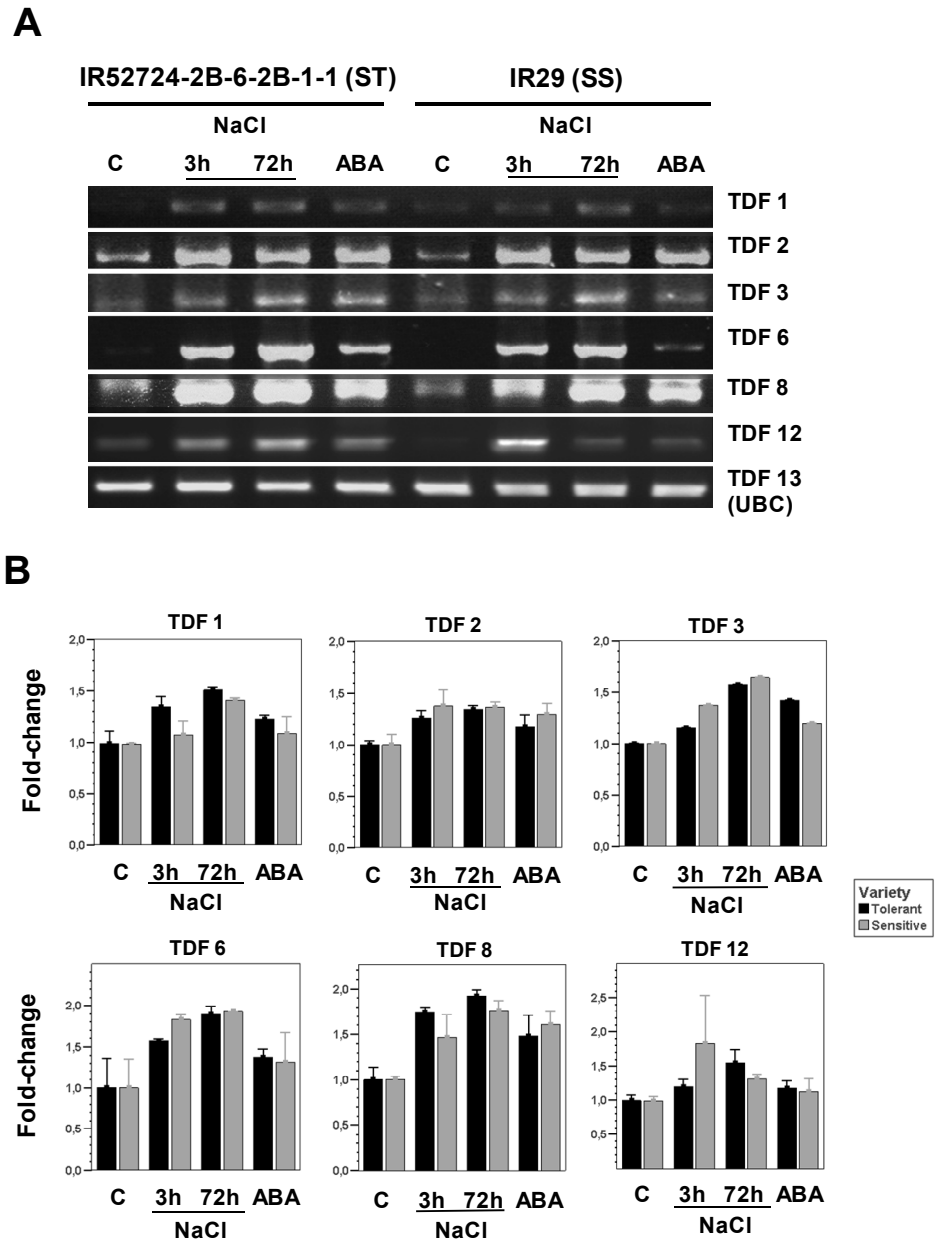

Figure 4. **RT-PCR analysis of water-stress regulated TDFs in response to salinity**. The expression profiles were compared between a salt tolerant (**ST**) rice line and a salt sensitive (**SS**) variety. **(A)** RT-PCR products from shoots of untreated (control; **C**) and treated seedlings with 200 mM NaCl (3 and 72 hours), and 50µM ABA (6 hours). **(B)** Band optical density was determined and fold-change ratios were calculated on the basis of normalised values of gene expression. The UBC gene (TDF13) was used as a control of constitutive expression to normalise mRNA levels of the different TDFs. Error bars show means of 2 independent replicates ± standard deviation.

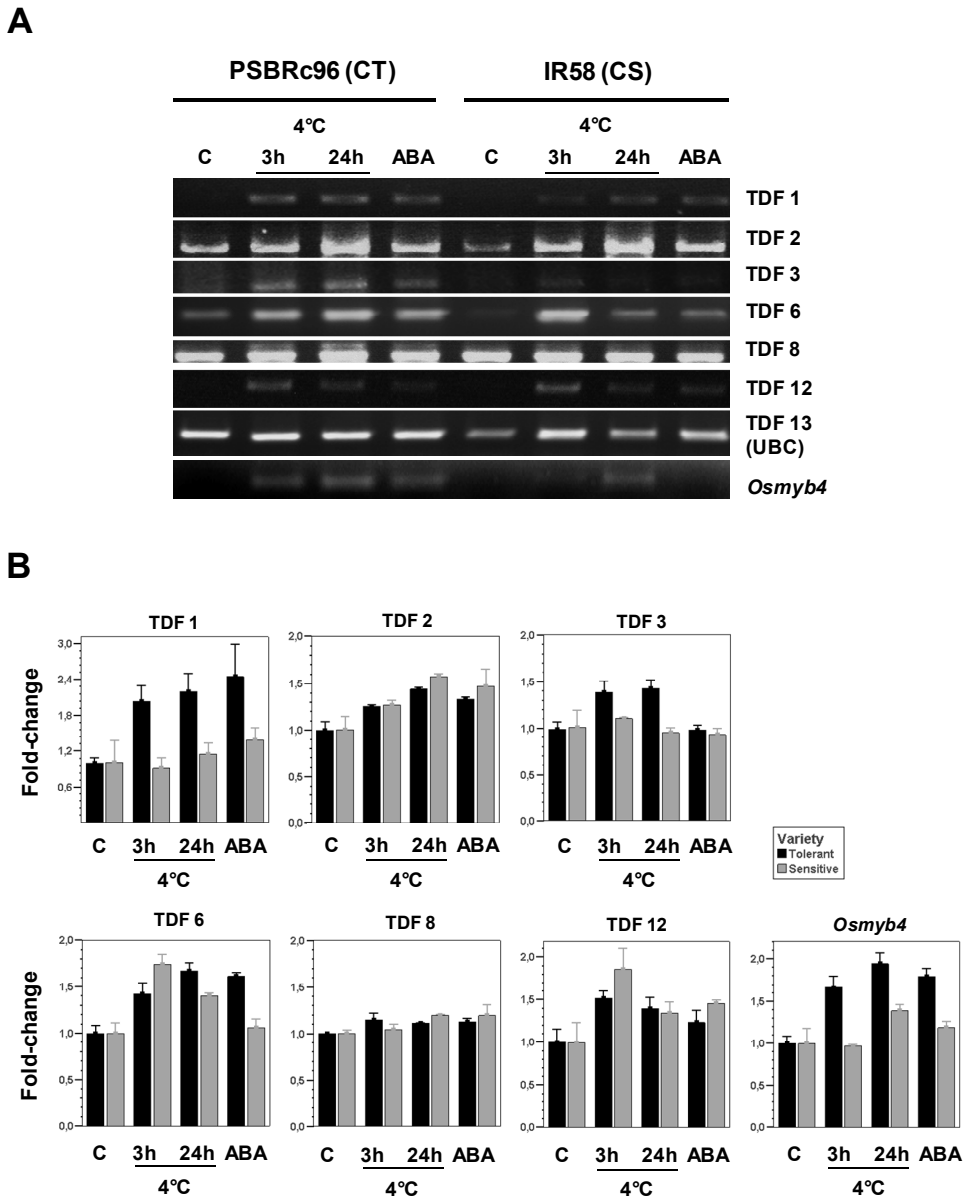

Figure 5. **RT-PCR analysis of water-stress regulated TDFs in response to cold**. The expression patterns were compared between a cold tolerant (**CT**) genotype and a cold sensitive one (**CS**). **(A)** RT-PCR products from shoots of seedlings maintained at room temperature (control; **C**) or at 4° C for 3 and 24 hours; and treated with 50µM ABA for 6 hours. **(B)** Band optical density was determined and fold-change ratios were calculated on the basis of normalised values of gene expression. The UBC gene (TDF13) was used as a control of constitutive expression to normalise mRNA levels of the different TDFs. The *Osmyb4* gene was used a gene marker for cold stress induction. Error bars show means of 2 independent replicates ± standard deviation.

4. Discussion

The analysis of gene expression in response to water deficit is crucial to understand the way that plants adapt to limited water resources, ensuring their survival and the completeness of the life cycle (Bray, 2004). The goal of this work was to identify rice genes expressed in the early events of water stress response, and whose expression could indicate a potential role in stress tolerance. We identified by cDNA-AFLP several clones that were differentially expressed under water-stress conditions, either in 'PSBRc1', a tolerant genotype adapted to drought-prone areas, and/or in 'IR64' - a high yielding variety released for favourable environments – and sensitive to drought.

The expression patterns of eight selected TDFs were analysed by reverse Northern and/or RT-PCR analysis, to validate the cDNA-AFLP transcript profiling. For most clones, the gene induction by water stress displayed in the cDNA-AFLP experiments, was validated through both techniques. The only exception was TDF6 that exhibited a constitutive expression by blot-analysis. Such apparent contradiction might be explained by the presence of different members of the sucrose synthase (SUS) gene family (SUS1, SUS2, SUS3) that could cross-hybridize by reverse Northern. Nevertheless, the amplification by RT-PCR using specific primers, confirmed that TDF6 was induced by water deficit. Meanwhile, we were not able to confirm by reverse Northern, the down-regulation of TDF5 and TDF9 in water stress conditions, observed in 'PSBRc1' by cDNA-AFLP. This discrepancy was most probably due to the presence of other mRNA more abundant and constitutively expressed that could cross-hybridise in the reverse Northern-blot. In the RT-PCR analyses, the only notable difference was the detection of TDF12 in 'IR64', since the transcript was not observed in this variety by cDNA-AFLP. Interestingly, in the cDNA-AFLP profiling of maritime pine samples submitted to water deficit, the qualitative variations proved to be quantitative by reverse northern or RT-PCR analysis, showing that there were no exclusive genes expressed either in control or stress conditions (Dubos and Plomion, 2003).

The overall comparison of the expression patterns obtained in the reverse Northern and RT-PCR analyses, validated the results of the cDNA-AFLP experiments, with most of the cDNA clones showing similar expression profiles, despite some minor discrepancies. Such differences across the transcript profiles provided by these three methodologies, were previously reported by other authors for few differentially expressed cDNA clones (Campalans *et al*., 2001; Milioni *et al*., 2002; Dubos and Plomion, 2003; Leymarie *et al*., 2007; Ventelon-Debout *et al*., 2008; Li *et al*., 2009).

Besides focusing on TDFs with differential gene expression, we also selected two TDFs constitutively expressed i.e. TDFC and TDF13. Interestingly, TDF C corresponded to a rice gene encoding OsPK4, a protein that belongs to group 3 of the SNF1-related protein kinase family (SnRK3) (Ohba *et al*., 2000). The constitutive expression of *OsPK4* in rice seedlings submitted to drought stress was previously reported by Ohba *et al*. (2000), thus supporting our results. Furthermore, Ohba and collaborators (2000) found that the expression of the *OsPK4* gene was not altered after salt or cold stress treatments. However, the transcript levels of its homologous genes in wheat (*WPK4*) and maize (*ZmPK4*) increased in low temperature conditions, suggesting that this protein kinase may exert different functions across cereals (Ohba *et al*., 2000). The mechanisms of transcriptional induction seem also to differ from those presented by other members of the SnRK3 group e.g., SALT OVERLY SENSITIVE 2 (SOS2)/SnRK3.1*,* an essential gene in the salinity stress response in *Arabidopsis* (Mahajan, 2008).

The differences in the expression of specific genes between stress-tolerant and stress-sensitive genotypes indicate that tolerance mechanisms are genetically encoded, and that the ability to use such genetic programs depends on the natural adaptation of genotypes to diverse environments (Bray, 1993; Bonhert and Jensen, 1996; Ergen *et al*., 2009). We compared by RT-PCR the expression profiles of six cDNA clones across genotypes adapted to drought, salinity and low-temperatures (i.e. stress-tolerant), to

those released for optimal growth conditions (i.e. stress-sensitive), trying to find genes with a more general role, or alternatively, a specific one, in the adaptation to abiotic stress. Indeed, in natural ecosystems, plants usually face diverse stressful conditions either concurrently, or separated in time, thus requiring complex regulatory pathways involving both common and stress-specific responses (Bray, 1997; Knight and Knight, 2001). Moreover, it is well-known that the disruption of plant water status occurs also at high concentrations of salt and at low temperatures (Bray, 1997; Verslues *et al*., 2006).

The sequence of TDF2 was highly homologous to a rice gene encoding a C-type cyclin. The eukaryotic cell cycle is controlled by a family of heterodimeric protein kinases composed by a catalytic subunit, termed cyclin-dependent kinase (CDK), and by an activating/regulatory subunit – the cyclin (Mironov *et al*., 1999). Plants as sessile organisms must have specific mechanisms in order to adjust their cell cycle in response to environmental cues (Peres *et al*., 2007). Abiotic and biotic stresses negatively affect plant growth through the inhibition of the cell cycle machinery (Peres *et al*., 2007). It was demonstrated that in the maize endosperm, cell proliferation and endoreduplication decreased in water deficit conditions (Setter and Flannigan, 2001). A rapid transient inhibition of the cell cycle was observed in *Arabidopsis* roots in response to salt stress (West *et al*., 2004). However, not all CDKs and cyclins have a role in the control of cell cycle (Mironov *et al*., 1999). Surprisingly, a rice C-type cyclin-dependent protein kinase (*CDKC;1*) was found to be strongly and rapidly induced by salinity and ABA (Huang *et al*., 2008). The authors proposed an involvement in the salt-response and ABA-signalling pathway, besides in developmental programs. The putative protein kinase activity would thus be responsible for regulating the salt-induced gene expression (Huang *et al*., 2008). We observed that TDF2 was induced after 3 hours of water-stress in the drought-tolerant genotype (cDNA-AFLP pattern; Fig. 1), and that after 3 days of stress, the transcript levels were higher in the tolerant than in the sensitive genotype.

The TDF2 cDNA clone was also induced by salinity and cold, but at very similar levels between the two genotypes with contrasting responses to these stresses. The data seems to indicate that TDF2 has a stronger implication in water-stress response/tolerance, as compared to the other stresses. Huang *et al*. (2008) verified that *CDKC; 1* was only induced in response to salt stress, but not to osmotic stress, reinforcing the hypothesis that these C-type cyclins are involved in more specific stress signalling responses.

The TDF12 cDNA clone presented homology at protein level to a calcium-binding EF hand family protein. In plants, calcium acts as a secondary messenger in cellular signal transduction, constituting a pivotal regulator in many different cellular processes, among them stress responses (Song *et al*., 2008). In eukaryotes, Ca^{2+} signalling is mediated by a large and functionally diverse family of calcium-binding proteins (CaBPs) that may fall into three categories: trigger/sensor (e.g., calmodulin), buffer proteins, or Ca^{2+}- stabilized proteins (Zhou *et al*., 2006; Wang *et al*., 2008). The EF-hands proteins can be found in each category and comprise more than 50% of all well-characterised CaBPs (Zhou *et al*., 2006). The TDF12 clone was induced by water-stress and ABA in the drought-tolerant variety within the first hours of stress, being still up-regulated after 3 days of stress, whereas no considerable changes occurred in the drought-sensitive genotype. The expression of TDF12 was constitutive in the salt-tolerant genotype, whereas it was strongly induced in the salt-sensitive one, thus resembling a 'stress-anticipating' mechanism (Gong *et al*., 2005). Meanwhile, in cold conditions, TDF12 was strongly induced in both cold-tolerant and cold-sensitive varieties in the first hours, though after 24 hours the levels were similar in both varieties. The expression pattern of TDF12 across the different genotypes suggests that this protein likely participates in cellular signal transduction leading to osmotic stress tolerance, though it can also be triggered by cold stress.

Changes in carbohydrate concentration have been demonstrated to be implicated in the responses to a number of different abiotic stresses (Ciereszko *et al*., 2001). Sucrose is not only necessary for plant growth and development, but it also acts as a signalling molecule in pathways that interact with those involved in stress responses, to modulate metabolism. The up-regulation of enzymes involved in sucrose synthesis and metabolism e.g., sucrose phosphate synthase (SPS) and sucrose synthase (SUS) by sugars, drought, salt and low temperature, has been previously reported (Déjardin *et al*., 1999; Ciereszko *et al*., 2001; Seki *et al*., 2002). The transcript levels of TDF6, a putative member of the sucrose synthase family strongly accumulated in the drought-sensitive variety after water-stress imposition. In the drought-tolerant variety, the transcript was already accumulated in pre-stress conditions, with no considerable change after water-deficit. Most probably, upon water-stress, the drought-tolerant variety may be less affected than the sensitive one, thus maintaining the same transcript levels as prior to stress. Under salinity and cold stress, TDF6 was induced at very similar levels across tolerant and sensitive genotypes, suggesting that sugar signalling is activated in both tolerant and sensitive genotypes in response to salt and low temperature.

The expression pattern of TDF8, a transcript with homology to a SC35-like splicing factor, was apparently more strongly induced in the drought-sensitive variety than in the drought-tolerant one, suggesting once again that the drought-tolerant genotype was apparently less affected by water-deficit than the sensitive one, by exhibiting lower levels of transcript induction. Under salt stress conditions, the levels of mRNA were similar between sensitive and tolerant genotypes. However, no change was observed in the accumulation of TDF8 upon cold stress, in any of the genotypes with differential cold stress adaptation. Hence, the expression of TDF8 seems to be induced by osmotic stress, but not by low temperature. The regulation at post-transcriptional level of gene expression in response to abiotic stress - more precisely the role of splicing factors – has recently emerged as a

crucial component in the acquisition of stress tolerance (Floris *et al*., 2009). Transgenic plants overexpressing splicing factors had significantly increased stress tolerance towards drought and salinity (Bourgon *et al*., 2007). Therefore, it is reasonable to speculate that TDF8 could have a major role in osmotic stress tolerance.

The mRNA levels of TDF1 and TDF3, both coding for hypothetical proteins, were markedly increased in the varieties with drought and cold stress tolerance. On the other hand, the transcripts of TDF1 and TDF3 were also more accumulated in the salt-tolerant than in the salt-sensitive variety, though the difference in expression was not so pronounced as in the other genotypes. The expression profiles of TDF1 and TDF3 suggest a possible role in stress tolerance, probably being involved in a more global stress response, rather than in a stress-specific one. Additional studies would be necessary to disclose the exact nature of these gene products, given that they could be involved in tolerance to multiple stresses. Furthermore, these results demonstrate the usefulness of cDNA-AFLP in the discovery of new genes putatively involved in abiotic stress response/tolerance.

In addition, most cDNA clones were induced by ABA, at least in one of the genotypes with differential stress tolerance. The response to ABA thus depended on the genotype, but coincided many times with the response to a particular abiotic stress. Nonetheless, the expression of the distinct TDFs in response to stress was not always related to ABA accumulation. This suggests the participation of ABA-dependent and ABA-independent signalling transduction pathways in the adaptive responses to the distinct types of stress, across both tolerant and sensitive genotypes.

In this work, despite the limited number of identified genes, we were able to identify stress-responsive genes not only involved in the water-deficit response, but also in the responses to salinity and cold. Most of the genes seemed to be implicated in stress signalling transduction, and point to a possible role in stress tolerance. Some genes were more strongly (or specifically) expressed in tolerant genotypes, whereas others were induced

under stress conditions in both tolerant and sensitive genotypes, hence revealing both specific and common responses. In sum, the whole data strongly suggests that the identified genes constitute good candidates for rice improvement aiming for multiple abiotic stress tolerance.

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6. Literature cited:

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389-3402.

Bachem CW, van der Hoeven R, de Bruijn SM, Vreugdenhil D, Zabeau M, Visser RG (1996) Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. 9: 745-753.

Bachem CWB, Oomen RJFJ, Visser RGF (1998) Transcript imaging with cDNA-AFLP: a step-by-step protocol. Plant Mol Biol Rep 16: 133-138.

Bassam BJ, Caetano-Anollés G, Gresshoff PM (1991) Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal Biochem 196**:** 80-83.

Bassam BJ, Gresshoff PM (2007) Silver staining DNA in polyacrylamide gels. Nat Protoc 2: 2649-2654.

Bonhert HJ, Jensen RG (1996) Strategies for engineering water-stress tolerance in plants. TIBTECH 14: 89-97.

Bourgon L, Amorós B, Naranjo MA, Vicente O (2007) Drought and salt tolerance conferred by overexpression of splicing factors in transgenic plants. Buletinul USAMV-CN, 64.

Bray EA (1993) Molecular responses to watet deficit. Plant Physiol 103: 1035-1040.

Bray EA (1997) Plant responses to water deficit. Trends Plant Sci 2(2): 48-54

Bray EA (2004) Genes commonly regulated by water-deficit stress in *Arabidopsis thaliana*. J Exp Bot 55(407): 2331-2341.

Breyne P, Zabeau M (2001) Genome-wide expression analysis of plant cell cycle modulated genomes. Curr Opin Plant Biol 4: 136-142.

Campalans A, Pagès M, Messeguer R (2001) Identification of differentially expressed genes by the cDNA-AFLP technique during dehydration of almond (*Prunus amygdalus*). Tree Physiol 21: 633-643.

CAWMA (Comprehensive Assessment of Water Management in Agriculture) (2007) In D Molden, ed, Water for food, water for life: a comprehensive assessment of water management in Agriculture. London: Earthscan, and Colombo: International Water Management Institute, pp. 1-20.

Ciereszko I, Johansson H, Kleczkowski LA (2001) Sucrose and light regulation of a cold-inducible UDP-glucose pyrophosphorylase gene via a hexokinase-independent and abscisic acid-insensitive pathway in *Arabidopsis*. Biochem J 354 (Pt 1): 67-72.

Cooper B, Clarke JD, Budworth P, Kreps J, Hutchison D, Park S, Guimil S, Dunn M, Luginbühl P, Ellero C, Goff SA, Glazebrook J (2003) A network of rice genes associated with stress response and seed development. Proc Natl Acad Sci 100**:** 4945-4950.

Degenkolbe T, Do PT, Zuther E, Repsilber D, Walther D, Hincha DK, Kohl KI (2009) Expression profiling of rice cultivars differing in their tolerance to long-term drought stress. Plant Mol Biol 69: 133-153.

Déjardin A, Sokolov LN, Kleczkowski LA (1999) Sugar/osmoticum levels modulate differential abscisic acid-independent expression of two stress-responsive sucrose synthase genes in *Arabidopsis*. Biochem J 344 Pt 2: 503-509.

Donson J, Fang Y, Espiritu-Santo G, Xing W, Salazar A, Miyamoto S, Armendarez V, Volkmuth W (2002) Comprehensive gene expression analysis by transcript profiling. Plant Mol Biol 48: 75-97.

Dubos C, Plomion C (2003) Identification of water-deficit responsive genes in maritime pine (*Pinus pinas*ter Ait.) roots. Plant Mol Biol 51: 249-262.

Ergen NZ, Thimmapuram J, Bohnert HJ, Budak H (2009) Transcriptome pathways unique to dehydration tolerant relatives of modern wheat. Funct Integr Genomics 9(3): 377-396.

Floris M, Mahgoub H, Lanet E, Robaglia C, Menand B (2009) Post-transcriptional regulation of gene expression in plants during abiotic stress. Int J Mol Sci 10(7): 3168-3185.

Gong Q, Li P, Ma S, Rupassara SI, Bohnert HJ (2005) Salinity stress adaptation competence in the extremophile *Thellungiella halophila* in comparison with its relative *Arabidopsis thaliana*. Plant J 44: 826-839.

Gorantla M, Babu PR, Lachagari VBR, Reddy AMM, Wusirika R, Bennetzen JL, Reddy AR (2007) Identification of stress-responsive genes in an *indica* rice (*Oryza sativa* L.) using ESTs generated from drought-stressed seedlings. J Exp Bot 58: 253-265.

Huang YW, Tsay WS, Chen CC, Lin CW, Huang HJ (2008) Increased expression of the rice C-type cyclin-dependent protein kinase gene, Orysa;CDKC;1, in response to salt stress. Plant Physiol Biochem 46(1): 71-81.

Knight H, Knight MR (2001). Abiotic stress signalling pathways: specificity and cross-talk. Trends Plant Sci 6(6): 262-267

Leymarie J, Bruneaux E, Gibot-Leclerc S, Corbineau F (2007). Identification of transcripts potentially involved in barley seed germination and dormancy using cDNA-AFLP. J Exp Bot 58 (3): 425-437.

Li Y, Tian Z, Liu J, Xie C (2009) Comparative cDNA-AFLP analysis reveals that DL-*β*-amino-butyric acid induces resistance through early activation of the host-defense genes in potato. Physiol Plantarum 136: 19-29.

Mahajan S, Pandey GK, Tuteja N (2008) Calcium- and salt-stress signaling in plants: shedding light on SOS pathway. Arch Biochem Biophys 471: 146–158.

Mewes HW, Frishman D, Gulldener U, Mannhaupt G, Mayer K, Mokrejs M, Morgenstern B, Munsterkotter M, Rudd S, Weil B (2002) MIPS: a database for genomes and protein sequences. Nucleic Acids Res 30 (1): 31-34.

Milioni D, Sado P-E, Stacey NJ, Roberts K, McCann MC (2002) Early gene expression associated with the commitment and differentiation of a plant tracheary element is revealed by cDNA–amplified fragment length polymorphism analysis. Plant Cell 14: 2813-2824.

Mironov V, De Veylder L, Van Montagu M, Inze D (1999) Cyclin-dependent kinases and cell division in plants -the nexus. Plant Cell 11(4): 509-522.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15: 473-497.

Nguyen NV (2008) Sustainable Intensification of Rice Production for food security in the near future – A Summary Report. FAO International Rice Comission (IRC) - Plant Production and Protection Division (AGP), *on World Wide Web URL:* http://www.fao.org/fileadmin/templates/agphome/documents/Rice/sustintriceprod.pdf

Ohba H, Steward N, Kawasaki S, Berberich T, Ikeda Y, Koizumi N, Kusano T, Sano H (2000) Diverse response of rice and maize genes encoding homologs of WPK4, an SNF1-related protein kinase from wheat, to light, nutrients, low temperature and cytokinins. Mol Gen Genet 263(2): 359-366.

Peres A, Churchman ML, Hariharan S, Himanen K, Verkest A, Vandepoele K, Magyar Z, Hatzfeld Y, Van Der Schueren E, Beemster GT, Frankard V, Larkin JC, Inzé D, De Veylder L (2007) Novel plant-specific cyclin-dependent kinase inhibitors induced by biotic and abiotic stresses. J Biol Chem 282(35): 25588-25596.

Rabbani MA, Maruyama K, Abe H, Khan MA, Katsura K, Ito Y, Yoshiwara K, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. Plant Physiol 133**:** 1755-1767.

Rodríguez M, Canales E, Borroto CJ, Carmona E, Lopez J, Pujol M, Borras-Hidalgo O (2006) Identification of genes induced upon water-deficit stress in a drought-tolerant rice cultivar. J Plant Physiol 163 (2006): 577-584.

Seki M, Narusaki M, Ishida J, Nanjo T, Fujita M, Oone Y, Kamiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Taji T, Shinozaki K Y, Carninci P, Kawai J, Hayashizaki K Y, Shinozaki K (2002) Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high salinity stresses using a full length cDNA microarray. Plant J 31: 279-292.

Setter TL, Flannigan BA (2001) Water deficit inhibits cell division and expression of transcripts involved in cell proliferation and endoreduplication in maize endosperm. J Exp Bot 52(360): 1401-1408.

Song WY, Zhang ZB, Shao HB, Guo XL, Cao HX, Zhao HB, Fu ZY, Hu XJ (2008) Relationship between calcium decoding elements and plant abiotic-stress resistance. Int J Biol Sci 4(2): 116-125.

Stumm GB, Vedder H, Schlegel J (1997) A simple method for isolation of PCR fragments from silver-stained polyacrylamide gels by scratching with a fine needle. Elsevier Trends Journals Technical Tips Online: T01115.

Vannini C, Locatelli F, Bracale M, Magnani E, Marsoni M, Osnato M, Mattana M, Baldoni E, Coraggio I (2004) Overexpression of the rice *Osmyb4* gene increases chilling and freezing tolerance of *Arabidopsis thaliana* plants. Plant J 37: 115-127.

Ventelon-Debout M, Tranchant-Dubreuil C, Nguyen TT, Bangratz M, Siré C, Delseny M, Brugidou C (2008) Rice yellow mottle virus stress responsive genes from susceptible and tolerant rice genotypes. BMC Plant Biology 8: 26 (http://www.biomedcentral.com/1471-2229/8/26)

Verslues PE, Agarwal M, Katiyar-Agarwal S, Zhu J, Zhu J-K (2006) Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. Plant J 45: 523–539.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Pelman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucl Acids Res 23: 4407-4414.

Vuylsteke M, Van Den Daele H, Vercauteren A, Zabeau M, Kuiper M (2006) Genetic dissection of transcriptional regulation by cDNA-AFLP. Plant J 45: 439-446.

Vuylsteke M, Peleman JD, van Eijk MJT (2007) AFLP-based transcript profiling (cDNA-AFLP) for genome-wide expression analysis. Nat Protoc 2(6): 1399-1413.

Wang H, Zhang H, Gao F, Li J,·Li Z (2007) Comparison of gene expression between upland and lowland rice cultivars under water stress using cDNA microarray. Theor Appl Genet 115: 1109-1126.

Wang SL, Fan KQ, Yang X, Lin ZX, Xu XP, Yang KQ (2008) CabC, an EF-hand calcium-binding protein, is involved in Ca2+-mediated regulation of spore germination and aerial hypha formation in *Streptomyces coelicolor*. J Bacteriol 190(11): 4061-4068.

West G, Inzé D, Beemster GT (2004) Cell cycle modulation in the response of the primary root of Arabidopsis to salt stress. Plant Physiol 135(2): 1050-1058.

Yang L, Zheng B, Mao C, Qi X, Liu F, Wu P (2004) Analysis of transcripts that are differentially expressed in three sectors of the rice root system under water deficit. Mol Gen Genomics 272: 433-442.

Zhou Y, Yang W, Kirberger M, Lee HW, Ayalasomayajula G, Yang JJ (2006) Prediction of EF-hand calcium-binding proteins and analysis of bacterial EF-hand proteins. Proteins 65(3): 643-655.

Zhou J, Wang X, Jiao Y, Qin Y, Liu X, He K, Chen C, Ma L, Wang J, Xiong L, Zhang Q, Fan L, Deng XW (2007) Global genome expression analysis of rice in response to drought and high-salinity stresses in shoot, flag leaf, and panicle. Plant Mol Biol 63**:** 591-608.

Supplemental data

Seed Germination

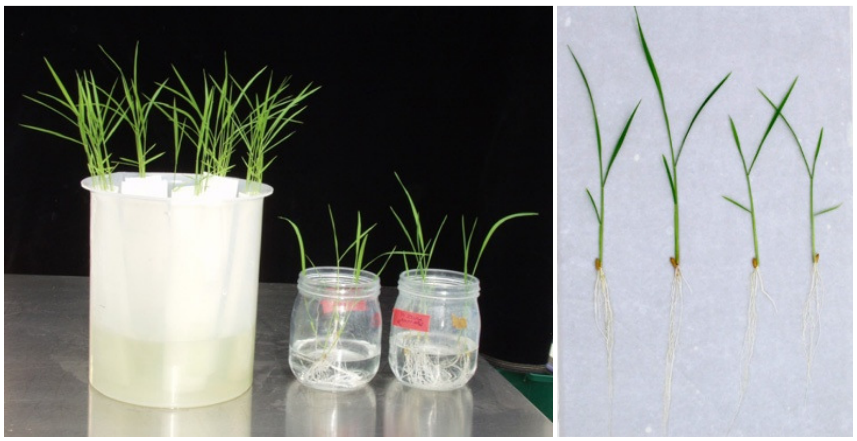
Test seeds were heat-treated 5 days in a convection oven at 50°C to break dormancy, before germination. Proper breaking of the seed dormancy is necessary, because the delay in the germination makes some seeds more sensitive to stress e.g., salinity (Gregorio *et al*., 1997)[9]. After dormancy breaking, seeds were surface sterilised with 0.1% (w/v) benlate fungicide during 15 min in 400 mL solution, using a magnetic stirrer. Seeds were then washed 3 times with bi-distilled water (250 mL) for 15 min. Further sterilisation with ethanol and sodium hipochloride was performed as described in section 2.1 in 'Materials and Methods'. Seedlings were grown for 14 days in a growth chamber (Fitoclima D1200 PI, Aralab) under fluorescent light (150 μE for shell, at 28°C/16 hours light and at 26°C/8 hours dark). Seeds were germinated on filter paper towels (dried after being autoclaved) partially submerged in 1:4 (v/v) Murashige and Skoog medium (1962) using 2.0 L gobelets covered with aluminium paper to prevent algae formation (Supplemental Fig. S1). General conditions:

- each gobelet may contain between 2 to 4 paper towels;
- 10 seeds per paper towel
- 1.5L nutrient solution.

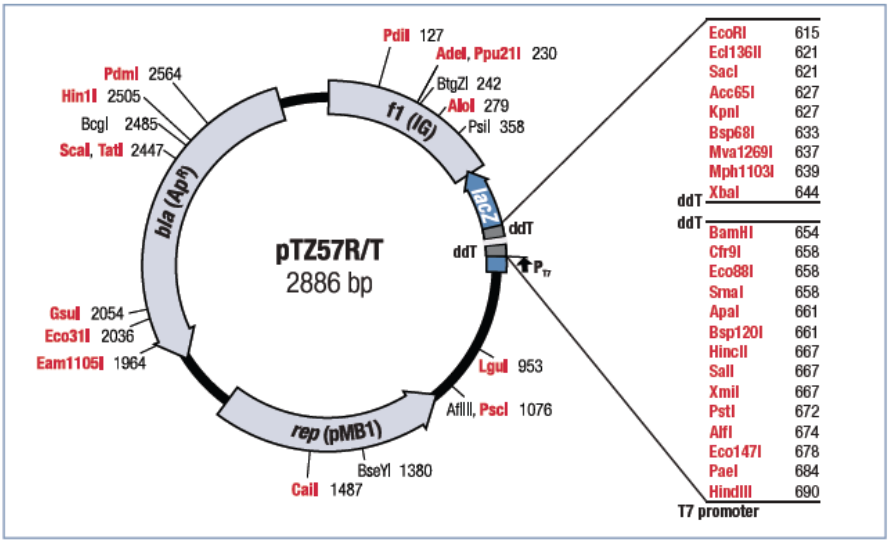
It was important to maintain and monitor the pH of the nutrient solution, because deviation (±1.0) of culture solution will make some nutrients toxic and others deficient (Gregorio *et al*., 1997). The pH was maintained at approximately 5.2 and checked every 2 days. It was also important to make up the nutrient solution volume with water, due to loss by evaporation (Gregorio *et al*., 1997). Nutrient solution was changed every 7 days and stress treatments were performed in fresh nutrient solutions. Seedlings

[9] Gregorio GB, Senadhira D, Mendoza RD (1997) Screening rice for salinity tolerance. IRRI discussion paper series no.22. International Rice Research Institute, Los Baños. Laguna, Philippines, 30 p.

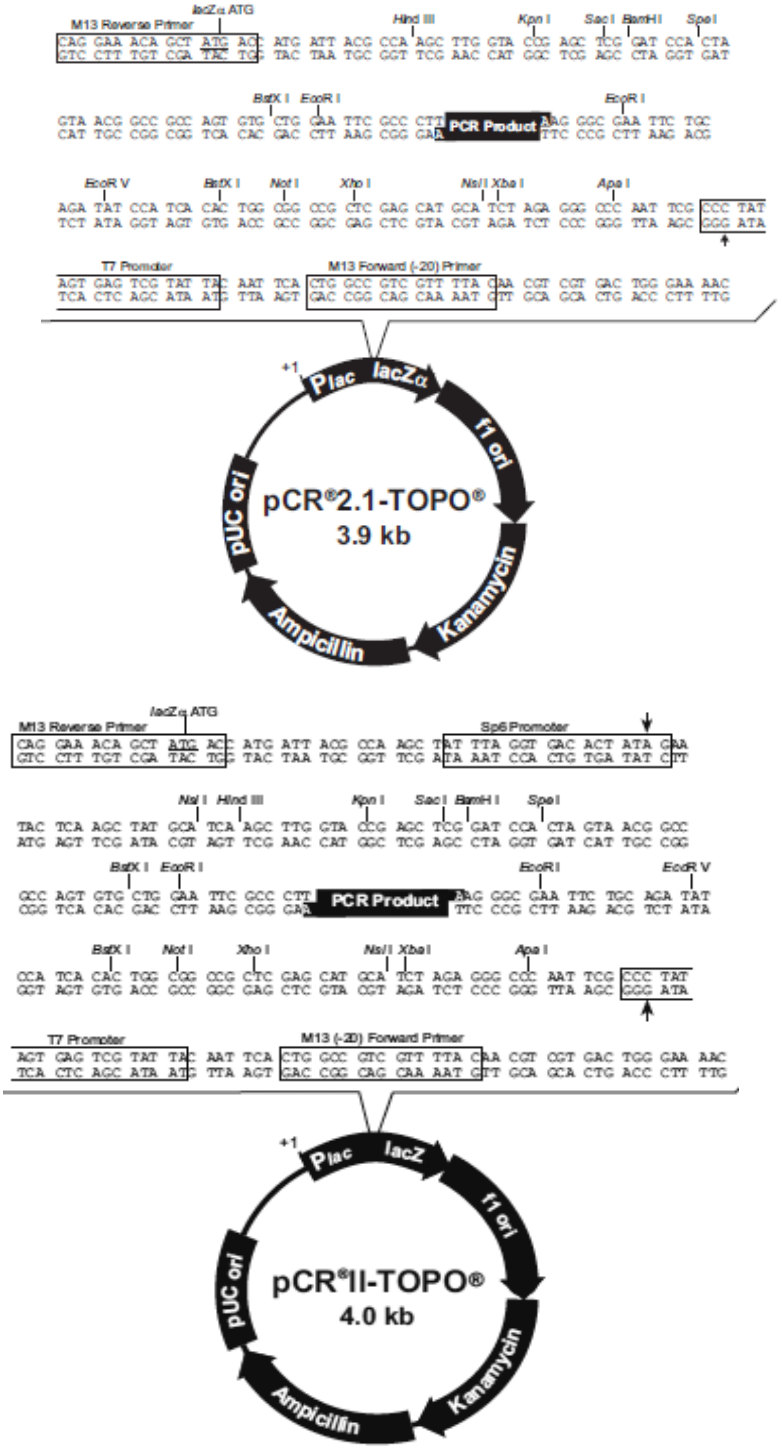
germinated for 14 days could be transferred to plastic containers filled with substrate mixture of peat moss and vermiculite (1:1) supplemented with osmocote (1g/L) for further growing until maturity, at greenhouse conditions.



Supplemental Figure S1. **Fourteen days-old rice seedlings germinated on filter paper towels.** Smaller glass flasks show seedlings submitted to stress treatments.



Supplemental Figure S2. **Restriction Map of vector pTZ57R (Fermentas).**



Supplemental Figure S3. Restriction Map of TOPO vectors (Invitrogen).

Chapter V

Concluding remarks and future perspectives

The comparison of the rice embryo proteome of varieties adapted to distinct environmental conditions, namely drought-prone uplands, saline soils, and cool-elevated areas, to those adapted to more favourable conditions, is here presented for the first time. The genetic differences between stress-tolerant and stress-sensitive genotypes were evidenced at the embryo proteome level. Based on embryo protein quantitative changes it was revealed that tolerant genotypes were more distantly related as compared to sensitive ones. This is consistent with the idea that tolerant genotypes are adapted to more diverse environmental conditions than sensitive genotypes, that in turn are adapted to more favourable and homogeneous ecosystems e.g., irrigated lands. A loss of genetic diversity seems to be associated with stress sensitivity, by contrast to high genetic diversity that probably confers a higher plasticity to tolerant genotypes once facing stressful environmental conditions, thus contributing for stress tolerance. The natural variation across rice landraces and wild varieties could therefore be more explored in the research of the mechanisms underlying abiotic stress tolerance. Proteomics is a very powerful tool to dissect such variation.

Rice embryo proteins implicated in diverse cellular processes e.g., protection against stress and nutrient reservoir activity, displayed a high level of genetic variability across the distinct rice genotypes. The expression patterns of these proteins were quite complex, and their qualitatitive and qualitative variations across the distinct embryos, could be related to diverse mechanisms contributing for desiccation tolerance during the late phase of seed maturation. The polymorphism displayed by the proteins identified in the rice embryo might be further explored for genetic mapping in populations derived from the varieties analysed in this study (e.g., ‘IR64’ and ‘PSBRc1’), aiming the control of traits either related to water stress tolerance or grain quality. On the other hand, future proteomic analyses in the vegetative tissues of ‘PSBRc1’ and ‘IR64’ in response to water-deficit, may contribute towards novel clues on drought tolerance. In addition, our results suggest

that seed storage proteins, in particular glutelin type-B 2, may be implicated in cellular protection against dehydration in the rice mature embryo. Therefore, future work could uncover the putative role of seed storage proteins in drought/desiccation tolerance.

For the first time, a clear difference was observed in the phosphorylation status of Rab21 across genotypes with contrasting stress tolerance. The protein was found to be more strongly phosphorylated in the embryos of sensitive varieties than in those of tolerant varieties. Up to eight isoforms were detected in the rice mature embryo. The results strongly suggest that in the embryos of the sensitive genotypes, most isoforms should be heavily phosphorylated, with only a very small proportion being less phosphorylated and/or unphosphorylated. By contrast, in the embryos of tolerant genotypes, as the protein was not so strongly phosphorylated, there should have a much higher proportion of less phosphorylated and/or unphosphorylated forms as compared to sensitive genotypes. This was supported by the detection of a most basic (hence less phosphorylated) form of Rab21 only in the embryos of tolerant genotypes. We propose that less phosphorylated and/or unphosphorylated forms of Rab21 have an important role in stress tolerance, most probably because, if necessary, they are readily accessible to further phosphorylation. In this context, under normal germination conditions, more densely phosphorylated isoforms of Rab21 could be rapidly degraded; but in the case of environmental constraints during germination, the less phosphorylated and/or unphosphorylated forms would be already accessible for further phosphorylation, without 'waiting' for *de novo* protein synthesis. Protein phosphorylation could thus trigger the signal for retarding seedling growth in adverse environmental conditions. The expression pattern of Rab21 seems to support the hypothesis that tolerant genotypes possess a higher preparedness to face environmental constraints when compared with sensitive ones. Future work regarding the function of Rab21 and its phosphorylation role in stress tolerance could focus on the

comparison of the accumulation of the protein between sensitive and tolerant genotypes, in vegetative tissues submitted not only to water deficit, but also to salinity and cold stresses. Not less important it would be the comparison of the protein expression under milder and more severe stress conditions, to check for a possible differential accumulation of the distinct forms of the protein.

A sequence corresponding to the putative rice DBF1 gene is referenced in GenBank since 2003 (Ac. no. AY297448). However, there is no work reporting the characterisation of this gene or its encoded protein until date. A 20-kD rice protein cross-reacted with an antibody raised against the maize DBF1 transcription factor, demonstrating to be induced by water-deficit, salinity, cold and ABA. Although we could not acertain the precise identity of this protein, the whole data indicates that it is homologous to DBF1. The expression pattern of this putative DRE-binding protein was evaluated between genotypes with contrasting drought tolerance, suggesting to take part in 'stress-anticipating' mechanisms in the drought-tolerant variety. Future work concerning protein identification, gene cloning and assays in transgenic plants could reveal its involvement in abiotic stress tolerance.

Finally, cDNA-AFLP allowed the identification of six water stress-responsive genes that proved to be also induced by salinity and cold stress. Most cDNA clones were implicated in stress signalling transduction, suggesting a role in stress tolerance. Some genes were more strongly (or specifically) expressed in tolerant genotypes under stress conditions, whereas others were induced in both tolerant and sensitive genotypes, hence revealing specific and common stress responses. Despite the limited number of identified genes, they seem to offer novel opportunities for rice improvement aiming for multiple abiotic stress tolerance. Transgenic approaches would be usefull to test their concrete role in abiotic stress tolerance.

APPENDIX

Review Article:

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Molecular Responses to Drought in Rice and Maize: Towards Genetic Engineering for Stress Tolerance

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Abstract. Rice and maize are the most important cereals for human consumption. Given the anticipated future increase of the human population, their production must increase to cover the increasing demand for these crops. A major factor that limits the world's cereal production is abiotic stress caused by drought, high salinity or low temperatures. Plants respond to these environmental changes through a number of homeostatic mechanisms that maintain the water balance and the integrity of tissues. This includes several regulatory mechanisms that activate the expression of tolerance effector genes. Therefore, the study of the molecular mechanisms by which plants tolerate osmotic stress is fundamental to improve crop production. Here, we review recent studies on molecular responses to drought and how this information suggests opportunities in genetic engineering for stress tolerance in crops.

Introduction

Rice (*Oryza sativa*) and maize (*Zea mays*) are two major cereal crops for food consumption worldwide. Total production (2000) was about 600 million tons each. These crops, together with wheat, will in the future account for nearly 80% of the cereal demand in developing countries [1,2]. Rice constitutes the staple food for more than half of the world's population. In Asia alone, more than two billion people obtain 60% to 70% of their daily calorie intake from rice [3]. It is the most rapidly growing food resource in Africa and its consumption is also increasing in Europe and North America [4]. In developing countries, maize is used for human consumption, while in the developed world it is mainly for industrial use and animal feed. The high yields in North and Central America outproduce countries such as Mexico, where maize is the staple cereal grain [5]. In the Near East and North Africa, wheat is the dominant staple and its demand for food supply is increasing in a wide number of developing countries [2].

Besides their importance for food security, rice and maize are model plants due to the advantages of rice genomics and maize genetics. Rice has the smaller genome (430 Mbp) compared with other monocots (3000 Mbp in maize and 16000 Mbp in wheat). The overall colinearity with other cereals make it an excellent model for cereal genomics [6]. Maize is the best-studied and most tractable genetic system among cereals, making it the most important system for cereal genetics [7]. Moreover, both genomes are being sequenced. Due to these advantages, in this review we will only focus on developments in rice and maize systems, despite the obvious importance of wheat.

1. Rice and Maize Sequencing Projects

December 2002, the public consortium "International Rice Genome Sequencing Project" (IRGSP) announced the completion of a high quality draft (at least phase-2 sequences) of the 12 chromosomes of the *japonica* cultivar "Nipponbare". The sequencing IRGSP progress presents chromosomes 7 and 10 as being completed, as well as the centromeres on chromosomes 4 and 8, besides chromosomes 1 [8] and 4 [9]. Monsanto [10] and Syngenta [11] have also focused on sequencing the *japonica* subspecies genome, sharing their sequencing data with the public project, whereas a Chinese genome research project has developed a draft sequence of the genome of the subspecies *indica,* cultivar *93-1.1,* the main subspecies grown in China and Southeast Asia [12]. A collection of over 28,000 full-length cDNA clones has been mapped to the genome of these two subspecies [13], in addition to expressed sequence tag (EST) mapping, containing 6591 entries [14].

September 2002, the National Science Foundation of America announced the launch of the 'Maize Genome Sequencing Project', representing a new challenge for sequencing given its size (about six times larger than that of rice) and complexity [7]. The maize genes comprise only about 20% of the whole genome and they are scattered throughout highly conserved and repetitive sequences [15]. Two deep-coverage bacterial artificial chromosome (BAC) libraries have already been generated [16,17] and an integrated genetic/physical map is also being produced. Considerable maize sequence data sets include ESTs, genome survey sequences from transposon-tagged sites, random clone insert sequences, among others [7].

Although the existence of so many genome-sequencing projects seems redundant, they are necessary to understand the differences in the mechanisms of species and subspecies-specific phenotypes and adaptations. Despite the conservation in gene order and content among the cereals' genome observed by both genetic and physical mapping [18], there is local rearrangements, such as tandem gene duplication, small inversions and translocations of one or a few genes between chromosomes [7]. Even the comparison between the genomes of rice subspecies *indica* and *japonica,* demonstrates the existence of significant numbers of rearrangements and polymorphisms [19]. All these sequencing data opens the way for the next steps in functional genomics and at the same time it will allow to engineer new crops more productive and hardier.

2. Importance of Genetic Engineering for Stress Tolerance Improvement

Breeding approaches for stress improvement were successful in the development of cultivars or hybrids more tolerant in the field. Rice varieties with tolerance to abiotic stresses, namely problem soils, adverse temperatures, and moisture stress, as well as resistance to diseases and insects, constitute 80 to 90% of the planted rice area in Asia. Wide-scale adoption of improved varieties contributed to increases in rice production and yield potential and stability [1]. However, despite the cultivation of improved varieties, rice production is facing serious constraints, including declining yield growth rates.

To face this problem and promote an increase in the production of rice, the United Nations launched last October the International Year of Rice 2004 under the motto "Rice is Life" (http://fao. org/rice2004/). The maize production in the United States has also shown a net increase in grain yield during the last century related with genetic improvements such as hybrid development and exploitation of heterosis. The selection strategies for improved yield in parental lines and improved yield stability in their hybrid progenies were ultimately related to an improved tolerance to abiotic stresses, especially to water-deficit [20].

Drought, like many other environmental stresses, has adverse effects on crop yield and water stress is one of the major causes for crop yield reductions. On the other hand, as water resources for agronomic uses become more limiting, the development of drought-tolerant lines becomes increasingly more important. Based on population projections (8,300 million people are expected in 2030) and improved consumption patterns in developing countries, world food grain production must increase by almost 50% in the next 30 years. The challenge for food security in the next decades, is thus to achieve an even greater cereal's production with less land, less labour, fewer chemicals and less water [1].
Genetic engineering or modification (GM) tools will contribute to meet this challenge, by introducing foreign genes conferring stress tolerance, in a much faster and efficient manner compared to that of conventional genetic methods. On the other hand, transgenic approaches are powerful tools to gain valuable information towards a better understanding of the mechanisms beyond the stress tolerance, testing how single or sets of genes confer stress tolerance in GM plants.

The ideal GM crop should possess a highly regulated stress-response capability that doesn't affect crop performance under normal conditions. Until date most of the genetically modified stress-tolerant plants still are non-agronomic plants and only few of them have been tested in field conditions [21]. The introduction of foreign genes into crop plants raises questions about the possible post-transcriptional and post-translational modifications that might occur and its impact on human health and on the environment.

In this review, we will discuss how transgenic approaches serve as powerful means to elucidate stress-response mechanisms. We summarise recent cases where stress-responsive genes proved to confer some kind of tolerance, with special emphasis on rice and maize in the framework of considerations about the biosafety of transgenic plants.

3. Plant Molecular Responses to Drought

The responses to abiotic stress are complex, involving many genes and biochemical-molecular mechanisms [22-29]. These stress-related genes can be grouped into two major categories: those that are involved in signalling transduction cascades and in the control of gene expression, and those that encode proteins that directly protect cellular structures against stresses [22,23]. The first group includes protein kinases e.g., calcium-dependent (CDPK), mitogen-activated (MAPK), SNF1-related protein kinases type 2 and 3 (SnRK2- and SnRK3); putative osmosensors, such as the transmembrane histidine kinase *AtHK1;* enzymes involved in phosphoinositide metabolism; and stress-inducible transcription factors belonging to a range of protein families, e.g. DREBs, ERFs, MYBs, bZIPs, NAC and others [22-26,28]. The second group of gene products includes heat shock proteins, chaperones, late embryogenesis abundant (LEA) proteins, osmoprotectants (proline, glycine betaine, trehalose) and antioxidants (e.g. catalase, ascorbate, carotenoids) [24,25,29].

3.1 Signal Perception and Transduction in Drought Responses

The perception is the first step in stress molecular responses, triggering a cellular signal transduction pathway that will translate a physical stress into a biochemical response. For example, there are several aspects of cellular water loss that can be sensed by the plant, such as the decrease on turgor, change in cell volume or membrane area and alterations in cell wall - plasma membrane connections [29]. In the case of osmotic stress, two osmosensors have been identified in yeast: one is a transmembrane protein that is activated under conditions of high osmolarity, and another is a complex of three proteins that is

inhibited in the same conditions [30]. In plants, the putative osmosensor *AtHK1* [31], a transmembrane histidine kinase, is thought to relay changes in osmotic potential outside the cell to the transduction pathway(s) inside the cell, regulating drought-inducible gene expression [28].

Following stress perception, a signalling mechanism must be activated to induce genes specifically involved in the stress tolerance acquirement. It is becoming clear that the linear pathways identified in this context are only part of a more complex signalling network with multiple functional interconnections [22,23,28]. For instance, many genes that are induced by cold are also induced by drought or ABA [22,32], probably because many cold-inducible genes encode proteins to protect the plant from the consequences of freezing stress, which includes dehydration. In nature, plants suffer multiple stresses either at the same time or separated temporally and they must present an integrated and co-ordinated response to them. This might explain the existence of crosstalk between different signalling pathways in response to stress [28].

Molecular and genetic approaches have greatly contributed to start elucidating about the components of signal transduction pathways induced by drought and other abiotic stresses. The gene *RD29A* from *Arabidopsis* (responsive to dehydration) [33], also known as COR78 (cold-regulated) or LTI78 (low-temperature-induced), is a cold and drought-regulated gene that has been used to examine the convergence of these two pathways. The promoter analysis of this gene has been found to contain two major *cis*-acting elements: the dehydration-responsive element/C-repeat (DRE/CRT) and the ABA-responsive element (ABRE), that function in ABA-independent and ABA-dependent gene expression in response to abiotic stress, respectively [23].

In *Arabidopsis* two groups of transcription factors were found to bind to the DREB/CBF *cis*-acting element and were designed as *CBF/DREB1* and *DREB2* [34,35]. The *CBF/DREB1* genes are quickly and transiently induced by cold, whereas the *DREB2* genes are induced by dehydration or salt (osmotic stress), leading to the expression of target genes involved in stress tolerance acquirement. In rice, it was isolated four genes homologues to *CBF/DREB1* and one homologue to *DREB2* [36]. Gene expression of *OsDREB1A* and *OsDREB1B* was induced by cold, whereas the expression of *OsDREB2A* was induced by dehydration and high salinity, in a similar way to what was described for *Arabidopsis*. The overexpression of *OsDREB1A* in transgenic *Arabidopsis* resulted in plants with a higher tolerance to drought, freezing and high salinity, indicating that is similar functional to *DREB1A* [37,38]. This is, therefore, a potentially useful gene for genetic engineering to generate transgenic cereal crop plants hat show tolerance to multiple abiotic stresses.

Detailed analyses of ABRE and DRE/CBF elements in the expression of the *RD29A* gene revealed that DRE/CBF acts co-operatively with ABRE as a coupling element in ABA-responsive gene expression, in response to drought stress [39]. This is indicative that crosstalk can occur at the level of different *cis*-DNA regulatory elements that mediate stress responses [22]. Other example comes from the *CBF/DREB1* genes that are mainly induced by cold, despite *CBF4/DREB1D* gene induction by osmotic stress [40], suggesting that *CBF4* can mediate the crosstalk between DRE2 and CBF/DREB1 regulatory systems. In maize, the DBF1 transcription factor binds to DRE2 element activating the *rab17* promoter in an ABA-dependent pathway, pointing to a differential regulation of stress-inducible genes in maize and *Arabidopsis* [41]. Such interactions constitute real advantages to the plant since they provide the crosstalk between different stress signalling pathways and the plasticity of the responses.

3.2 Importance of ABA in Drought Responses

ABA is synthesised *de novo* in response to drought and high salinity, but not in response to cold. More than half of the drought-inducible genes identified by cDNA microarrays are also induced by salt and/or by ABA, while only 10% of the drought inducible genes were also induced by cold [22]. Some genes that codify for enzymes involved in ABA biosynthesis [42] were strongly induced by drought. Furthermore, the overexpression of bZIP transcription factors that bind to the ABRE *cis*-element e.g., *ABF3* and *AREB2/ABF4*, caused ABA hypersensitivity, reduced transpiration rate and enhanced drought tolerance in transgenics [43]. All these data indicates the existence of significant crosstalk among the drought and ABA pathways that seems greater than that of ABA and cold [22].

4. Gene Function Analyses

4.1 Transgenic Approaches

The use of transgenic model plants is one of the most powerful tools to ascribe a function to a particular gene. The insertional mutagenesis either through heterologous maize transposons or *Agrobacterium*-mediated T-DNA insertions, have been quite valuable for the identification and isolation of genes displaying a mutant phenotype. However, many genes don't display a phenotype when mutated, being necessary to engineer the insertion sequences to monitor or change the expression pattern of adjacent genes. Also the employment of various forms of gene silencing technology (*e.g.* RNAi mediated gene silencing) allows recovering of knockout phenotypes for genes with redundant function. These transgenic approaches can be addressed by high throughput reverse genetics methods that help to provide functions to the genes discovered by genome sequencing [44].Rice can provide powerful insertion mutagenesis and reverse genetics tools for functional genomics. Rice can now be transformed in a more efficient and routine way than any other cereal [45]. The efficient rice transformation procedure for generating large numbers of T-DNA plants recently published facilitates routine integration of these genomics technologies [46]. With respect to maize, collections with a knockout in each gene of the genome (not including lethal mutations) derived by insertional mutagenesis will be made available in databases.

4.2 Transcriptomics and Proteomics

Microarray technology using cDNAs or oligonucleotides, among other transcript-profiling techniques, is extremely important to analyse the patterns of gene expression in response to abiotic stress [47,48,49,50,51]. Despite the fact that knowing when and where a gene product (RNA and/or protein) is expressed thus providing important clues about its biological function, it doesn't necessary prove a causal relationship between gene sequences and their function. Other factors than the mRNA alone, such as protein modifications, determine the activity of a gene product at cellular level. Therefore, the true picture of gene expression can be offered by proteomics that addresses the protein expression of a cell type and the post-translational modifications. The recent developments in mass spectrometry and the creation of algorithms for automatic spot quantification, along with a better resolution and reproducibility of two-dimensional polyacrylamide gelelectrophoresis, have simplified protein analysis and their characterisation. On the other hand, the availability of genome sequences has also contributed to simplify and speed up the identification of proteins.

Proteomics is a valuable tool to analyse drought stress response. Studies on the regulation of gene expression during maize embryogenesis through two-dimensional PAGE analysis, revealed the expression of a set of polypeptides that were rapidly induced by ABA in young embryos [52]. These polypeptides accumulated along with maturation, coinciding with the period when the endogenous level of ABA attained a maximum [53]. These polypeptides corresponded to the maize LEA proteins, Rab17 and Rab28 [54,55]. Furthermore, proteomics offers an interesting new approach to analyse the modifications that occur on gene products when overexpressed in transgenic plants.

5. Genes Conferring Drought Tolerance in Transgenic Plants

Most genetic engineering involving abiotic stress-tolerance has been made in non-agronomic plants, like *Arabidopsis* and tobacco. This fact is related to their greater facility in transforming and reduced life cycles compared with crop plants. Following below, we report some examples of rice transgenic plants that were engineered for drought tolerance, based on different mechanisms of stress response. All these experiments make reference to genes encoding a single specific stress-protective protein, although other approaches, such as the use of transcription factors, confer improvement to several abiotic stresses simultaneously [38]. For a detailed review of this subject, see [56].

5.1 Osmoprotectants

Organic compounds of low molecular weight are known as compatible solutes, osmolytes or osmoprotectants. They accumulate in plants in response to water stress and high salinity. The primary function of compatible solutes is to maintain cell turgor and consequently the adjustment of the osmotic potential in the cytoplasm [57]. Recent studies indicate that they can also act as free-radical scavengers or chemical chaperones by directly stabilising membranes and/or proteins (for review, see [24]). Compatible solutes are grouped into three major classes: amino acids (*e.g.* proline), quaternary amines (*e.g.* glycine betaine) and polyol/sugars (*e.g.* mannitol, trehalose).

5.1.1 Proline

The enzyme P5CS (Δ^1–pyrroline-5-carboxylate synthetase) is involved in the biosynthesis of proline. In rice, a mothbean (*Vigna aconitifolia*) gene codifying for this enzyme was overexpressed under a stress and ABA-inducible promoter complex (e.g. AIPC-ABA), leading to the accumulation of proline. The transgenic plants accumulated up 1.6 to 2.5-fold more proline under drought conditions, as compared to control plants. The second generation of transgenic plants showed an increase in biomass under drought and salt stress in respect to the non-transformed plants, reflected as higher levels of fresh shoot and root weight. Thus, the extent of drought and salt stress tolerance in transgenic plants were correlated to the levels of proline accumulated [58].

5.1.2 Glycine Betaine

The compatible solute glycine betaine is biosynthesised by different pathways. The choline oxidase (*codA*) gene from the bacterium soil *Arthrobacter globiformis* is involved in a single-step pathway and was transferred to rice to enhance salt and cold tolerance [57]. In transgenic plants, the levels of glycine betaine were as high as 1-5 μm per gram fresh

weight of leaves. Although treatments with 150 mM NaCl inhibited the growth of both wild type and transgenic plants, the last recovered earlier after the elimination of the stress. The authors suggested that this tolerance could be related with the stabilisation of the structure/function of proteins, since the levels of concentration of this solute were rather low for osmotic adjustment.

5.1.3 Trehalose

It is known that trehalose has a high water retention activity, which maintains the fluidity of membranes under dry conditions [59]. Dicotyledonous transgenic plants expressing the trehalose-6-phosphate (T-6-P) synthase (TPS) and/or T-6-P phosphatase (TPP) genes from bacteria, exhibited an increased drought tolerance, but also strong developmental alterations. However, two recent works on rice expressing the TPS and TPP bacterial genes under constitutive [59] and tissue/stress inducible promoters [60], respectively, showed that the overproduction of trehalose resulted in the improvement of abiotic stress tolerance, without stunting growth. Maize transgenic plants overexpressing an *Arabidopsis* gene e.g., *AtTPS* codifying for TPS, under the CaMV35S promoter were obtained and are tested for drought stress improve-ment (A.M. Almeida, personal communication).

5.2 LEA Proteins

Plants activate the expression of specific stress-associated proteins, such as the LEA proteins, in addition to the accumulation of compatible solutes. These proteins accumulate in high concentrations in mature seeds and are induced by ABA in immature embryos and in vegetative tissues, by abiotic stress and/or ABA, [61]. Although their function still remains unknown, they have been proposed several roles in protecting cellular structures from dehydration e.g., hydration buffer, sequestering ions, protection of other proteins and renaturation of unfolded proteins [62]. In rice, the overexpression of a LEA protein from barley (*HVA1*) under a constitutive promoter, conferred drought and salt tolerance that was correlated with the high levels of accumulation of this protein. [63]. Transgenic plants expressing LEA proteins from maize under a constitutive promoter, also presented a higher tolerance to drought and salinity in respect to control plants. These studies support the role of LEA proteins in protecting plants against drought and/or high salinity stress.

6. Biosafety considerations

The Green Revolution in the 1960s allowed the introduction of higher-yielding varieties of rice, maize and wheat, leading to an increase in food production to keep pace with population growth. Nowadays, a "Doubly Green" Revolution is necessary. Future agriculture must be both more productive and more environmentally friendly in terms of the conservation of natural resources and the avoidance of pollution, than was achieved in the first Green Revolution [64]. As discussed above, genetic engineering tools may help to meet this challenge.

In 1999, 40% of all cotton, 35% of soy and 25% of maize grown in the United States, were genetically modified (GM) [65]. In 2001, world production of GM crops occurred mainly in the United States, Brazil, Argentina, Canada and China [66]. In Europe, almost none GM crops were cultivated probably due to the highly restricted release of GM crops and their controversy. However, European Union (EU) generally permits the release and commercialisation of GMOs [67]. Many research studies about biosafety have been

conducted in the last 15 years. The most important environmental concerns include the possibility of: (1) the novel traits increasing the weediness or invasiveness of crop plants or related species; (2) the spread of the novel genes and traits through hybridisation with wild, sexually compatible relatives, thus potentially increasing the fitness levels of these wild species and causing the disturbance or loss of rare populations; (3) novel insect resistance traits, and (4) the evolution of pests with increased resistance that could necessitate the use of higher doses of pesticides [68]. Moreover, environmental safety assessment should exist also with respect to the novel traits that are achieved through mutagenesis and selective breeding, which also introduces genetic modifications. The need of a constant assessment of the risks associated with their environmental release is important to control biosafety.

Another type of concern is related with the use of antibiotics and herbicides as selectable markers in transformation procedures. These markers raise questions about the possible antibiotic resistance on humans and herbicide resistance into wild types. However, these kind of problems associated with the presence of marker genes in plants have been known for quite some time and various studies have been carried in order to remove these genes [69]. Therefore, the future transgenic crops will have this problem obviated.

Establishing the biosafety of the drought-resistant GM crops discussed above will be particularly challenging in terms of risks and benefits. On the one hand, such plants will have an extended habitat with possibly undesired ecological consequences. Also, the changes brought about in overall plant physiology will have to be assessed for their consequences for food, feed and ecology. There may be unexpected or unintended changes accompanying GM drought tolerance. Novel developments in ecological genomics and all other 'omics' approaches will prove helpful, if not crucial, for the acceptable assessment of the biosafety of GM drought tolerance in crops. On the other hand, however, drought resistance in crops clearly aims to help solving or alleviating a pressing problem in current-day and future agriculture. At the end of the day, it should therefore be a proper risk-benefit analysis that will allow a balanced and informed decision on the acceptability and desirability of the use of GM drought tolerance in crops in a given area. This way, genetic modification should help to meet the challenge of the "Doubly Green" Revolution. We believe that the future and foreseeable problems of notably the lack of proper water in agriculture should be an important issue in such a risk-benefit analysis.

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References

[1] Khush, G. (2003). Productivity improvements in rice. Nutr. Rev. 61, S114-116.
[2] Hoisington, D., Khairallah, M., Reeves T., Ribaut J.M., Skovmand, B., Taba, S. et al. (1999). Plant Genetic Resources: What can they contribute toward increased crop productivity? Proc. Natl Acad. Sci. USA 96, 5937-5943.
[3] FAO (2003). The International Year of Rice 2004. Concept Paper. Rome, Italy: FAO (www.fao.org/rice2004/en/f-sheet/concept.pdf).
[4] Nguyen, V.N. and Tran, D.V (2002). Rice in Producing Countries. FAO Rice Information, Vol. 3, Chapter III, Rome, Italy: FAO (www.fao.org/DOCREP/005/Y4347E/y4347e04.htm#bm04).
[5] FAO (1992). Maize in Human Nutrition. Rome, Italy: FAO (www.fao.org/docrep/T0395E/T0395E00.htm).
[6] Goff, S.A. (1999). Rice as a model for cereal genomics. Curr. Opin. Plant Biol. 2, 86-89.
[7] Chandler, V.L. and Brendel, V. (2002). The maize genome sequencing project. Plant Physiol. 130, 1594-1597.
[8] Sasaki, T., Matsumoto, T., Yamamoto, Sakata, K. K., Baba, T., Katayose, Y. et al. (2002). The genome sequence and structure of rice chromosome 1. Nature 420, 312-316.

[9] Feng, Q., Zhang Y, Hao, P., Wang, S., Fu, G., Huang Y. et al. (2002). Sequence and analysis of rice chromosome 4. Nature 420, 316-320.
[10] Barry, G. (2001). The use of the Monsanto draft rice genome sequence in research. Plant Physiol. 125, 1161-1165.
[11] Goff , S.A., Ricke, D., Lan, T.H., Presting, G., Wang, R. Dunn, M. et al. (2002). A draft sequence of the rice genome (*Oryza sativa* L. ssp *japonica*). Science 296, 92-100.
[12] Yu, J., Hu, S., Wang, J, Wong, G.K.S., Li, S. Liu, B. et al. (2002). A draft sequence of the rice genome (*Oryza sativa* L. ssp *indica*). Science 296, 79-91.
[13] Kikuchi, S., Satoh, K., Nagata, T., Kawagashira, N., Doi, K., Kishimoto, N. et al. (2003). Collection, mapping, and annotation of over 28,000 cDNA clones from *japonica* rice. Science 301, 376-378.
[14] Wu, J., Maehara, T., Shimokawa, T., Yamamoto, S., Harada, C., Takazaki, Y. et al. (2002). A comprehensive rice transcript map containing 6591 expressed sequence tag sites. Plant Cell 14, 525-535.
[15] San Miguel, P., Tikhonov A., Jin, Y.K., Motchoulskaia N., Zakharov, D., Melake-Berhan, A. et al. (1996). Nested retrotransposons in the intergenic regions of the maize genome. Science 274, 765-768.
[16] Cone, K., McMullen, M., Bi, I.V., Davis, G. Yim, Y.S., Gardiner, J. et al. (2002). Genetic, physical and informatics resources on maize: on road to an integrated map. Plant Physiol. 130, 1598-1605.
[17] Tomkins, J.P., Davis, G., Main, D., Yim, Y., Duru, N. Musket, T. et al. (2002). Construction and characterization of a deep-coverage bacterial artificial chromosome library for maize. Crop Sci. 42, 928-933.
[18] Gale, M.D. and Devos, K.M. (1998). Comparative genetics in the grasses. Proc. Natl Acad. Sci. USA 95, 1971-1974.
[19] Sasaki, T. and Sederoff, R. (2003). Genome studies and molecular genetics. The rice genome and comparative genomics of higher plants. Curr. Opin. Plant Biol. 6, 97-100.
[20] Bruce, W.B., Edmeades, G.O. and Barker, T.C. (2002). Molecular and physiological approaches to maize improvement for drought tolerance. J. Exp. Bot. 53, 13-25.
[21] Dunwell, J.M. (2000). Transgenic approaches to crop improvement. J. Exp. Bot. 51, 487-496.
[22] Shinosaki, K, Yamaguchi-Shinosaki, K. and Seki, M. (2003). Regulatory network of gene expression in the drought and cold stress responses. Curr. Opin. Plant Biol. 6, 410-417.
[23] Seki, M., Kamei, A., Yamaguchi-Shinosaki, K. and Shinosaki. K. (2003). Molecular responses to drought, salinity and frost: common and different paths for plant protection. Curr. Opin. Biotechnol. 14, 194-199.
[24] Wang, W., Vinocur, B. and Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta 218, 1-14.
[25] Sung, D.Y., Kaplan, F., Lee, K.J. and Guy, C.L. (2003). Acquired tolerance to temperature extremes. Trends Plant Sci. 8, 179-187.
[26] Hazen, S.P., Wu, Y. and Kreps, J.A. (2003). Gene expression profiling of plant responses to abiotic stress. Funct. Integr. Genom. 3, 105-111.
[27] Zhu, J.K. (2001). Plant salt tolerance. Trends Plant Sci. 6, 66-71.
[28] Knight, H. and Knight, M.R. (2001). Abiotic stress signalling pathways: specificity and cross-talk. Trends Plant Sci. 6, 262-267.
[29] Bray, E.A. (1997). Plant responses to water deficit. Trends Plant Sci. 2, 48-54.
[30] Posas, F., Wurgler-Murphy, S.M., Maeda, T., Witten, E.A., Thai, T.C. and Saito, H. (1996). Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 "two component" osmosensor. Cell 86, 865-875.
[31] Urao, T., Yakubov, B., Satoh, R., Yamaguchi-Shinozaki, K., Seki, M., Hirayama, T. et al. (1999). A transmembrane hybrid-type histidine kinase in Arabidopsis functions as an osmosensor. Plant Cell 11, 1743-1754.
[32] Shinosaki, K. and Yamaguchi-Shinosaki, K. (2000). Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signalling pathways. Curr. Opin. Plant Biol. 3, 217-223.
[33] Yamaguchi-Shinosaki, K. and Shinosaki, K. (1994). A novel cis-acting element in *Arabidopsis* gene is involved in responsiveness to drought, low temperature, or high-salt stress. Plant Cell 6, 251-264.
[34] Liu, X., Kasuga, M. Sakuma, Y. Abe, H., Miura, S., Yamaguchi-Shinosaki, K. et al. (1998). Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 binding domain separate two cellular signal transduction pathways in drought- and low temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell 10, 1391-1406.
[35] Sakuma, Y., Liu Q., Dubouzet, J.G., Abe, H., Shinosaki, K. and Yamaguchi-Shinosaki, K. (2002). DNA binding specifity of the ERF/AP2 domain of *Arabidopsis* DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. Biochem. Biophys. Res. Comm. 290, 998-1009.
[36] Dubouzet, J.G., Sakuma, Y., Ito, Y., Kasuga, M., Dubouzet, E.G. Miura, S. et al. (2003). *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt and cold-responsive gene expression. Plant J. 33, 751-763.

[37] Gilmour, S.J., Sebolt, A.M., Salazar, M.P., Everard, J.D. and Thomashow, M.F. (2000). Over-expression of the *Arabidopsis* CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimatation. Plant Physiol. 124, 1854-1865.
[38] Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinosaki, K. and Shinosaki, K. (1999). Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat. Biotechnol. 17, 287-291.
[39] Narusaka, Y., Nakashima, K, Shinwari, Z.K. Sakuma, Y., Furihata, T. Abe, H. et al. (2003). Interaction between two *cis*-acting elements, ABRE and DRE, in ABA-dependent expression of *Arabidopsis rd29A* gene in response to dehydration and high salinity stresses. Plant J. 34, 137-148.
[40] Haarke, V. Cook, D., Riechmann, J.L, Omaira, P., Thomashow, M.F. and Zang, J.Z. (2002) Transcription factor CBF4 is a regulator of drought adaptation in *Arabidopsis*. Plant Physiol. 130, 639-648.
[41] Kizis, D. and Pagès. M. (2002). Maize DRE-binding proteins DBF1 and DBF2 are involved in *rab17* regulation through the drought responsive element in an ABA-dependent pathway. Plant J. 30, 679-689.
[42] Iuchi, S., Kobayashi, M., Taji, T., Naramoto, M,, Seki, M., Kato, T. et al. (2001). Regulation of drought tolerance by gene manipulation of 9-cis epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*. Plant J. 27, 325-333.
[43] Kang, J.Y., Choi., H.I., Im, M.Y. and Kim, S.Y. (2002). *Arabidopsis* basic leucine zipper proteins that mediate stress-responsive abscisic acid signalling. Plant Cell 14, 343-357.
[44] Pereira, A. (2000). A transgenic perspective on plant functional genomics. Transg. Res. 9, 245-260.
[45] Delseny, M. (2003). Towards an accurate sequence of the rice genome. Curr. Opin. Plant Biol. 6, 101-105.
[46] Sallaud, C., Meynard, D., Boxtel, J., Gay, C., Bès, M., Brizard, J.P. et al. (2003). Highly efficient production and characterisation of T-DNA plants for rice (*Oryza sativa* L.) functional genomics. Theor. Appl. Genet. 106, 1396-1408.
[47] Matsumura, H., Nirasawa, S. and Terauchi, R. (1999). Transcript profiling in rice (*Oryza sativa* L.) seedlings using serial analysis of gene expression (SAGE). Plant J. 20, 719-726.
[48] Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinosaki, K., Carninci, P. et al. (2001). Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. Plant Cell 13, 61-72.
[49] Kawasaki, S., Borchert, C., Deyholos, M., Wang, H., Brazille, S., Kawai, K. et al. (2001). Gene expression profiles during the initial phase of salt stress in rice. Plant Cell 13, 889-905.
[50] Yu, L.X. and Setter, T.L. (2003). Comparative transcriptional profiling of placenta and endosperm in developing maize kernels in response to water deficit. Plant Physiol .131, 568-582.
[51] Cooper, B., Clarke, J.D., Budworth, P., Kreps, J., Hutchison, D., Park, S. et al. (2003). A network of rice genes associated with stress response and seed development. Proc. Natl Acad. Sci. USA 100, 4945-4950.
[52] Sanchez-Martinez, D., Puigdomenech, P. and Pagès, M. (1986). Regulation of gene expression in developing *Zea mays* embryos. Plant Physiol. 82, 543-549.
[53] Goday, A., Sánchez-Martinez, D., Gómez, J., Puigdomenech, P. and Pagès, M. (1988). Gene expression in developing *Zea mays* embryos: regulation by abscisic acid of a highly phosphorylated 23-25-kD group of proteins. Plant Physiol. 88, 564-569.
[54] Vilardell, J., Goday, A., Freire, M.A., Torrent, M., Martinez, M.C., Torné, J.M. et al. (1990). Gene sequence, developmental expression and protein phosphorylation of RAB-17 in maize. Plant Mol. Biol. 14, 423-432.
[55] Pla, M., Gómez, J., Goday, A. and Pagès, M. (1991). Regulation of the abscisic acid-responsive gene rab28 in maize viviparous mutants. Mol. Gen. Genet. 230, 394-400.
[56] Zhang, J.Z. (2003). Overexpression analysis of plant transcription factors. Curr Opin. Plant Biol. 6, 430-440.
[57] Sakamoto, A., Murata, A. and Murata, N. (1998). Metabolic engineering of rice leading to biosynthesis of glycine betaine and tolerance to salt and cold. Plant Mol. Biol. 38, 1011-1019.
[58] Zhu, B., Su, J., Chang, M., Verma, D.P., Fan, Y.L. and Wu, R. (1998). Overexpression of a Δ^1–pyrroline-5-carboxylate synthetase gene and analysis of tolerance to water-and salt-stress in transgenic rice. Plant Sci. 139, 41-48.
[59] Jang, I.C., Oh, S.J., Seo, J.S., Choi, W.B., Song, S.I.K., Kim, C.H. et al. (2003). Expression of a bifunctional fusion of the *Escherichia coli* genes for trehalose-6-phsphate synthase and trehalose-6-phosphate phosphatase in transgenic rice plants increases trehalose accumulation and abiotic stress tolerance without stunting growth. Plant Physiol. 131, 516-524.
[60] Garg, A.K., Kim, J.K., Owens, T.G., Ranwala, A.P., Choi, Y.D., Kochian, L.V. et al. (2002). Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc. Natl Acad. Sci. USA 99, 15898-15903.

[61] Dure III, L. (1997). LEA proteins and the desiccation tolerance of seeds. In Cellular and Molecular Biology of Plant Seed Development. (Larkins, B.A. and Vasil, I.K. Eds). The Netherlands: Kluwer Academic Publishers, pp. 525-543.
[62] Wise, M.J. and Tunnacliffe, A. (2003). POPP the question: what do LEA proteins do? Trends Plant Sci. 124, 1-5.
[63] Xu, D., Duan, X., Wang, B., Hong, B., Ho, T.D. and Wu, R. (1996). Expression of a late embryogenesis abundant protein gene, *HVA1*, from barley confers tolerance to water deficit and salt stress in transgenic rice. Plant Physiol. 10, 249-257.
[64] Abelson, P.H. and Hines, P.J. (1999). The plant revolution. Science 285, 367-368.
[65] Borlaug, N.E. (2000). Ending world hunger, the promise of biotechnology and the threat of antiscience zealotry. Plant Physiol. 124, 487-490.
[66] Wisniewski, J.P., Frangne, N., Massonneau, A. and Dumas, D. (2002). Between myth and reality: genetically modified maize, an example of sizeable scientific controversy. Biochim. 84, 1095-1103.
[67] Saeglitz, C. and Bartsch, D. (2003). Regulatory and associated political issues with respect to Bt transgenic maize in the European Union. J. Invertebr. Pathol. 83, 107-109.
[68] Macdonald, P. and Yarrow, S. (2003). Regulation of Bt crops in Canada. J. Invertebr. Pathol. 83, 93-99.
[69] Scutt, C.P., Zubko, E. and Meyer P. (2002). Techniques for the removal of marker genes from transgenic plants. Biochimie 84, 1119-1126.