Universitat de Lleida

MOLECULAR AND PHYSIOLOGICAL APPROACHES TO ENHANCE THE PERFORMANCE AND PRODUCTIVITY OF RICE

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DOCTORAL DISSERTATION

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The photos of the cover

In front: Rice field in Vietnam, part of the photo, taken from QT Luong, www.terragalleria.com

Behind from left to right and from up to down:

1. *Rice field in Oki province, view of O-Yama,* Utagawa Hiroshige, taken from http://en.wikipedia.org/wiki/File:Riziere_Oki.jpg
2. *Oryza Sativa* L., taken from www.plant-pictures.de
3. Rice grains, part of the image collection of www.irri.org
4. Aerial view of terrace rice fields in Yuanyang, Yunnan Province, southern China taken from Jialiang Gao, www.peace-on-earth.org

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Paul Christou, Doctor in Plant Biochemistry, Professor of Plant Biotechnology and Head of the Applied Plant Biotechnology group at the University of Lleida, and Teresa Capell, Doctor in Plant Physiology, Professor of Plant Biotechnology and Co-leader of the Applied Plant Biotechnology group at the University of Lleida, both attached to the department of Plant Production and Forest Science of the University of Lleida during the course of the thesis experimental work,

We hereby state,

That Svetlana Dashevskaya, who majored in Biology at the Tel-Aviv University, has performed under our direction and supervision, and within the Applied Plant Biotechnology group from the department of Plant Production and Forest Science, the experimental work entitled "Molecular and physiological approaches to enhance the performance and productivity of rice",

That the work accomplishes the adequate conditions in order to be defended in front of the corresponding Thesis Committee and, if the opportunity arises, to obtain the Doctor degree by the University of Lleida,

And we sign the current document that this may be officially recorded, to complete formalities deemed necessary.

Lleida, December 2011

Dr. Paul Chrisou Dra. Teresa Capell

In memory of my mother who
has never ignored my “why?” questions

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Part A

Bioregulators as a tool for the improvement of rice growth and productivity under abiotic stress conditions

Abbreviations

AM symbiosis	Arbuscular mycorrhizal symbiosis
bp	Base pair(s)
BSA	Bovine serum albumin
CaMV 35S	Cauliflower mosaic virus 35S
dNTP mix	Mix of deoxynucleotide 5`-triphosphate
EDTA	Ethylene diamino tetra acetic acid
e.g.	For example
h	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hpt	Hygromycin phosphotransferase gene
IRRI	International Rice Research Institut
IT	Infection thread
kDa	Kilodalton(s)
kg	Kilogram(s)
LB	Luria Burtoni medium
mg	Milligrams
MgATP	Magnesium adenosine triphosphate
min	Minutes
mL	Milliliter(s)
mM	Millimolar
mm	Millimeter(s)
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
ng	Nanogram(s)
Os	Oryza sativa (rice)
PAGE	Polyacrylamide gel
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
RNS	Root nodule simbiosis
RT-PCR	Reverse transcription polymerase chain reaction
s	Second(s)
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
SSC	Saline sodium citrate
SYM	Symbiotic component
T0	Primary transformants
T1	First transgenic generation
UV	Ultra violet
µg	Microgram(s)
µl	Microliter(s)
ψ_w	Water potential

Abstract

Abiotic stresses such as drought and salinity are important environmental factors that limit crop productivity. Strategies to improve the growth of crops under abiotic stress conditions include conventional breeding, marker-assisted breeding, genetic engineering and the application of natural and synthetic bioregulators. Natural bioregulators include plant hormones such as abscisic acid (ABA), cytokinins, gibberellins and jasmonic acid, whereas synthetic bioregulators are chemical products that improve plant growth under field conditions. Safeners are synthetic bioregulators that selectively protect crop plants but not weeds from herbicides by coordinately inducing entire herbicide detoxification pathways. In addition to their direct role in detoxification, some safeners can also improve plant growth particularly under abiotic stress conditions.

I evaluated a number of potential synthetic bioregulators alone or in combination with ABA to select the combinations that improved rice growth and productivity under mild but long-term drought and salinity stress conditions optimized in a growth chamber. Cyprosulfamide alone or in combination with ABA was found to promote rice growth under salinity stress, resulting in earlier flowering and the appearance of new tillers. These responses were either absent or much more limited in untreated plants under stress.

Experiments to unravel the molecular basis of cyprosulfamide activity resulted in the identification of genes that were potentially responsible for the improved performance of rice plants under salinity stress. Proteomic analysis of untreated rice plants and those treated with cyprosulfamide and/or ABA in the presence or absence of salinity stress allowed us to identify stress-response genes with roles in defense, detoxification, energy, metabolism, photosynthesis, protein folding and protein transport among others. Interestingly, many proteins that were modulated by exposure to salinity stress were also modulated by cyprosulfamide or cyprosulfamide plus ABA. We propose that cyprosulfamide induces abiotic stress-response genes and pre-adapts the plants to withstand abiotic conditions. Three genes encoding a late embryogenesis abundant group 3 protein (OsLEA3), a putative fumarylacetoacetate hydrolase (FAH) and a mitochondrial import inner membrane translocase (TIM) were strongly upregulated by salinity stress as well as by cyprosulfamide or cyprosulfamide plus ABA in the absence of stress. There was good agreement between gene expression profiles determined by

quantitative real-time reverse transcriptase polymerase chain reaction and the proteomic data.

Resum

Estressos abiòtics com la sequera i la salinitat són els factors ambientals més importants que limiten la productivitat dels cultius. Estratègies per millorar el creixement dels cultius sota condicions d'estrès abiòtic inclouen la millora convencional, la selecció assistida per marcadors, l'enginyeria genètica i l'aplicació de bioreguladors naturals o sintètics. Els bioreguladors naturals són hormones vegetals com l'àcid abscísic (ABA), citoquinines, giberelines i l'àcid jasmònic, mentre que els bioreguladors sintètics són productes químics que milloren el creixement de les plantes en condicions de camp. Els fitoprotectors són bioreguladors sintètics que selectivament protegeixen les plantes del cultiu, però no les males herbes dels herbicides induint de forma coordinada les vies de detoxificació d'herbicides. A més del seu paper directe en la detoxificació, alguns fitoprotectors també poden millorar el creixement de plantes en particular sota condicions d'estrès abiòtic.

He avaluat un nombre de potencials bioreguladors sintètics sols o en combinació amb ABA per seleccionar les combinacions que milloren el creixement i la productivitat de l'arròs en condicions de sequera (o estrès hídric) moderada, però a llarg termini i d'estrès per salinitat, optimitzat en una cambra de creixement. L'agent fitoprotector ciprosulfamida sol o en combinació amb ABA s'ha trobat que promou el creixement de l'arròs sota condicions d'estrès salí, donant lloc a una floració avançada i a l'aparició de tiges noves. Aquestes respostes van estar absents o van ser molt més limitades en les plantes no tractades sotmeses a estrès.

Experiments per desentranyar les bases moleculars de l'efecte de la ciprosulfamida han resultat en la identificació de gens que són potencialment responsables de la millora del rendiment de les plantes d'arròs sota estrès per salinitat. L'anàlisi proteòmic de les plantes d'arròs sense tractar i de les tractades amb ciprosulfamida i / o ABA en presència o absència d'estrès per salinitat ens ha permès identificar gens de resposta a l'estrès amb funcions relacionades amb la defensa, la destoxificació, l'energia, el metabolisme, la fotosíntesi, el plegament i el transport de proteïnes, entre d'altres. Curiosament, moltes proteïnes que eren modulades per l'exposició a estrès per salinitat també van ser modulades per ciprosulfamida o ciprosulfamida més ABA. Proposem que l'agent fitoprotector ciprosulfamida indueix gens de resposta a l'estrès abiòtic i pre-adapta les plantes per suportar les condicions d'estrès abiòtic. Tres gens que codifiquen per late embryogenesis abundant group 3 protein (OsLEA3), putative

fumarylacetoacetate hydrolase (FAH) i mitochondrial import inner membrane translocase (TIM) estan induïdes fortament per l'estrès salí, així com per ciprosulfamida o ciprosulfamida més ABA en l'absència d'estrès. Es va trobar una correlació positiva entre els perfils d'expressió génica determinada per la reacció en cadena de la polimerasa quantitativa o en temps real amb la transcriptasa inversa (qRT-PCR) i les dades de proteòmica.

Resumen

Estreses abióticos como la sequía y la salinidad son los factores ambientales más importantes que limitan la productividad de los cultivos. Estrategias para mejorar el crecimiento de los cultivos bajo condiciones de estrés abiótico incluyen la mejora convencional, la selección asistida por marcadores, la ingeniería genética y la aplicación de biorreguladores naturales o sintéticos. Los biorreguladores naturales son hormonas vegetales como el ácido abscísico (ABA), citoquininas, giberelinas y ácido jasmónico, mientras que los biorreguladores sintéticos son productos químicos que mejoran el crecimiento de plantas en condiciones de campo. *Safeners* son biorreguladores sintéticos que selectivamente protegen a las plantas del cultivo pero no a las malas hierbas de los herbicidas, induciendo de forma coordinada todas las vías de detoxificación de herbicidas. Además de su papel directo en la detoxificación, algunos *safeners* también pueden mejorar el crecimiento de plantas en particular bajo condiciones de estrés abiótico.

He evaluado diversos biorreguladores sintéticos candidatos, solos o en combinación con ABA para seleccionar las combinaciones que mejoren el crecimiento y la productividad del arroz en condiciones de sequía o salinidad moderada, pero a largo plazo, optimizado en una cámara de crecimiento. Cyprosulfamide solo o en combinación con ABA se ha encontrado que promueve el crecimiento del arroz bajo estrés por salinidad, dando lugar a una floración temprana y a la aparición de tallos nuevos. Estas respuestas eran ausentes o mucho más limitadas en las plantas no tratadas sometidas a estrés.

Experimentos para desentrañar las bases moleculares de la actividad de cyprosulfamide resultaron en la identificación de genes que son potencialmente responsables de la mejora del rendimiento de las plantas de arroz bajo estrés por salinidad. El análisis proteómico de las plantas de arroz sin tratar y las tratadas con cyprosulfamide y / o ABA en presencia o ausencia de estrés por salinidad nos ha permitido identificar genes de respuesta a estrés con funciones relacionadas con la defensa, la detoxificación, la energía, el metabolismo, la fotosíntesis, el plegamiento de proteínas y el transporte de proteínas, entre otros. Curiosamente, muchas proteínas que eran moduladas por la exposición a estrés por salinidad también eran moduladas por cyprosulfamide o cyprosulfamide más ABA. Nosotros proponemos que el *safener* cyprosulfamide induce genes responsables de estrés abiótico y adapta a las plantas para soportar las condiciones de estrés abiótico. Tres genes que codifican late embryogenesis abundant

group 3 protein (OsLEA3), putative fumarylacetoacetate hydrolase (FAH) y mitochondrial import inner membrane translocase (TIM) fueron inducidas fuertemente por el estrés de salinidad, así como por cyprosulfamide o cyprosulfamide más ABA en la ausencia de estrés. También se vió una correlación positiva entre los perfiles de expresión genética determinada por la reacción en cadena de la polimerasa en tiempo real o cuantitativa con transcriptasa inversa (qRT-PCR) y los datos de proteómica.

Chapter 1

General introduction

1.1. Abiotic stress

Abiotic stresses are environmental factors that exert harmful effects on living organisms. Among the most common abiotic stresses are drought, salinity and extreme temperatures. Animals are affected by abiotic stresses, but plants are much more vulnerable because of their inability to move and constant exposure to detrimental environmental conditions. Environmental stresses reduce crop productivity (Verslues et al., 2006) and are increasing due to salinization by irrigation and the lower level of rainfall (Vinocur and Altman, 2005; Verslues et al., 2006). Some land has deteriorated to the extent that it can no longer be used for agriculture. In addition growing world populations means that more agricultural land is being set aside for urbanization. The availability of food is therefore threatened, and what arable land remains must increase in productivity to maintain food security (Christou and Twyman, 2004). One solution is to create plants that are more tolerant towards abiotic stresses (Takeda and Matsuoka, 2008). The discovery of genes with potential roles in abiotic stress adaptation has accelerated thanks to "omics" technologies that allow the global analysis of gene and protein functions in plants under stress (Mochida and Shinozaki, 2010).

1.1.1. Drought

Drought is the major abiotic stress threatening agricultural productivity (Yang et al., 2010). Drought can be defined as a period of below-normal precipitation that limits productivity by introducing a water deficit, and thus a lower water potential in the plant (Verslues et al., 2006). About 28% of the world's soil is constitutively affected by drought, and up to 50% is affected periodically due to shallowness, poor water holding capacity and other factors (Salekdeh et al., 2009). Drought is a major contributor to food insecurity and poverty (FAO, http://www.fao.org/nr/water/docs/waterataglance.pdf).

Rice is one of the world's most important food crops, but it is very sensitive to drought stress because of its limited ability to adapt to water-deficit conditions (Yang et al., 2010). In rain-fed ecosystems (approximately one-third of all rice crops) drought reduces productivity by 13–35% (Degenkolbe et al., 2009). Maize is another staple crop

that is highly sensitive to water deficit, especially during pollination and embryo development (Yang et al., 2010).

The first processes to be affected by drought are cell growth and photosynthesis (Chaves 1991). Photosynthesis can be affected directly by the reduced availability of CO_2 due to stomatal closure or oxidative stress.

1.1.2. Salinity

Soil salinity is the second most important abiotic stress factor affecting agricultural productivity, particularly in South and South-East Asia and in arid and semi-arid regions with a limited water supply and a hot dry climate (Hakim et al., 2009). Today approximately 20% of the world's farmland and nearly 50% of all irrigated land is affected by salinity. Salinity is expected to increase in the future, resulting in the loss of 30% of arable land by 2025 and 50% by 2050 (Wang et al., 2003).

Species vary in their tolerance to salt stress (**Figure 1.1**). Rice is the most sensitive among the cereals (Munns et al., 2008) although the sensitivity of maize varies according to the developmental stage, and is highest during early vegetative growth (Fortmeier and Schubert, 1995).

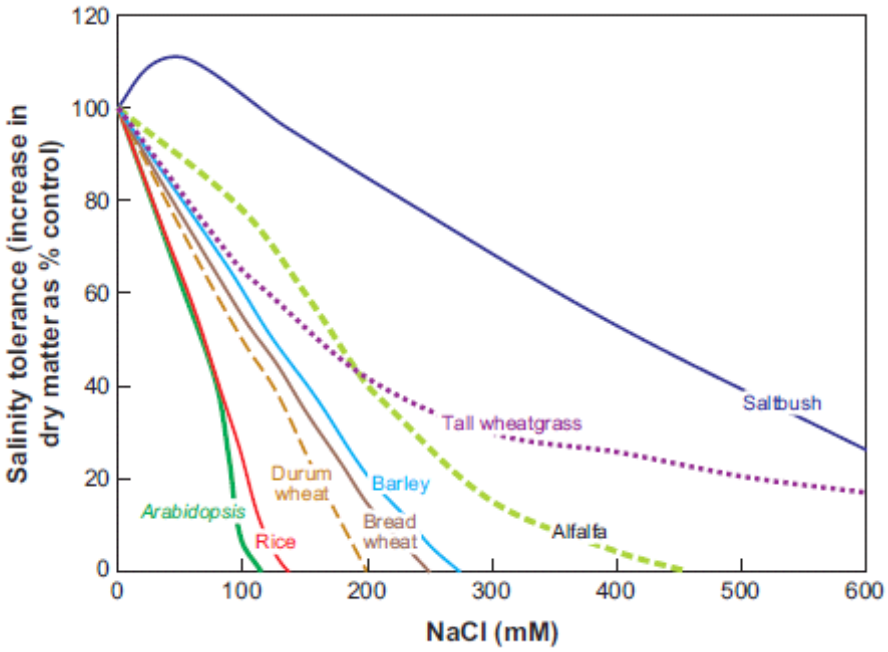

Figure 1.1. Tolerance of different plant species to salt stress, expressed as the increase in dry mass at different salt concentrations (Munns et al., 2008).

Salt stress affects plants by two mechanisms (Munns and Tester, 2008):

- High concentrations of salt in the soil reduce water uptake by the roots, producing similar effects to drought. This is called the osmotic effect and it has an immediate impact on plant growth and development.
- Salt accumulating inside the plant (particularly in leaves) has a toxic effect. Because it takes some time for salt to accumulate inside the plant this ionic effect is not immediate.

Root growth in *Arabidopsis thaliana* seedlings is strongly affected by salt stress but not by drought stress (Verslues et al., 2006). Root inhibition is most likely caused by the direct toxicity of Na^+. The resulting ion imbalance and hyperosmotic stress often induce secondary stresses such as oxidative damage (Zhu 2001).

The osmotic effect of salinity stress reduces photosynthesis and cell growth by the same mechanisms as drought (Chaves et al., 2009). If the stress is prolonged, however, additional stress-response genes are activated, reflecting the combined effects of dehydration, osmotic stress and ion imbalance. Microarray analysis of plants exposed to salt and dehydration has also indicated substantial differences between the gene

expression profiles elicited by these stresses (Seki et al., 2002). Strategies to induce salt stress tolerance include the elimination of sodium ions from the cytoplasm and the accumulation of low-molecular-weight protective compounds known as osmolytes or compatible solutes (compatible because they do not inhibit normal metabolic functions). Such molecules include glycine betaine, trehalose, proline, sorbitol, mannitol and ectoine (Hasegawa and Bressan, 2000).

1.1.3. Cold

Low temperature also limits plant growth, and this has a major impact on grasses by inducing vernalization and causing low-temperature damage at anthesis (Tester and Bacic, 2005). Cold stress can be divided into chilling and freezing stress depending on the temperature. Chilling stress occurs at temperatures below the plant's normal growth temperature but not low enough to form ice crystals (Levitt, 1972). The primary impact of chilling is to cause membrane leakiness if membranes cannot retain their fluidity at low temperatures (Beck et al., 2004). In contrast, freezing stress results from the formation of ice crystals in the extracellular space, initially causing dehydration but in many cases also structural damage through expansion. Prolonged cold stress slows down metabolism and leads to the formation of free radicals, which induce oxidative stress. Because cold and drought both induce dehydration as a primary effect, they share more common features compared to salinity (Verslues et al., 2006; Beck et al., 2007).

Plants vary greatly in their ability to survive freezing temperatures. At one extreme, plants from tropical and subtropical regions (such as soybean, rice and maize) have little or no frost tolerance and suffer chilling injury at 0–10ºC. In contrast, plants from temperate regions (such as rye and wheat) can survive at temperatures down to –30°C, depending on the species and variety.

Low but non-freezing temperatures induce a response known as cold acclimation which involves the induction of COR (cold-regulated) genes that cause a variety of biochemical and physiological changes (Guy 1990), including the accumulation of hydrophilic polypeptides that promote freezing tolerance by inhibiting ice crystal formation (Thomashow, 1998; Jaglo-Ottosen et al., 1998).

1.1.4. Plant water potential and changes during stress

Water is required by all life forms as a medium for biochemical reactions. In plant cells, water-generated turgor pressure is also a driving force for cell expansion. However, vegetative growth of plants can only occur when the free energy state of water molecules lies within a particular physiological range, and this is expressed as the water potential (ψ_w). In any plant cell, ψ_w consists of pressure and osmotic potential. While maintaining a positive turgor pressure, plant cells usually adjust their osmotic potential to balance the water budget and thus meet the requirements of the whole plant (Bernstein, 1961). Substantial changes in the environmental water potential therefore cause osmotic stress, which disrupts normal cellular activities and eventually kills the plant. All abiotic stresses reduce the water potential of plant cells. High salinity and drought are the major causes of osmotic stress in plants (Xiong and Zhu, 2002), but chilling and freezing also cause osmotic stress by reducing water absorption and inducing dehydration (Zhu et al., 1997; Verslues et al., 2006). Upon exposure to osmotic stress, plants exhibit a wide range of responses at the molecular, cellular and whole plant levels (Hasegawa et al., 2000; Xiong and Zhu, 2002).

1.1.5. Plant responses to low water potential

Plants respond immediately to declining water potential by closing their stomata, which reduces water loss by transpiration. If the stress is prolonged, plants accumulate solutes to increase their osmotic potential, stiffen their cell walls to counter the loss of turgor pressure (Boyer, 1995), and reduce the rate of shoot and root growth (**Figure 1.2**).

Under severe stress conditions it becomes increasingly difficult to avoid dehydration, and mechanisms that allow the tolerance of reduced water content become important. Most of the dehydration tolerance mechanisms studied thus far function primarily to protect the cellular structure from the effects of dehydration (Verslues et al., 2006). These mechanisms include the accumulation of osmolytes that protect macromolecular structures from conformational changes (Turner and Jones, 1980), the accumulation of protective proteins and the detoxification of free radicals (Xiong and Zhu, 2002; Degenkolbe et al., 2009).

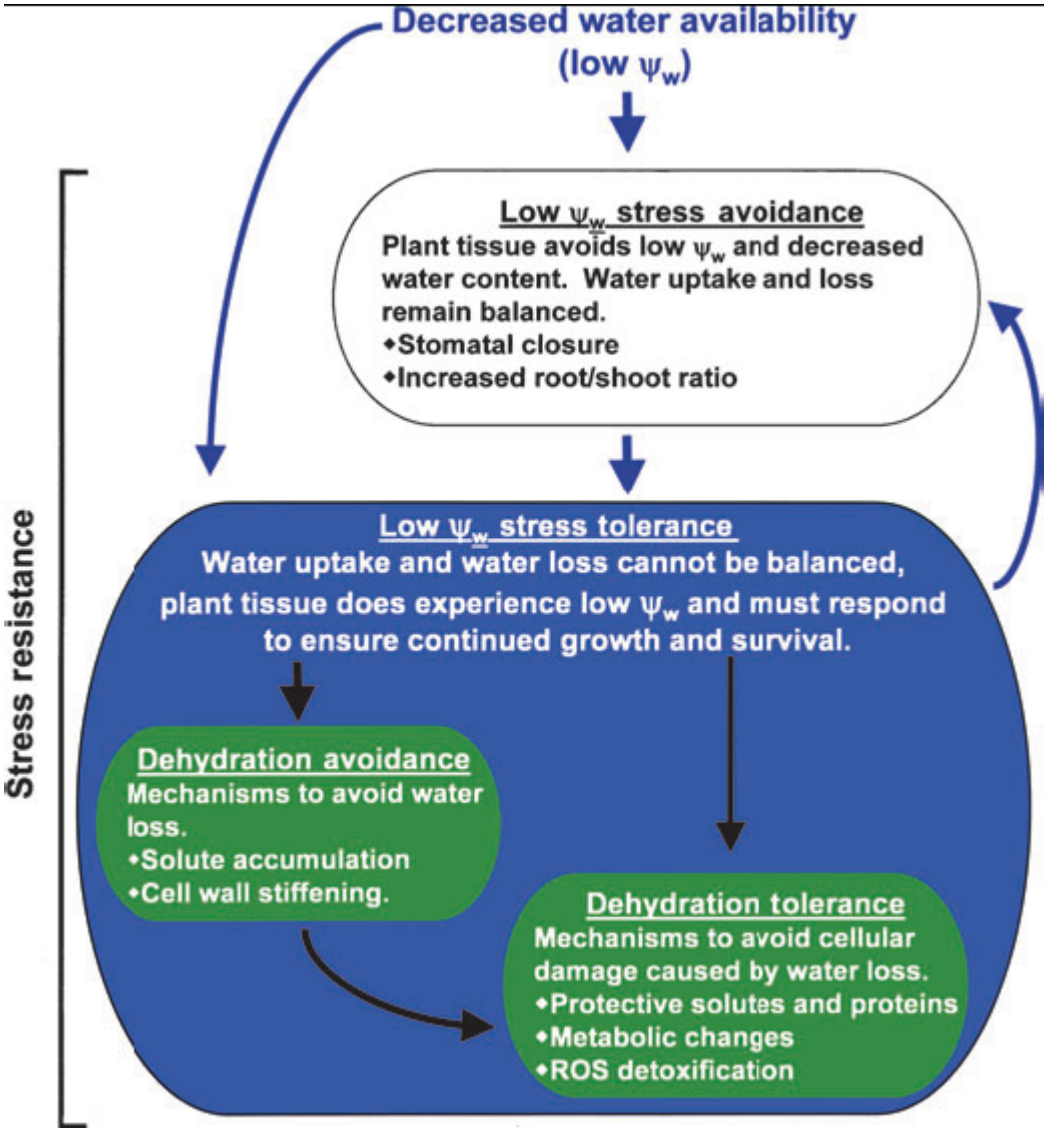

Figure 1.2. Plant responses to low water potential (Verslues et al., 2006).

1.1.6. Importance of stress intensity and duration

Abiotic stresses vary in their intensity and duration, and plants respond in different ways to mild vs severe stress and to transient vs long-term stress (Degenkolbe et al., 2009). Short periods of severe stress often induce short-term but ultimately unsustainable responses of the "wait and see" variety, whereas long-term stress requires the induction of more extravagant avoidance mechanisms that require significant developmental changes. For example, closing stomata and accumulating osmolytes is a suitable response to transient stress but is difficult to maintain, whereas plants facing

long-term dehydration may invest resources in increasing the absorption of water by deeper roots and increasing the conduction capacity of the root system (Levitt 1972).

Most studies thus far have focused on short-term responses to abiotic stress but there is a need to investigate long-term adaptation strategies as this is more similar to field conditions and will be more useful for the development of stress-tolerant crops (Vinocur and Altman, 2005).

1.1.7. Recovery from stress

Under natural conditions, plants usually experience cycles of stress and recovery (e.g. dehydration followed by rehydration as part of seasonal weather variations or agricultural practices). The degree of recovery from stress, which also has a molecular basis, is therefore as relevant as the initial stress response (Vinocur and Altman, 2005).

1.2. Secondary stresses in plants (osmotic and oxidative stress)

Primary stresses, such as drought, salinity, cold and heat, are often interconnected because they cause cellular damage and induce secondary stresses, such as osmotic and oxidative stress. Osmotic stress is caused by the insufficient availability of water, whereas oxidative stress is caused by the accumulation of reactive oxygen species (ROS).

The consequences of abiotic stress are reduced growth, abnormal development and ultimately death. However, many plants show a degree of tolerance to certain types of stress, and in each case that has been studied it has been shown that the stress induces a cascade of gene expression culminating in a physiological response that counters the stress and allows near normal growth.

1.2.1. Plant stress response mechanisms

Signal transduction pathways are induced when a signal is perceived by multiple primary sensors that vary in different stresses and provide the cell with different information (Xiong et al., 2002). For example, cold stress causes mechanical constraints, changes in membrane fluidity and changes in the activities of

macromolecules, whereas salt stress induces both ionic and osmotic effects. Different primary sensors probably control different aspects of the stress condition, so a primary sensor that detects changes in membrane fluidity will induce a certain signaling cascade whereas a primary sensor that detects changes in protein conformation will induce a different cascade. Primary signals generate secondary messengers such as inositol phosphates and ROS. Secondary messengers can modulate intracellular Ca^{2+} levels (**Figure 1.3**). Cold, drought and salinity have all been shown to induce a transient Ca^{2+} influx into the cytoplasm (reviewed by Sanders et al., 1999; Knight, 2000). These three stresses all induce the accumulation of ROS such as superoxide, hydrogen peroxide, and hydroxyl radicals (Hasegawa et al., 2000). Although ROS may activate downstream signal cascades via Ca^{2+} (Price et al., 1994), it is also possible that they can be sensed directly by key signaling proteins such as a tyrosine phosphatase through the oxidation of conserved cysteine residues (Xiong and Zhu, 2002).

Secondary messengers initiate a protein phosphorylation cascade that finally targets proteins directly involved in cellular protection or transcription factors controlling specific sets of stress-regulated genes (Xiong et al., 2002). The products of these genes may participate in the generation of regulatory molecules such as the plant hormones abscisic acid (ABA), ethylene and salicylic acid (SA). These regulatory molecules can, in turn, initiate a second round of signaling that may follow the above generic pathway (Alcázar et al., 2010). In Arabidopsis for example, cold stress induces ABA synthesis, leading to the activation of polyamine biosynthesis genes (Kasinathan and Wingler, 2004), but polyamines are also signaling molecules, leading to the activation of ABA biosynthesis genes (Cuevas et al., 2008; 2009).

Once damaged proteins are beyond repair, they must be eliminated to prevent aggregation. The major proteolytic system in eukaryotes is ubiquitin-mediated degradation in the proteasome (Ciechanover et al., 2000). The expression of genes encoding ubiquitin-related proteins and various proteases is induced by drought stress (Lee et al., 2009).

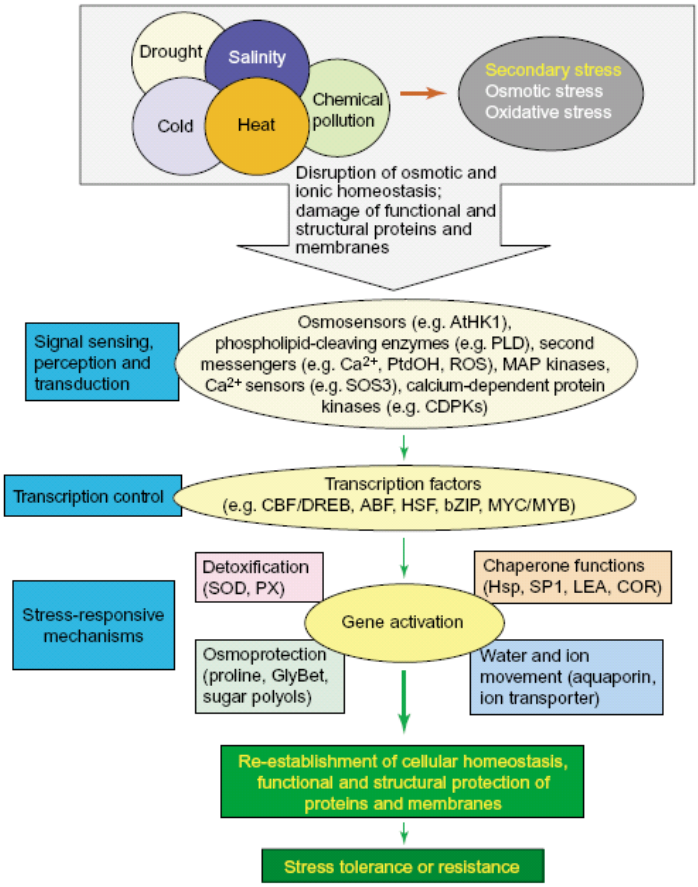

Figure 1.3. The complexity of the plant response to abiotic stress (Vinocur and Altman, 2005).

1.2.1.1. Detoxification

Plant cells produce ROS such as oxygen free radicals and peroxide ions continuously as part of normal metabolism, but stress increases the rate at which ROS are produced, causing damage to the structural components of cells as well as to DNA, resulting in the typical damage seen in stressed plants (stunting, chlorosis, wilting and ultimately death). Plants produce a number of enzymes whose specific function is to detoxify ROS, and these are induced by stress usually as a direct response to elevated ROS levels (de Pinto et al., 2006). Such enzymes include superoxide dismutase (SOD) (Bowler et al., 1992), ascorbate peroxidase (APX) (de Pinto et al., 2006), peroxidase (POD) (Lotfi et al., 2010), catalase (CAT) (Willekens et al., 1995), and enzymes that synthesize low molecular weight antioxidants such as ascorbic acid, glutathione and phenolic compounds (Gill and Tuteja, 2010).

SOD is a very important enzyme that participates in the first step of ROS scavenging. SODs are classified according to their metal cofactor, which can be copper, zinc, manganese or iron. Plants generally contain Cu/ZnSOD in the cytosol, FeSOD and/or Cu/ZnSOD in the chloroplasts and MnSOD in mitochondria. SOD not only combats oxidative stress but also drought and saline stress. Some plants, known as halophytes, thrive in saline environments and tend to have much higher levels of SOD than salt-sensitive plants. For example, the mangrove *Avicennia marina* has a SOD activity 40 times higher than pea (Cheeseman et al., 1997).

1.2.1.2. Water and ion movement

As well as inducing oxidative stress, salinity can directly affect plant survival by raising the intracellular Na^+ concentration to the point where this interferes with normal physiological processes (Serrano et al., 1999). Plants use three strategies to maintain Na^+ at physiologically normal levels: exclusion, compartmentalization and export, the last two achieved by the activity of membrane channels known as Na^+/H^+ antiporters (Apse and Blumwald, 2002). There are two classes: plasma membrane antiporters, which export Na^+ from the cytosol to the appoplast or external medium, and vacuolar antiporters, which transfer Na^+ ions across the tonoplast into the vacuole, wherein the Na^+ accumulates. The overexpression of the vacuolar Na^+/H^+ antiporter *AtNHX1* in *A. thaliana* allowed the transgenic plants to grow in media containing high concentrations of salt (Apse et al., 1999).

1.2.1.3. Osmoprotection

Common osmolytes include sugars (e.g. mannitol and trehalose), amino acids (e.g. proline), quaternary and other amines (e.g. glycine betaine and polyamines). These compounds are small molecules that tend not to affect the functions of macromolecules, even at very high concentrations (Xiong and Zhu, 2002). Osmolytes protect plants from osmotic stress by lowering the cellular osmotic potential to facilitate water absorption and restore intracellular salt concentrations (Wang et al., 2003). Drought, salt and cold stresses stimulate the accumulation of compatible osmolytes (Hasegawa et al., 2000). Halophytes synthesize large amounts of osmolytes to protect them against dehydration, but most crops lack this ability (Bhatnagar-Mathur et al., 2008).

1.2.1.4. Signaling cascades

Signal transduction pathways can be activated by specific or multiple stresses, but they tend to induce multiple responses including the direct modulation of protein activities (e.g. by phosphorylation) and the regulation of downstream genes. Many signal transduction components have been overexpressed in an attempt to increase abiotic stress tolerance (**Table 1.1**).

As stated earlier, primary abiotic stresses often induce secondary stress through the production of ROS, and the signaling pathways that help to eliminate ROS are therefore very useful for the improvement of stress tolerance. For example, nucleoside diphosphate kinase (NDPK) plays an important role in the oxidative stress signal transduction pathway (Moon et al., 2003). Small amount of ROS such as H_2O_2 can induce the expression of protective genes, such as mitogen-activated protein kinase kinase kinase (MAPKKK) in *A. thaliana*, which is part of the mitogen-activated protein kinase (MAPK) cascade (Kovtun *et al*., 2000).

Exogenous H_2O_2 or ABA-induced H_2O_2 activates Ca^{2+} channels in guard cells, resulting in stomatal closure, and this is probably mediated by the MAPK cascade (Pei *et al*. 2000). There is increasing evidence demonstrating that MAPKs play an important role in signal transduction pathways related to both biotic and abiotic stresses (Shou et al 2004a, 2004b).

In *A. thaliana*, RAC/ROP-like proteins are also involved in abiotic stress signaling. Small RAC/ROP-family G proteins regulate both development and stress responses (Christensen et al., 2003). Transgenic barley plants expressing the constitutively activated RACB mutant *racb-G15V* under the control of the maize ubiquitin-1 promoter had a higher transpiration rate and lost water rapidly (Schultheiss et al., 2005). Water loss in the mutant was associated with reduced responsiveness to ABA compared to wild type plants.

1.2.1.5. Transcriptional control

All signal transduction pathways induce direct responses at the protein level to alter cell behavior, but in many cases the target proteins are transcription factors that modulate gene expression. Stress response networks in plants can be divided into those dependent on ABA and those that do not involve this hormone (**Figure 1.4**) (Yamagushi-Shinosaki

and Shinozaki, 2005). The ABA-dependent pathway involves the induction of genes via the bZIP/ABRE and MYC/MYB system. In contrast, transcription factor DREB/CBF (dehydration responsive element binding protein/C-repeat binding factor) is ABA-independent (Agarwal et al., 2006). NAC transcription factors are known to regulate both ABA-dependent and ABA-independent genes (Agarwal and Jha, 2010) and are also involved in salinity tolerance (Olsen et al., 2006).

The regulation of downstream genes depends on interactions between these transcription factors and cis-acting response elements in the corresponding promoters (Chen et al. 2008). There are two response elements in the promoter of the ABA-inducible gene *rd29A*, an ABA-response element (ABRE) and a dehydration and cold response element (C-repeat/DRE). Basic leucine zipper (bZIP) transcription factors bind specifically to ABREs and trigger the expression of ABA-dependent genes. The bZIP proteins are involved in responses to UV light, salinity and drought stress. The transcription factors that bind the DRE element (DREBs) regulate the expression of stress-related genes in an ABA-independent manner (Shinozaki and Yamaguchi-Shinozaki, 2000; Agarwal et al., 2006).

More than 1,500 genes encoding transcription factors have been identified in the *A. thaliana* genome, including bZIP, Myb/c, WRKY, DBF, and ERF factors (Riechmann et al. 2000). Microarray experiments have demonstrated the upregulation of target genes containing the DRE, including 35 genes induced by the rice DREB1A protein (Ito et al., 2006).

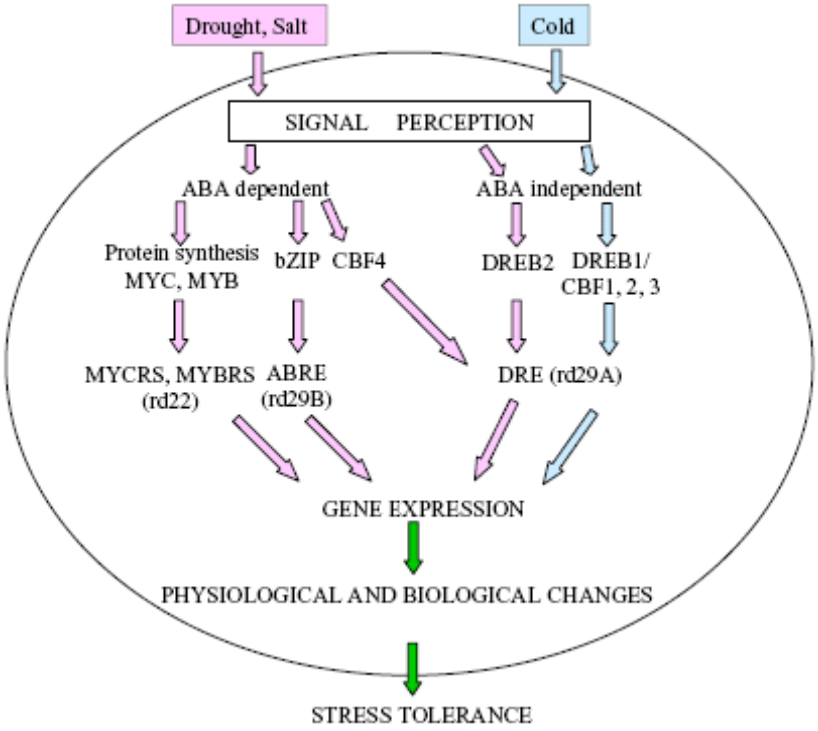

Figure 1.4. ABA-dependent and ABA-independent signal transduction in abiotic stress responses (Agarwal et al., 2006).

1.2.1.6. Role of ABA in the regulation of stress response genes

Abscisic acid is undoubtedly the plant hormone most intimately involved in stress signal transduction. ABA is produced in response to drought, salinity and low temperatures, and plays an important role in stress tolerance (Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2002). Many stress-responsive genes are upregulated by ABA (Xiong et al., 2002). For example, ABA regulates the synthesis of late embryogenesis abundant proteins (LEA), which are important for dehydration tolerance (Xu et al., 1996).

Because ABA was shown to induce H_2O_2 production (Guan et al., 2000; Pei et al., 2000), ROS may act as intermediate signals for the activation of catalase 1 (*cat1*) gene expression (Guan et al., 2000), the induction of heat tolerance (Gong et al., 1998), the activation of Ca^{2+} channels in guard cells (Pei et al., 2000), stomatal closure (e.g., Pei et al., 2000; Zhang et al., 2001), and even ABA biosynthesis (Zhao et al., 2001).

Previous studies have suggested that ABA inhibits cell division or DNA synthesis by inducing the expression of ICK1, a cyclin-dependent protein kinase inhibitor (Wang et al., 1998). The inhibition of growth by osmotic stress may be mediated by the production of ABA, which inhibits cell division by inducing ICK1.

1.2.1.7. The LEA proteins and their role in abiotic stress tolerance

LEA proteins are evolutionarily conserved proteins, found in many plants and cyanobacteria, as well as non-photosynthetic bacteria, yeast and several invertebrates (Tunnacliffe and Wise, 2007). They were first identified in the maturation and desiccation phases of cotton seed development (Dure and Chlan, 1981). They are highly hydrophilic and thermostable, and they are composed largely of glycine, alanine and glutamine, lacking cysteine and tryptophan (Iturriaga, 2008).

There are seven groups of LEA proteins based on the amino acid sequence (Baker et al., 1989; Dure et al., 1989). Groups 1, 2, 3, 4, 6 and 7 are hydrophilic proteins whereas group 5 includes proteins with hydrophobic characteristics. The major groups (1, 2 and 3) include two groups (1 and 2) found only in plants, whereas group 3 LEA proteins are widely distributed (Wang et al., 2007; Iturriaga, 2008). Group 1 LEA proteins possess an internal 20-amino-acid signature motif repeated up to four times depending on the species and a high proportion of glycine, glutamine acid and glutamine residues. Group 2 (the dehydrins) posess a highly-conserved 15-amino-acid lysine-rich sequence called the K-segment, with the consensus EKKGIMDKIKEKLPG. Dehydrins are induced by dehydration-related stresses such as low temperature, drought and high salinity (Close, 1996; Verslues et al., 2006). They are thought to act as chaperons, and thus to stabilize vesicles, proteins and membrane structures in stressed plants (Close, 1996). Group 3 LEA proteins possess tandem repeats of an 11-amino-acid motif that can form an amphiphilic α-helix structure that readily binds ions (Dure et al. 1989). This structure forms in the presence of glycerol, ethylene glycol and methanol, and after fast drying, but in aqueous solutions the group 3 proteins lack a secondary structure (Tolleter et al., 2007; Iturriaga, 2008).

The rice genome contains 34 *OsLEA* genes with diverse expression profiles, some constitutive, some spatiotemporally regulated and some related to stress tolerance (Wang et al., 2007). Two conserved motifs (CACGTA and CACGCACG) are located

within 1 kb of the transcriptional start site in ABA-induced and drought-induced *OsLEA* genes. Group 3 LEA genes were found to be expressed at higher than normal levels in the roots of salt-tolerant indica rice plants (Moons et al., 1995), and the different levels of LEA proteins in different indica rice varieties suggested there are multiple regulatory mechanisms (Moons et a., 1997a). The roots of severely dehydrated wheat seedlings lacking group 3 LEA proteins could not grow even after rehydration, whereas the shoots and scutella accumulated high levels of the proteins and were able to survive (Ried and Walker-Simmons, 1993).

The exact role of LEA proteins in dehydration is not clear, but some studies suggest they prevent the formation of damaging protein aggregates (Zhu, 2001; Xiong and Zhu, 2002; Goyal et al., 2005). LEA proteins may also protect macromolecules such as enzymes and lipids (Shinozaki and Yamaguchi-Shinozaki, 1999). Group 3 LEA proteins are able to protect enzymes such as LDH, malate dehydrogenase, citrate synthase, fumarase and rhodanase against partial dehydration (Battaglia et al., 2008). LEA proteins may provide a water-rich environment for their target enzymes, preventing inactivation by maintaining protein integrity during periods of dehydration. LEA proteins also protect membrane structures (Bravo et al., 2003; Hara et al., 2001). Seedlings of the saline-tolerant rice variety Bura Rata accumulated LEA proteins when exposed to 100 mmol/L NaCl and growth was arrested, but the LEA proteins were degraded during recover (Chourey et al., 2003). Shorter-term stress resulted in faster recovery and LEA degradation whereas longer-term stress resulted in slower and incomplete recovery with high levels of LEA proteins still present in the surviving plants.

1.2.1.8. Interactions between LEA proteins and ABA

Salinity and dehydration enhances ABA levels in many plants including rice. Several rice LEA genes are regulated by ABA (Bray 1997; Zhu et al. 1997) and contain ABREs in their promoters (Ono et al., 1996; Wang et al., 2007). The exogenous application of ABA and/or exposure to salt shock (150 mM NaCl) rapidly induces the accumulation of *Oslea3* mRNA in rice seedling roots, whereas the application of jasmonic acid does not (Moons et al., 1997a). Group 2 and 3 LEA proteins were expressed in response to ABA in rice and finger millet (*Eleusine coracana* Gaertn) seedlings (Jayaprakash et al., 1998). An ABA-inducible dehydrin is strongly expressed in saline-tolerant rice varieties

(Moons et al., 1995). Based on microarray data and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis, 16 LEA genes appeared to be upregulated by ABA in the japonica rice variety Nipponbare (Wang et al., 2007). Group 3 LEA genes are also induced by ABA in wheat seedlings (Ried and Walker-Simmons, 1993).

1.2.2. Responses and adaptation of rice plants to abiotic stress

Drought resistance is complex, involving physiological and biochemical processes at the cell, tissue, organ and whole-plant levels, depending on the developmental stage, and the severity, duration and timing of the stress (Farooq et al., 2009a). Leaf and root growth and development can be severely affected, but further responses include stunting, reduced leaf area and reduced biomass accumulation, tiller abortion, and changes in root dry matter and rooting depth (Farooq et al., 2009b). Rice plants respond to drought by reducing the number of new tillers and leaves, reducing leaf elongation, rolling existing leaves, and promoting leaf death. Photosynthesis is one of the main metabolic processes underlying productivity, and is directly affected by drought because stomatal closure is an early and effective response to water deficit (Chaves et al., 2002). Dehydration causes cell shrinking and a subsequent decline in volume. This results in viscous cellular contents, increasing the probability of protein–protein interactions, their aggregation and denaturation. Increased concentrations of solutes may become toxic, thereby affecting the activity of some enzymes, including those required for photosynthesis (Hoekstra et al., 2001).

Rice plants respond to drought by accumulating ABA, and exogenous ABA therefore mimics the effects of water stress and pre-adapts the plants to better withstand stress conditions (Davies and Jones, 1991). Higher ABA levels and lower cytokinin levels during dehydration favor stomatal closure and reduce water loss through transpiration.

Plants reduce water loss by increasing stomatal resistance, and increase water uptake by developing a deep and extensive root system, and accumulating osmolytes and osmoprotectants. Silicon also plays an important role in rice drought resistance (Farooq et al., 2009a). In rice, Si can represent up to 10% of the dry shoot weight (Ma et al., 2001) and the roots express a specific transporter (Lsi1) which is responsible for Si

uptake (Ma et al., 2006). It is thought that Si reduces cuticular transpirational water loss by accumulating beneath the cuticle (Agarie et al., 1998).

1.2.2.1. Genes expressed during salinity stress in rice

Salinity stress triggers the expression of many stress-response genes and proteins in rice, including *SalT* (encoding a mannose-binding jacaline-like lectin; Zhang et al. 2000), *Em* (encoding a hydrophilic LEA protein; Bostock and Quatrano 1992), *rMip1* and *rTip1* (encoding the major intrinsic proteins of the plasma and tonoplast membranes; Liu et al. 1994), *sodA1, sodCc1* and *sodCc2* (encoding superoxide dismutases; Sakamoto et al. 1995), *OsNHX1* (encoding a Na^+/H^+ exchanger; Fukuda et al. 1999). Certain enzymes are specifically induced by salinity stress in rice, including arginine decarboxylase which is involved in polyamine biosynthesis (Chattopadhyay et al. 1997), peroxidases which alleviate oxidative stress (Moons et al. 1997b) and pyruvate orthophosphate dikinase (PPDK), which plays an important role in C4 photosynthesis (Moons et al. 1998). Some of the genes and proteins involved in saline stress response signal transduction pathways, such as the ABRE-binding factor (Gupta et al. 1998) and the Ca^{2+}-dependent protein kinase OsCDPK7 (Saijo et al. 2000) have also been identified in rice. Rice also accumulates several stress proteins in response to saline stress, such as the RAB family (Mundy and Chua 1988), the dehydrin family (Bradford and Chandler 1992) and the LEA family (Moons et al. 1995, 1997a).

1.3. Approaches to improve plant performance under abiotic stress

Tolerance to abiotic stress is a complex quantitative trait influenced by the coordinated expression of many genes. Different approaches to improve the performance of plants under abiotic stress are described below.

1.3.1. Conventional breeding

Traditional approaches for the generation of crops with improved abiotic stress tolerance have so far met with limited success (Tester and Bacic, 2005). This is due to a number of contributing factors, including: (1) the focus has been on yield rather than specific traits; (2) breeding for tolerance traits is complicated by genotype x

environment (G × E) interactions and the relatively infrequent use of simple physiological traits as measures of tolerance; and (3) the desired traits can only be introduced from closely-related species.

Responses to drought or salinity stress in plants can be divided into stress avoidance and stress tolerance mechanisms. Stress avoidance involves morphological and physiological changes at the whole-plant level, e.g. the development of deeper and thicker roots, root pulling resistance, greater root penetration, osmotic adjustment and membrane stability (Fukai and Cooper, 1995; Nguyen *et al*., 1997). Conventional breeding may be used to improve these traits and to select varieties with better abiotic stress adaptations. However, understanding the physiological and molecular basis of crop stress tolerance is very important and may help target key yield-limiting traits.

1.3.2. Marker assisted breeding

Genetic markers allow polymorphisms to be tracked through populations, and if such markers are linked to important traits such as abiotic stress tolerance, they can be used to select resistant plants for breeding and follow donor introgressions in segregating populations. Many rice traits are quantitative in nature, i.e. controlled by multiple genes and environmental interactions. Mapping quantitative trait loci (QTLs), which are chromosomal regions that contain one or more genes contributing to a given trait, requires comprehensive genetic maps that allow phenotypes to be linked to markers near each QTL (Thomson, 2009). Although many QTLs affecting major traits have been mapped, important traits are often controlled by multiple small-effect QTLs that are masked by complex environmental interactions and/or differing genetic background effects.

QTLs associated with saline stress tolerance in rice were identified by Lin et al. (2004), who crossed a salt-tolerant indica variety with a susceptible japonica variety and mapped QTLs conferring seedling survival under stress, which correlated well with the degree of leaf damage and Na^+ accumulation in the shoots.

1.3.3. Genetic engineering

Although whole-plant avoidance of stress can be improved by conventional and marker assisted breeding, stress tolerance conferred at the cellular and molecular levels is easier to address by genetic engineering (Vinocur and Altman, 2005). The identification of genes involved in stress tolerance, and their introduction into susceptible plants, can transfer stress resistance traits to crops making them more productive in harsh environments.

There are two major forms of stress response genes: those encoding enzymes or structural proteins required for the direct protection of cells (e.g. enzymes for the biosynthesis of osmoprotectants, detoxifying enzymes, and proteins involved in the maintenance of homeostasis), and those encoding the components of signaling pathways that respond to stress (e.g. receptors, intracellular signaling molecules and transcription factors) (Shinozaki and Yamagushi-Shinosaki, 2000). The latter group may be more useful as targets for genetic engineering because they, in turn, regulate a larger number of protective genes (Bhatnagar-Mathur et al., 2007).

1.3.3.1. Detoxification

The overexpression of SOD in susceptible plants improves their tolerance towards a number of environmental stresses. For example, when *Nicotiana plumbaginifolia* MnSOD and *Arabidopsis* FeSOD were overexpressed in the mitochondria and chloroplasts of alfalfa, this increased photosynthetic activity by approximately 20% in the transgenic plants (Rubio et al., 2002). In another study, Cu/Zn SOD and APX were expressed in potato chloroplasts under the control of the oxidative stress-inducible *SWPA2* promoter, and the transgenic plants were four times more resistant to stress than control plants (Tang et al., 2006). Cu/Zn SOD and APX were also expressed in tall fescue plants, reducing chlorophyll degradation, low peroxide accumulation and low solute leakage compared to controls under oxidative stress (Lee et al., 2007). Similarly, tomato Cu/Zn SOD protected sugar beet from oxidative stress (Tertivanidis et al., 2004). Additional studies are listed in **Table 1.2**.

1.3.3.2. Water and ion movement

Several reports describe the overexpression of antiporters in transgenic plants as a means to enhance tolerance towards salinity (**Table 1.3**) For example, transgenic canola plants expressing the *A. thaliana* vacuolar Na^+/H^+ antiporter AtNHX1 were able to grow normally and produce seeds in the presence of 200 mM NaCl, a level sufficient to kill wild type plants after a few days (Zhang et al., 2001). The leaves and roots of transgenic plants contained higher levels of NaCl than normal, but the seeds were unaffected. Tall fescue plants transformed with the same gene germinated more successfully and grew more robustly than wild type plants under salinity stress (Zhao et al., 2007).

1.3.3.3. Osmoprotection

The transfer of genes for osmoprotectant synthesis can confer stress tolerance on susceptible crops, and some examples of this strategy are listed in **Table 1.4.** One of the most widely used osmoprotectants is glycine betaine (GB), a quaternary amine that has been used to increase the salinity tolerance of numerous crops (Sakamoto *et al* 1998, 2002; Mohanty *et al* 2002). The most efficient strategy for synthesizing GB in transgenic plants was described by Park et al. (2004, 2007). They generated tomato plants constitutively expressing a bacterial choline oxidase gene (*codA*), which converts choline to GB in a single step, in contrast to the typical eukaryotic GB pathway where two steps are required. The recombinant enzyme was targeted to the chloroplast, which provides better salinity tolerance than the same enzyme accumulating in the cytosol (Park et al., 2007).

Sugars and sugar-alcohols such as mannitol and trehalose are used as osmoprotectants by a number of halophytes, and the transfer of the corresponding metabolic pathways to cereal crops can also confer stress tolerance (Penna, 2003). For example, transgenic wheat plants accumulating mannitol were more tolerant than controls, although excessive mannitol synthesis was harmful (the plants were stunted and infertile) perhaps because the mannitol disrupted the normal osmolytic balance of the cells (Abebe et al., 2003). Trehalose synthesis in plants has been achieved by importing the *Escherichia coli* enzymes trehalose-6-phosphate synthase and phosphatase (TPS and TPP), which catalyze consecutive steps and generate trehalose from the precursors UDP-glucose and glucose-6-phosphate. The simplest way to implement this is to express a fusion gene

encoding a protein with both enzyme activities, *tpsp* (Garg et al., 2002; Jang et al., 2003).

Proline also protects transgenic plants against water deficit, as shown by Vendruscolo et al. (2007) who created transgenic wheat plants overexpressing pyrroline-5-carboxylate synthetase under the control of a stress-inducible promoter. Although proline levels correlated with stress tolerance, the underlying mechanism appears to involve protection against oxidative stress rather than osmotic adjustment.

1.3.3.4. Signal transduction

Transgenic potato plants expressing the *A. thaliana* nucleoside diphosphate kinase AtNDPK2 showed enhanced tolerance to high temperatures and salinity stress, and more effective stress tolerance was achieved by placing the gene under the control of the stress-inducible *SWPA2* promoter than the constitutive CaMV 35S promoter (Tang et al., 2007) (**Table 1.1**). AtNDPK2 confers tolerance towards multiple forms of stress probably through activation of the MAPK cascade (Moon et al., 2003).

Nicotiana protein kinase (NPK1) is a member of MAPKKK family, which conferred enhanced tolerance towards drought stress when expressed in transgenic maize plants (Shou et al., 2004a). NPK1 overexpression induced multiple stress-response genes, including those encoding a GST, a small HSP, PR1, alcohol dehydrogenase, a blue copper protein and DREB1.

1.3.3.5. Transcription factors

Numerous reports have shown that the overexpression of DREB genes can enhance tolerance towards drought, salinity and/or chilling in cereals (Oh et al., 2005, 2007; Ito et al., 2006; Pellegrineschi et al., 2004), tomato (Hsieh et al., 2002), peanut (Bhatnagar-Mathur et al., 2007) and forage grasses (James et al., 2008). Stress-inducible promoters are preferred for the regulation of DREB genes because the constitutive activity of DREB transcription factors can cause stunting and chlorosis, although there were no such effects when DREB genes were expressed constitutively in rice (Oh et al., 2005, 2007). Additional examples of transcription factors that enhance abiotic stress tolerance are listed in **Table 1.5**.

1.3.4. Model plants

Arabidopsis thaliana is a useful model for studying abiotic stress mechanisms and signaling networks because information from its fully sequenced genome provides insight into the mechanisms operating in other plants. However *A. thaliana* can only tolerate relatively low salt concentrations so there is a need for other model plants that tolerate osmotic stress better than *A. thaliana*. *Thellungiella halophila* is a promising model halophyte because it can grow normally in 300 mM NaCl, compared with ~75 mM for *A. thaliana* (Zhu, 2001). *T. halophila* has many traits that are similar to *A. thaliana*, such as a small stature, short life cycle, self-pollination and a small genome. Like *A. thaliana*, it can be transformed by *Agrobacterium*-mediated flower-dipping (Li et al., 2007). One additional advantage is that, at the cDNA level, *T. halophila* and *A. thaliana* genes share >90% identity (Zhu, 2001). Therefore, genetic analysis in *T. halophila* can benefit from the availability of the *A. thaliana* genome sequence.

Another disadvantage of *A. thaliana* is that, as a dicotyledonous plant, it is not a good model for cereals, which are monocotyledonous and therefore show fundamental differences in development and anatomy. It is therefore better to study stress pathways and tolerance mechanisms directly in cereals. Rice is a model species in cereal genomics because it is diploid, has a relatively small genome (~430 Mb), and is closely related to other important cereal crops (Yamaguchi-Shinozaki and Shinozaki, 2004). The rice genome has been fully sequenced, allowing the functional analysis of stress-response genes, expression profiling and comparison between monocot and dicot plant systems (Rabbani et al., 2003).

1.3.5. Crop plants

Rice is grown in at least 95 countries and is a staple food for more than half of the world's population (IRRI, 2002; Coats, 2003). Rice is grown in a wide range of environments including areas where other crops might fail. The demand for rice will reach 480 million metric tons by 2025 and 525 million metric tons by 2050 due to the projected increase in the world's population (FAO, 2002). Meeting this 35% increase in demand requires significant improvements in productivity despite dwindling water resources (Bouman *et al.*, 2007). Over 50% of the land used to grow rice is rain-fed, and it produces only 25% of total output (McLean et al., 2002).

In rain-fed ecosystems, drought is the major obstacle affecting rice production. For example, in the eastern Indian states of Jarkhand, Orissa and Chhattisgarh, yield losses from severe drought (on average once every five years) averaged 40% and cost an estimated $650 million (Pandey et al., 2005). Currently, there is no economically viable means of increasing rice yield under drought conditions. The development of rice plants that are resistant to drought is considered a promising approach that will help to satisfy the increasing demand for food, particularly in the emerging economies of Asia and Africa (Farooq et al., 2009).

Genetic engineering is a useful approach for the improvement of drought and salinity tolerance in rice (Yamaguchi-Shinozaki and Shinozaki, 2004). Some studies have focused on the overexpression of single genes that control the synthesis of osmoprotectants, modify membrane lipids, encode LEA proteins or detoxification enzymes, or that regulate stress signal transduction pathways. The overexpression of transcription factors that regulate LEA gene expression significantly improves the tolerance of rice plants towards various abiotic stresses (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999).

Despite the progress described above, the single gene approach is often ineffective because drought and salinity stresses are complex traits and plants have a tendency to restore homeostasis (Konstantinova et al., 2002). The transfer of multiple genes, e.g. to modulate an entire metabolic pathway, can therefore be a more promising approach (Cominelli and Tonelli, 2010). It is also imperative to evaluate engineered plants under field conditions, because laboratory tests may not represent a realistic environment. It should also be borne in mind that from an agricultural perspective, the most important property of stress-tolerant plants is their yield, but it is also necessary to monitor stress sensitivity during the life cycle. For example, rice is less sensitive to drought and salinity during vegetative growth but more susceptible during flowering and seed development (Khatun and Flowers, 1995). Finally, it is important to evaluate the characteristics of transgenic plants quantitatively, and to ensure that enhanced tolerance traits are stable over multiple generations (Flowers, 2004).

1.3.5.1. Transgenic rice plants with enhanced drought stress tolerance

Many regulatory proteins with roles in stress tolerance have been characterized in rice (Zhu, 2002) and some have been shown to improve drought stress tolerance when overexpressed (**Table 1.6**). Rice plants expressing various transcription factors have been evaluated under laboratory conditions but only a few have also been tested in the field. Transgenic rice plants overexpressing OsDREB1A were tolerant towards high temperature and drought (Dubouzet et al., 2003) and those overexpressing OsDREB1G and OsDREB2B were more resistant to dehydration (Chen et al., 2008). Rice plants overexpressing NAC family transcription factors were comparable to those expressing DREB1A (Hu et al. 2006, 2008; Nakashima et al. 2007). The overexpression of OsDREB1F conferred tolerance to salinity, drought and chilling (Wang et al., 2008).

Hu et al. (2006) carried out field tests of transgenic rice plants expressing NAC1, and found they tolerated severe drought and salinity stress during the sensitive reproductive stage. The expression of *AtDREB1A/CBF3* driven by the *OsHVA22p* promoter significantly improved the performance of rice in rain-free fields, increasing spikelet fertility by 42% and the yield per plant by 11%, whereas the same gene driven by the constitutive *OsActin1* promoter conferred only a marginal improvement (Xiao et al., 2009). Rice plants expressing the APETELA2 (AP2) homolog AP37, a transcription factor involved in flower development, spikelet meristem determinacy, plant growth and stress tolerance (Haake et al., 2002; Dubouzet et al., 2003), were also protected from salinity and drought stress in the field, with yields 16–57% higher than controls (Oh et al., 2009). The root-specific expression of OsNAC10 also conferred drought tolerance at the reproductive stage in field-grown rice plants, increasing the yield by 25–42% under drought conditions and by 5–14% under normal conditions (Jeong et al., 2010).

Some signal transduction pathways induce genes responsible for the elimination of ROS, e.g. NDPK is a key component of the oxidative stress response pathway. The overexpression of *AtNDPK2* in rice plants induced a number of antioxidant genes, including those encoding the enzymes peroxidase, thioredoxin reductase, peroxiredoxin, glutathione reductase and glutathione transferase (Seong et al., 2007b). Many Ca^{2+}-sensing protein kinases are involved in stress responses, and rice plants expressing calcineurin B-like protein-interacting protein kinase (CIPK) were protected against chilling, drought and salinity, mediated in part by the accumulation of higher amounts

of proline and sugars than control plants (Xiang et al., 2007). The overexpression of *OsLEA3-1* driven either by the drought-inducible HVA1-like promoter or the constitutive CaMV 35S promoter conferred drought tolerance in rice under field conditions and also improved the yield (Xiao et al., 2007).

Polyamines accumulate in rice in response to several forms of stress, and transgenic plants expressing *Datura* arginine decarboxylase produced much higher levels of putrescine under stress, promoting spermidine and spermine synthesis and protecting the plants from drought (Capell et al., 2004). Other genes that conferred drought stress tolerance when introduced into rice are listed in **Table 1.6**.

1.3.5.2. Transgenic rice plants with enhanced salinity stress tolerance

As stated above, salinity tolerance in plants is achieved either by reducing the uptake of salt into the plant, or by removing salt from the cytoplasm by export or vacuolar sequestration through the activity of Na^+/H^+ antiporters (Munns, 2002). Saline-tolerant rice plants have been generated by overexpressing the *E. coli* plasma membrane Na^+/H^+ antiporter *nhaA*, although the yield of these plants was lower than that of controls (Wu et al., 2005). In contrast, the root-specific expression of the *A. thaliana* selective Na^+/H^+ antiporter AtHKT1 produced rice plants that gained more biomass under salinity stress compared to controls and that had a lower concentration of Na^+ in the shoots (Plett et al., 2010). The *Pennisetum glaucum* homolog PgNHX1 also conferred high levels of salinity tolerance when expressed in rice, with the transgenic plants achieving a higher grain yield than controls in the presence of 60 mM NaCl (Verma et al., 2007; Islam et al., 2010).

Transcription factors that confer drought tolerance in rice also confer salinity tolerance, at least under laboratory conditions, as shown by the overexpression of DREB, NAC, zinc finger, CBF and ERF family transcription factors (Hu et al., 2008; Zheng et al., 2009; Xiang et al., 2008; Wang et al., 2008; Gao et al., 2008; Chen and Guo, 2008; Oh et al., 2007). These studies are listed in **Table 1.7**.

Some signal transduction proteins that confer drought tolerance when expressed in transgenic plants have also been shown to confer salinity tolerance. For example, rice *OsMAPK5* was either over-expressed or suppressed in transgenic rice plants, with overexpression conferring resistance to cold, drought and salinity stress, and

suppression by RNAi increasing the sensitivity of the plants to these stresses (Xiong and Yang, 2003). Other signaling proteins have a more specific effect on salinity tolerance, e.g. the sucrose non-fermenting I-related protein kinase2 (SnRK2) protein SAPK4, which reduced the accumulation of NaCl and improved the efficiency of photosynthesis under saline stress condotions (Diedhiou et al., 2008). SAPK4 was shown to regulate genes involved in ion homeostasis and the response to oxidative stress, such as vacuolar H^+-ATPase, the Na^+/H^+ antiporter NHX1, the Cl^- channel OsCLC1 and a catalase.

Rice plants overexpressing the barley LEA3 gene *hva1* were protected from chilling and salinity stress, and were also more resistant to dehydration stress via cell membrane protection (Xu et al., 1996; Babu et al., 2004). The accumulation of OsLEA3 in the vegetative tissues of transgenic rice plants also conferred tolerance towards salinity and dehydration stress (Hu et al., 2008).

The trehalose synthesis fusion gene *tpsp* conferred a high degree of tolerance towards salinity, drought and freezing stress in rice when driven by a stress-inducible or a light-regulated promoter, even though the increase in trehalose levels was only modest (Garg et al., 2002). Transgenic plants were also more tolerant towards solarization, with a 5–10% higher rate of photosynthesis than controls.

Transgenic rice plants expressing cytosolic Sod1 from the mangrove *Avicennia marina* were much more tolerant towards oxidative, salinity and drought stress than untransformed plants (Prashanth et al., 2007). The overexpression of SOD in the chloroplast achieved greater oxidative stress tolerance than cytosolic expression, perhaps because chloroplasts and mitochondria are rich sources of ROS. Cytosolic SOD was more effective against drought and salinity stress (Prashanth et al., 2007).

Other genes that confer salinity stress tolerance when expressed in rice are listed in **Table 1.7.** All these studies were carried out under laboratory conditions rather than in the field, therefore other environmental factors that prevail in saline environment such as alkaline soil pH, high diurnal temperatures, low humidity, the presence of salts other than NaCl salts and higher concentrations of selenium and boron were not taken in account. Field testing for salinity stress tolerance is more difficult to carry out because of these complicating factors, and because the concentrations of different salts vary widely in different soils (Yamaguchi and Blumwald, 2005). Even so, robust field testing will be necessary before commercialization.

1.3.6. Natural and synthetic bioregulators

Natural and synthetic bioregulators have been identified that influence all aspects of plant development, from seed germination to senescence and death (Considine, 1986; Hedin, 1990; Nickell, 1994). The use of chemicals to enhance plant growth has benefited the agrochemical industry for many years, but should also underpin basic research to improve our understanding of how exogenous chemicals interact with key genes and proteins that influence plant productivity. In this project, we hypothesized that the improvement of plant productivity under the influence of bioregulators will correlate with the modulation of multiple genes and proteins that can be identified using a suite of state-of-the-art genomic technologies in combination with biochemical, physiological and morphological assays. These genes can then be used as global markers in screening assays to find new bioregulators.

Well known examples of compounds with stress-reducing and yield-enhancing effects include fungicides from the strobilurine and azole classes and the insecticide imidacloprid (trade name: Trimax). Strobilurines such as azoxystrobine, fluoxastrobine, kresoxim-methyle, picoxystrobine, pyraclostrobine and trifloxystrobine, enhance CO_2 assimilation and general plant metabolism (Gerhard et al., 1998; Grossmann et al., 1999). They also inhibit protein and chlorophyll degradation, thereby delaying senescence (greening effect) (Bryson et al., 2000). Azoles affect ethylene biosynthesis, which also delays senescence (Habermeyer et al., 1998). The insecticide imidacloprid has positive effects on cotton yield especially under drought stress conditions (Gonias *et al*., 2006). Although some data are available concerning the metabolic processes induced by strobilurines and azoles (Beck, 2005; Clark, 2003; Gerhard, 2001; Siefer and Grossmann, 1996), little is known about the active mechanism of imidacloprid (Gonias *et al*., 2006) or other compounds which have been identified recently and which show anti-stress effects (Bayer CropScience, personal communication). For example, the effect of imidacloprid on the growth and yield of cotton was evaluated in field and growth chamber studies (Gonias et al., 2006). Treated plants were shown to grow more vigorously, mature earlier, and produce more lint than untreated control plants. The increased rate of biomass accumulation and carbohydrate synthesis was attributed to the higher rate of photosynthesis, reduced chlorophyll fluorescence and lower membrane leakage. The treated plants also had a lower level of antioxidant enzyme activity,

suggesting they were less susceptible to stress than controls, perhaps indicating that imidacloprid is an antioxidant that directly promotes stress tolerance.

The molecular basis of bioregulator and phytohormone activity has been investigated in *A. thaliana* through the overexpression of certain target genes (Tang et al., 2005) and the isolation of mutants that respond inappropriately to these chemicals (Dharmasiri et al., 2006). Many phytohormone target genes have been identified by these techniques (for reviews see Etheridge *et al*., 2006; Gray, 2004; Wang and He, 2004) and have helped to determine how bioregulators work. Transcription factors have been identified as the master regulators of many pathways induced by bioregulators, but more work needs to be done to understand the precise mechanisms of action and the cross-talk between the corresponding signaling pathways.

The impact of the auxin herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) on the *A. thaliana* transcriptome was recently determined using a whole-genome microarray (Raghavan et al., 2005, 2006). Approximately 25% of the genes modulated by 2,4-D had no known function, indicating that these kinds of experiments can deliver candidates that are not obvious from searches in the literature and databases.

Our current knowledge of hormone signal transduction pathways and their associated regulatory networks in plants is therefore incomplete. None of the known hormone transduction pathways has been fully elucidated and we have only a rudimentary understanding of the underlying molecular events and corresponding genes. Therefore, the development of synthetic bioregulators that can activate single or multiple signal transduction pathways involved in yield and quality traits is currently a hit-and-miss affair based on trial and error.

Genes	Genes induced	Stress	Source	Host	References
AtNDPK2	*pBC442, pBC601, OsAPX1, OsAPX2, OsSodB*	Chilling, oxidative	Arabidopsis	Rice	Seong et al., 2007b
OsCIPK	*No information*	Cold, drought, salt	Rice	Rice	Xiang et al., 2007
OsMAPK5	*No information*	Cold, drought, salt	Rice	Rice	Xiong &Yang, 2003
SAPK4	Vacuolar H^+-ATPase, Na^+/H^+ antiporter NHX1, Cl^- channel OsCLC1 and a catalase.	Salt	Rice	Rice	Diedhiou et al., 2008
RACB	*No information*	Water	Barley	Barley	Schultheiss et al., 2005
NDPK2 (inducible SWPA2 promoter and 35S)	*No information*	Oxidative, high temperature, salt stress	Arabidopsis	Potato	Tang et al., 2007
MAPKKK (NPK1) 35S promoter	HSP	Drought	Tobacco	Maize	Shou et al., 2004a
MAPKKK (NPK1) 35S promoter	GST, HSP, PR1	Freezing	Tobacco	Maize	Shou et al., 2004b

Table 1.1. Overexpression of plant protein kinases enhance tolerance to abiotic stress

Genes	Subcellular location	Promoter	Stress	Host plant	References
Nicotiana plumbaginifolia MnSOD and *Arabidopsis* FeSOD	Mitochondria, chloroplast	CaMV 35S	Water	Alfalfa	Rubio et al., 2002
Cassava Cu/Zn SOD and pea APX	Chloroplast	Inducible SWPA2 promoter	Oxidative, high temperature	Potato	Tang et al., 2006
SOD and APX	Chloroplast	Inducible SWPA2 promoter	Oxidative	Tall fescue	Lee et al, 2007
Pea MnSOD	Chloroplast	Inducible SWPA2 promoter	Drought, oxidative	Rice	Wang, 2005
Tomato Cu/ZnSOD	Cytosol, chloroplast	CaMV 35S	Oxidative	Sugar beet	Tertivanidis et al., 2004
Cu/ZnSOD from *Avicennia marina* (Sod1)	Cytosol	Ubiquitin promoter	Oxidative, salt, drought	Rice	Prashanth et al., 2007

Table 1.2. Overexpression of detoxifying genes in different plants

Antiporter Na^+/H^+	Source	Host	Stress	References
AtNHX1	*Arabidopsis*	Canola	Salt	Zhang et al., 2001
AtNHX1	*Arabidopsis*	Tall fescue	Salt	Zhao et al., 2007

Table 1.3. Overexpression of water and ion movement genes in different plants

Genes	Expression	Osmolyte accumulation	Stress tolerance	Secondary effects	Host plant	References
Choline Oxidase	Constitutive CaMV 35S	Glycine betaine	Chilling, salinity, oxidative stress	Not reported	Tomato	Park et al., 2007
Choline Mono-Oxygenase	Constitutive Ubi-1	Glycine betaine	Salinity, low temperature	Growth stem	Rice	Shirasawa et al., 2006
Choline Oxidase	Constitutive CaMV 35S	Glycine betaine	Chilling	Not reported	Tomato	Park et al., 2004
Spermidine Synthase	Constitutive CaMV 35S	Polyamines	Salinity, osmotic, Heavy metal (Cu)	Not reported	Pear	Wen et al., 2008
Arginine Decarboxylase	Constitutive Ubi-1	Polyamines	Drought	Not reported	Rice	Capell et al., 2004
Mannitol-1 P-dehydro-Genase	Constitutive Ubi-1	Mannitol	Drought, Salinity	Not reported	Wheat	Abede et al., 2003
Trehalose-6 Phosphate Synthase	Stress Inducible StDS2	Trehalose	Drought	None	Potato	Stiller et al., 2008
Trehalose-6 Phosphate Synthase/ Phosphatase	Constitutive Ubi-1	Trehalose	Drought, Salinity, Low temperature	None	Rice	Jang et al., 2003
Trehalose-6 Phosphate Synthase/ Phosphatase	Stress Inducible (ABA) Tissue Specific (rdcS)	Trehalose	Drought, Salinity, Low temperature	None	Rice	Garg et al., 2002
Pyrroline-5 Carboxylate Synthetase	Stress Inducible (AIPC-ABA)	Proline	Drought	Not reported	Wheat	Vendruscolo et al., 2007

Table 1.4. Overexpression of enzymes that synthesize osmoprotectants to enhance stress tolerance

Genes	Expression	Stress tolerance	Secondary effects	Target stress induced genes	Host plant	References
DREB1A	Stress-inducible HVA1	Salinity, drought	Not reported	Not reported	Bahia grass	James et al., 2008
*Hv*CBF4	Constitutive Ubi-1	Salinity, drought, low temperature	None	15 identified	Rice	Oh et al., 2007
DREB1A	Stress-inducible rd29A	Drought	None	Not reported	Peanut	Bhatnagar et al., 2007
DREB1A	Stress-inducible rd29A	Drought	Not reported	Not reported	Wheat	Pellegrineschi et al., 2007
OsDREB1A DREB1A	Constitutive Ubi-1; 35S	Salinity, drought, low temperature	Growth retardation	35 identified	Rice	Ito et al., 2006
DREB1A	Constitutive Ubi-1	Salinity Drought,	None	12 identified	Rice	Oh et al., 2005
DREB1A	Constitutive 35S	Chilling Oxidative stress	Growth retardation	1 identified	Tomato	Hsieh et al., 2002
OsNAC6	Constitutive Ubi-1	Salinity Drought,	Growth retardation, low reproductive yield	14 identified	Rice	Nakashima et al., 2007
OsNAC6	Stress-inducible OsNAC6,LIP 9	Salinity	None	14 identified	Rice	Nakashima et al., 2007
SNAC1	Constitutive 35S	Salinity Drought,	None	~ 40 identified	Rice	Hu et al., 2006
SNAC2	Constitutive Ubi-1		None	36 identified	Rice	Hu et al., 2008
CaKR1 Ankyrin Zinc finger	Constitutive 35S	Salinity, Oxidative stress	Thick leaves	Not reported	Tomato	Seong et al., 2007a
ABF3	Constitutive Ubi-1	Drought	None	7 identified	Rice	Oh et al., 2005

Table 1.5. Overexpression of plant transcription factors to enhance abiotic stress tolerance

Functional group	Overexpressed genes	Effects under drought stress	Evaluation	References
Transcription factor	*OsDREB1A, OsDREB1B, OsDREB1C, OsDREB1D, OsDREB2A*	Improved growth and photosynthetic capacity in transgenic plants than controls under drought stress.	GH	Dubouzet et al., 2003
	OsDREB1G, OsDREB2B	Increased tolerance to water stress	GH	Chen et al., 2008
	SbDREB2	Higher number of panicles as compared to the wild-type	GH	Bihani et al., 2011
	OsNAC6	Oxidative stress protection	GH	Nakashima et al., 2007
	SNAC1	Transgenic plants had higher seed setting and yield under stress conditions than controls.	field	Hu et al., 2006
	OsNAC10	Enhanced drought tolerance at the reproductive stage with increased grain yield under field conditions	field	Jeong et al., 2010
	AtABF3	Less leaf rolling and wilting	GH	Oh et al., 2005
	AtHARDY	Enhanced root system and improved water use efficiency in transgenic plants.	GH	Karaba et al., 2007
	AtZat10	High spikelet fertility, high yield of transgenic plants	field	Xiao et al., 2009
	OsWRKY11	Slower leaf-wilting and less-impaired survival rate of green parts of plants and slower water loss in detached leaves.	GH	Wu et al., 2009
	AP37	Enhanced drought tolerance in the field with increased grain yield.	field	Oh et al., 2009
	TSRF1	Better growth performance with increased levels of proline, soluble sugars and photosynthesis-related genes.	GH	Quan et al., 2010
	JERF1	Enhanced drought tolerance with increased levels of proline and ABA biosynthesis key enzyme.	GH	Zhang et al., 2010
Farnesylation	*OsCDPK7*	Improved glycine rich and LEA proteins under drought stress in transgenic plants than controls. Overexpression of OsCDPK7 had less wilting than controls.	GH	Saijo et al., 2000
Protein phosphorylation	*OsCIPK03, OsCIPK12, OsCIPK15*	Higher proline and sugar accumulation, less leaf rolling	GH	Xiang et al., 2007
	OsCIPK23	Improved pollination and seed set in transgenic rice than controls under drought stress.	GH	Yang et al., 2008
	NPK1	High spikelet fertility, high yield of transgenic plants	field	Xiao et al., 2009
	DSM1	Increased tolerance to dehydration stress at the	GH	Ning et al., 2010

		seedling stage by regulating scavenging of ROS.		
	OsSIK1	Increased drought tolerance by enhanced activities of peroxidase, superoxide dismutase and catalase	GH	Ouyang et al., 2010
Heat shock protein	*OsHSP17.7*	Transgenic plants with higher expression levels of sHSP17.7 protein recovered upon rewatering after stress.	GH	Sato and Yokoya, 2007
Late embryogenesis abundant protein	*HVA1*	Transgenic plants maintained higher growth rates than controls under drought.	GH	Xu et al.,1996
	HVA1	After drought stress, transgenic lines showed increased stress tolerance (cell integrity and growth) compared to controls.	GH	Rohila et al., 2002
	HVA1	Higher leaf RWC and tolerance to water stress by protecting cell membrane.	GH	Babu et al., 2004
	OsLEA3-1	Increase in drought stress tolerance in the field conditions and higher grain yield	GH, field	Xiao et al., 2007
	PMA80 and *PMA1959*	Accumulation of either PMA80 or PMA1959 correlated with increased drought tolerance.	GH	Cheng et al., 2002
Proline	*p5cr*	Faster shoot and root growth observed in transgenic seedlings than controls under drought stress. Stress-inducible expression of *p5cs* transgene had greatest effect.	GH	Su and Wu, 2004
	p5cs	Transgenic plants had faster shoot and root growth and higher proline accumulation compared with non-transformed plants under drought stress.	GH	Su and Wu, 2004
Glycine betaine (GB)	*Coda*	Majority of transgenics survived an episode of acute drought stress. Under cycles of drought/recovery, transgenics had higher biomass and were taller than controls.	GH	Sawahel, 2003
	COX	Increased GB synthesis improved plant growth response under stress in transgenics compared with controls.	GH	Su et al., 2006
Trehalose	*TPS*	Transgenic lines showed more sustained plant growth, less photo-oxidative damage, more trehalose and more favorable mineral balance under drought stress than controls.	GH	Garg et al., 2002
	TPS and *TPP*	Better growth performance and photosynthetic capacity in transgenic plants than controls under stress.	GH	Jang et al., 2003
Polyamine	Oat *adc*	Increased endogenous putrescine level improved drought tolerance in transgenic plants.	GH	Capell et al., 2004
SOD	*MnSOD*	Transgenic plants exhibited less membrane injuries than controls	GH	Wang et al., 2005

Ubiquitin ligase	*OsSDIR1*	Better performance and more closed stomatal pores.	GH	Gao et al., 2011
Unknown	*TaSTRG*	Higher survival rate, fresh weight and chlorophyll content, accumulated higher proline and soluble sugar contents, and had significantly higher expression levels of putative proline synthetase and transporter genes than the control.	GH	Zhou et al., 2009

Table 1.6. Transgenic rice plants with improved tolerance to drought stress. Modified table based on Farooq et al. (2009a). GH-greenhouse.

Functional group	**Overexpressed genes**	**Effects under salinity stress**	**Evaluation**	**References**
Transcription factor	*SNAC2*	Higher germination and growth rate than w.t. and increased sensitivity to ABA	GH	Hu et al., 2008
	ONAC045	Enhanced salt tolerance to salt treatments	GH	Zheng et al., 2009
	OsbZIP23	Improved salt tolerance and increased sensitivity to ABA	GH	Xiang et al., 2008
	OsDREB1F	Enhanced tolerance to salinity stress	GH	Wang et al., 2008
	TERF1	Improved salt tolerance	GH	Gao et al., 2008
	OPBP1	Better survival under 250 mM of NaCl	GH	Chen and Guo, 2008
	HvCBF4	High-salinity tolerance	GH	Oh et al., 2007
Protein phosphorylation	OsMAPK5	Improved salt tolerance	GH	Xiong and Yang, 2003
	SnRK2	Less Na^+ and Cl^- in transgenic plants and improved photosynthesis under salinity stress	GH	Diedhiou et al., 2008
	OsSIK1	Increased salt tolerance by enhancing peroxidase, superoxide dismutase and catalase. Less accumulation of H_2O_2.	GH	Ouyang et al., 2010
Na^+/H^+ antiporter	*nhaA*	Germination rate, growth and average yield per plant of transgenic lines were better than those of control lines under salt or drought stress. Moreover, sodium and proline content of transgenic lines under drought stress was also higher than in controls.	GH	Wu et al., 2005
	OsVP1 and OsNHX1	Higher photosynthetic activity and increase in root biomass.	GH	Liu et al., 2010
	OsNHX1	Improved salt tolerance with delayed appearance and development of damage or death caused by salinity stress, as well as improved recovery upon removal from salinity stress	GH	Chen et al., 2007
	PgNHX1	More extensive root system, flowering and seeds setting under stress of 150 mM NaCl	GH	Verma et al., 2007
	PgNHX1	Better physiological status and flowering and seeds setting as well asunder salt stress, higher grain yields.	GH	Islam et al., 2010
	AtHKT1;1	Less damage when exposed to long-term treatment with 100 mM NaCl, less Na+ and malondialdehyde in the leaves, higher photosynthetic activity. Increase in root biomass, which enabled more water uptake. Improved Na^+ exclusion under conditions of salinity stress.	GH	Plett et al., 2010

Late embryogenesis abundant protein	HVA1	Improved salt tolerance	GH	Xu et al., 1996
	OsLEA3	Enhanced tolerance to salinity stress	GH	Hu, 2008
Proline	*P5CSF129A*	Enhanced proline accumulation, better biomass production and growth performance under salt stress, and lower extent of lipid peroxidation and higher proline accumulation.	GH	Kumar et al., 2010
Glycinebetaine (GB)	*OsBADH1*	Better survival rate under high salinity conditions. Enhanced glycine betaine accumulation led to increased salt tolerance in immature and mature transgenic rice seedlings.	GH	Hasthanasombut et al., 2011
Trehalose	*tpsp*	Higher photosynthesis rate, enhanced tolerance	GH	Garg et al., 2002
Antioxidant	*Sod1*	Better performance under salinity stress	GH	Prashanth et al., 2008
	Glyoxalase II	Sustained growth and more favorable ion balance	GH	Singla-Pareek et al., 2008
Cyclophilin	OsCYP2	Better tolerance to salt stress, lower levels of lipid peroxidation products and higher activities of antioxidant enzymes	GH	Ruan et al., 2011
Hydrolase	*OsSbp*	Enhanced tolerance at the young seedlings stage	GH	Feng et al., 2007
Stress Associated protein	*OsiSAP8*	Tolerant to salinity stress during anthesis stage without any yield penalty	GH	Kanneganti and Gupta, 2008
Unknown	*TaSTRG*	Transgenic plants under salt stress had a lower intracellular Na^+/K^+ ratio, higher survival rate, fresh weight and chlorophyll content, accumulated higher proline and soluble sugar contents, and had significantly higher expression levels of putative proline synthetase and transporter genes than the control plants.	GH	Zhou et al., 2009

Table 1.7. Transgenic rice plants with improved tolerance to salinity stress. GH-greenhouse.

1.4. References

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Objectives

1. Evaluate potential bioregulators for their positive effect on rice growth under abiotic stress conditions.

2. Determine the molecular mechanisms of bioregulator activity.

3. Develop a novel cell-based assay to screen for new candidate bioregulators using suitable molecular marker genes.

Chapter 2

Development of reproducible assays to evaluate drought and salinity stress in rice callus and plants

2.1. Introduction

Drought and high salinity are major abiotic stresses that reduce agricultural productivity and crop yields every year (Vinocur and Altman, 2005; Yang et al., 2010). The constantly growing population and diminishing agricultural resources require land to be used more efficiently, particularly through the development of plants that can adapt to survive under harsh conditions. Two major approaches have been used to improve plant stress tolerance: a transgenic approach, in which plants are engineered to express particular genes that confer stress tolerance, and a genomics-assisted breeding approach, using marker-assisted selection, mutation and micropropogation. Natural and synthetic bioregulators have also been used for many years to enhance plant growth and productivity (Nickell, 1979).

Rice is one of the world's most important crops (Coats, 2003) but it is severely affected by drought (Farooq et al., 2009) and salinity stress (Munns et al., 2008). We chose rice as a model to investigate whether natural and synthetic bioregulators could be used to increase productivity under these abiotic stress conditions because the entire genome sequence is available and is significantly smaller than that of other cereals. Before investigating the potential impact of bioregulators, however, it was important to develop optimized abiotic stress assays to ensure reliability and reproducibility. Even though our experiments were carried out in a growth chamber with a precisely-controlled photoperiod, and constant temperature and humidity, it was not easy to achieve uniformity among the plants in terms of the life cycle and the response to abiotic stresses. The key factors required to develop an optimized and reproducible assay were the availability of a large population of plants from which a uniform subpopulation could be selected, and the use of a large number of replicates per treatment. Only when both these conditions were met could the impact of stress and bioregulator treatments be studied in a meaningful way.

2.2. Materials and Methods

2.2.1. Callus assays to measure drought stress

Rice seeds (*Oryza sativa* ssp. *japonica* cv EYI105) were dehusked and sterilized with 70% ethanol for 5 min and 5% sodium hypochlorite for 20 min before rinsing three times with sterile distilled water. Callus was induced on MS basal medium (Murashige and Skoog 1962) supplemented with 2.5 mg/l 2,4-D and 3% (w/v) sucrose, pH 5.8. Ten seeds were placed on each plate of medium and were maintained in a dark growth chamber at 24ºC. Expanded mature embryos were separated from the endosperm 7 days after incubation and transferred to a fresh MS medium. After two weeks, the growing callus was used for further propagation.

Small and uniform pieces of callus were chosen for the drought stress experiment, and were transferred to MS medium supplemented with 100–400 mM mannitol to impose the drought stress. Three replicate treatments were used for each treatment. Every plate contained five pieces of uniform callus and they were transferred to fresh medium every two weeks. The experiment was terminated after 49 days.

2.2.2. Callus assays to measure salinity stress

This experiment was set up as above for drought stress, except the medium was supplemented with 50–200 mM NaCl rather than mannitol to impose the salinity stress. The experiment was terminated after 47 days.

2.2.3. Drought stress assays in rice plants

Wild type rice plants (*Oryza sativa* ssp. *japonica* cv EYI105) were grown in the growth chamber under carefully controlled conditions (16-h photoperiod at 900 μmohn m^{-2} s^{-1} photosynthetically active radiation, temperature 26 ± 2°C and 80% relative humidity). The stress experiments were carried out on six-week-old plants by exposing them to different concentrations of polyethylene glycol with molecular weight of 8000 (PEG-8000) to impose the drought stress. PEG-8000 is a high-molecular-weight, non-ionic, non-plasmolysing and non-penetrating osmotic agent that simulates drought stress conditions. As previously established, 20% PEG is sufficient to impose drought stress in six-week-old rice plants, causing wilting in 2-3 days (Capell et al., 2004). More

prolonged but less severe stress conditions were achieved using 5, 10 and 15% PEG. The experiment was terminated when the plants began to wilt.

2.2.4. Salinity stress assays in rice plants

This experiment was established as above for drought stress, except the plants were transferred to separate trays containing 50–200 mM NaCl. The NaCl solution was maintained at the same level in the trays throughout the experiment to prevent the roots drying. After 15 days, the shoots were separated from the roots and dried at 65°C for 48 h.

2.2.5. Measurement of root length

Roots were washed to remove soil, stained with bromophenol blue and arranged on a glass plate. The plate was scanned at high resolution scanner and the WinRHIZO Pro software package was used to calculate the total length by summing the lengths of all pieces with varying diameters.

2.2.6. Recovery from stress

Drought and salinity stress were imposed until the plants began to wilt, at which point they were transferred to trays containing water for recovery. The transfer time was determined by observing the health of the plants to avoid lethal overstressing. Similarly, the duration of recovery was determined by observation. After recovery, the shoots were separated from the roots and dried at 65°C for 48 h.

2.2.7. Statistical analysis

Drought and salinity experiments with callus were carried out with five callus pieces per plate and three plates per condition (n = 15). For drought and salinity stress experiments with plants, we used five plants per treatment (n = 5) and the experiment was carried out three times. Data were analyzed by two-way analysis of variance (ANOVA) followed by Student's *t*-test using the residual mean square in the ANOVA as the estimate of variability.

2.3. Results

We carried out preliminary experiments with rice callus and plants to determine the appropriate stress conditions and optimize these for subsequent experiments with bioregulators. Callus experiments were carried out to provide insight into the underlying physiological and biochemical mechanisms at the cellular level, and to develop a screening platform for additional potential bioregulators.

2.3.1. Callus assays to measure drought stress

In preliminary experiments we subjected rice callus to drought stress by culturing callus pieces on medium supplemented with 100–400 mM mannitol. After 49 days we measured the callus fresh weight. We found that callus growth was inhibited by mannitol and that the severity of the effect was proportional to the mannitol concentration (**Figure 2.1**). Treatment with 400 mM mannitol resulted in a callus growth rate that was significantly different from controls, and we therefore used this concentration for subsequent experiments (**Figure 2.2**).

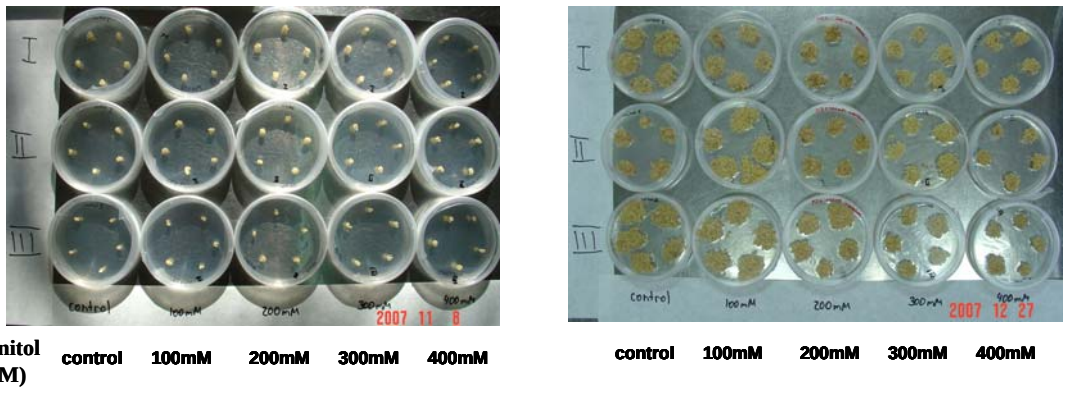

Figure 2.1. Rice callus growth under different concentrations of mannitol. First day of the experiment shown on the left, and last day on the right.

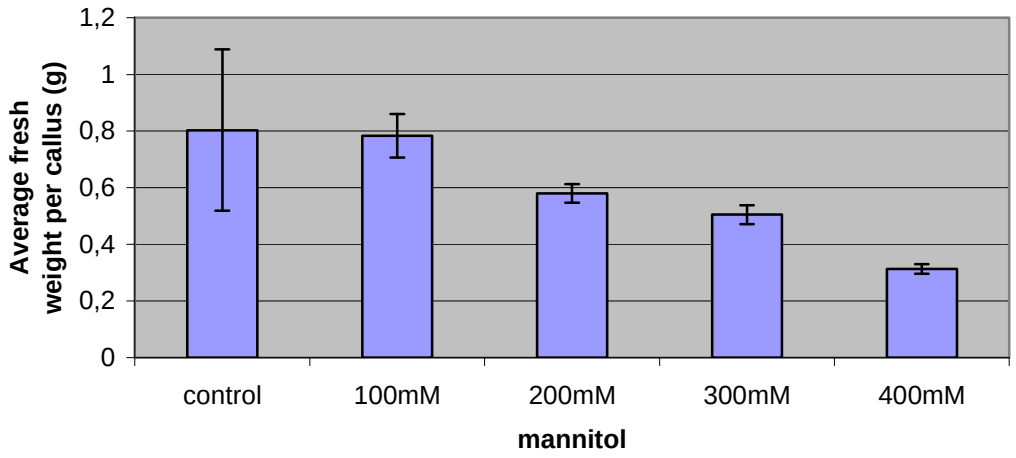

Figure 2.2. Fresh weight of rice callus after 49 days on different concentrations of mannitol.

2.3.2. Callus assays to measure salinity stress

The salinity stress experiment was carried out as described above for drought stress, with the rice callus cultured on media containing 50–200 mM NaCl. After 47 days, we measured the callus fresh weight and found that NaCl suppressed callus growth. Again, the severity of the effect was proportional to the NaCl concentration (**Figure 2.3**). All the treatments caused a statistically significantly difference in the growth rate compared to untreated callus (**Figure 2.4**) and we chose 150 mM NaCl as the working concentration for subsequent experiments.

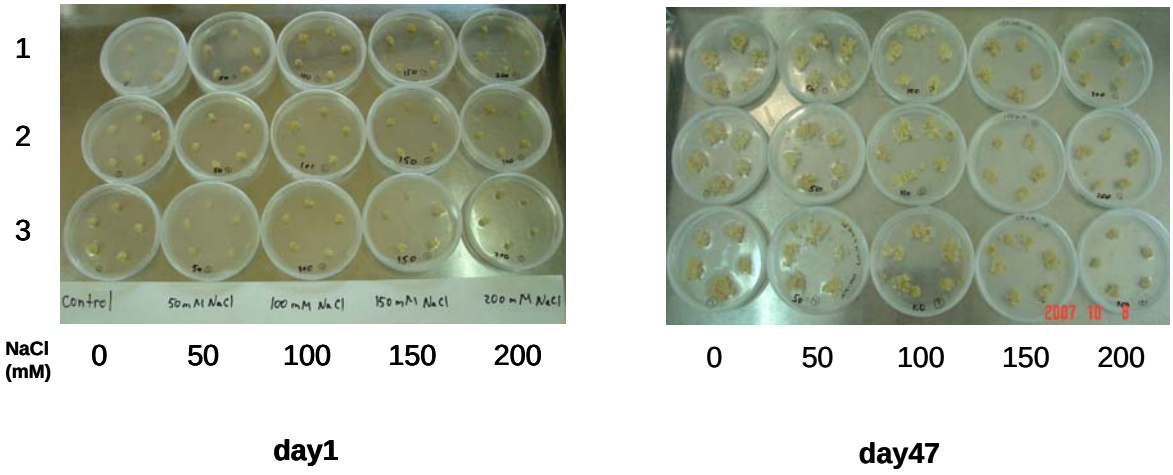

Figure 2.3. Rice callus growth in different concentrations of NaCl. First day of the experiment shown on the left, and last day on the right.

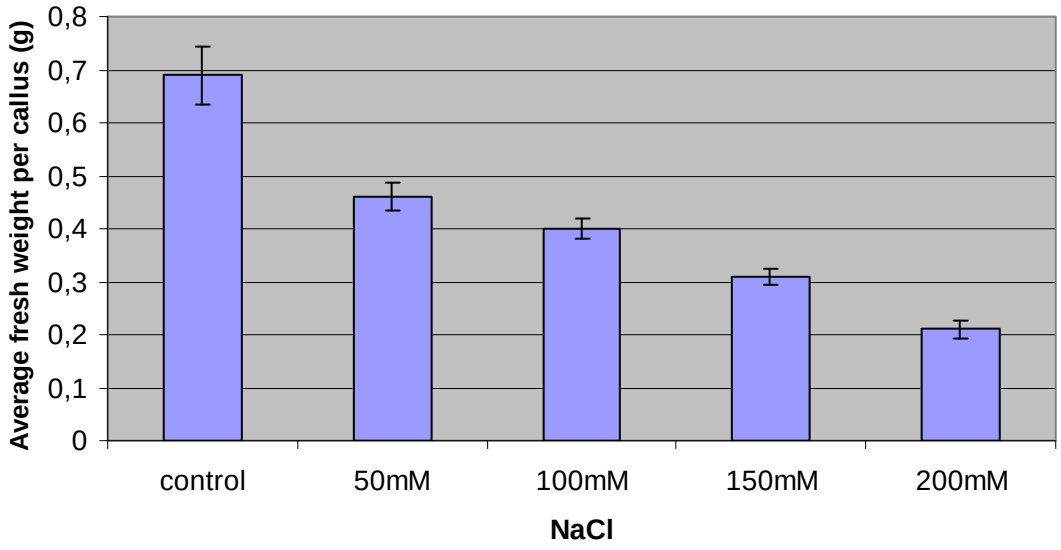

Figure 2.4. Fresh weight of rice callus after 47 d exposed to different concentrations of NaCl

2.3.3. Drought stress assays in rice plants

We treated rice plants with 5, 10, 15 and 20% PEG (M_r 8000; Perez-Molphe-Balch et al., 1996) to find a concentration sufficient to induce drought stress. After 22 days, plants treated with 5 and 10% PEG had wilted but had not died (**Figure 2.5**). The shoots of plants treated with 10 and 20% PEG were significantly shorter than control shoots, whereas the shoots of plants treated with 5% PEG were not, so it appeared that 5% PEG was tolerated (**Figure 2.6**). There was also a reduction in root length proportional to the PEG concentration but the differences in dry root weight were not significant (data not shown).

Additional experiments were carried out with modifications to confirm the results. This time, a 5% PEG treatment was not included, and 15% PEG was used as an intermediate value between the 10% and 20% treatments. We also used shorter six-week-old plants as the starting population. After 20 days, these plants did not react as observed in the previous experiment. Those treated with 10% PEG showed stress tolerance, those treated with 15% PEG were mildly affected. And those treated with 20% PEG were severely affected (**Figure 2.7**). These results probably reflect the fact that the smaller plants had a less active photorespiratory metabolism and therefore a slower response to the drought stress. The size of the plants affects the physiological response to stress and

it is therefore very important to choose the plants of a uniform size. The different responses or large and small plants are summarized in **Table 2.1**.

For the subsequent experiments with bioregulators, we selected large and well-developed plants and these were exposed to 10% PEG (**Table 2.2**) because this allows the observation of gradual changes in plant responses and the impact of bioregulators is therefore easier to determine.

Figure 2.5. Drought stress in rice plants subjected to different concentrations of PEG for 22 days.

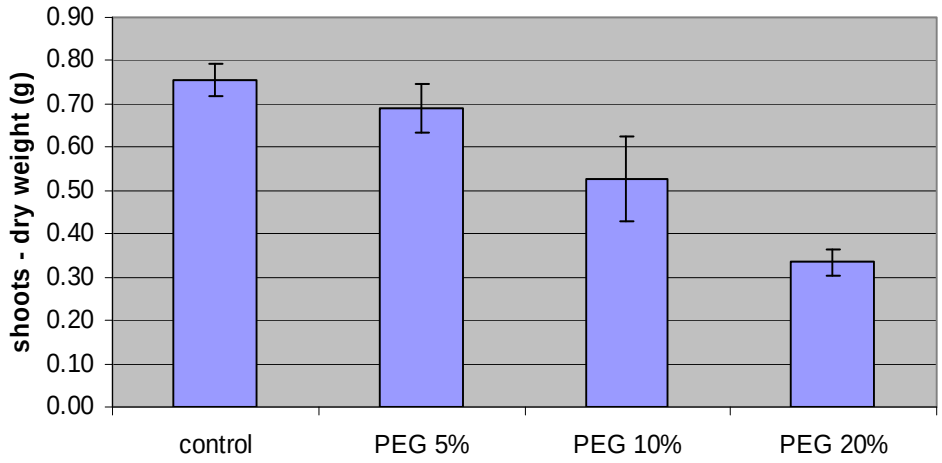

Figure 2.6. Dry weight of rice shoots subjected to different concentrations of PEG.

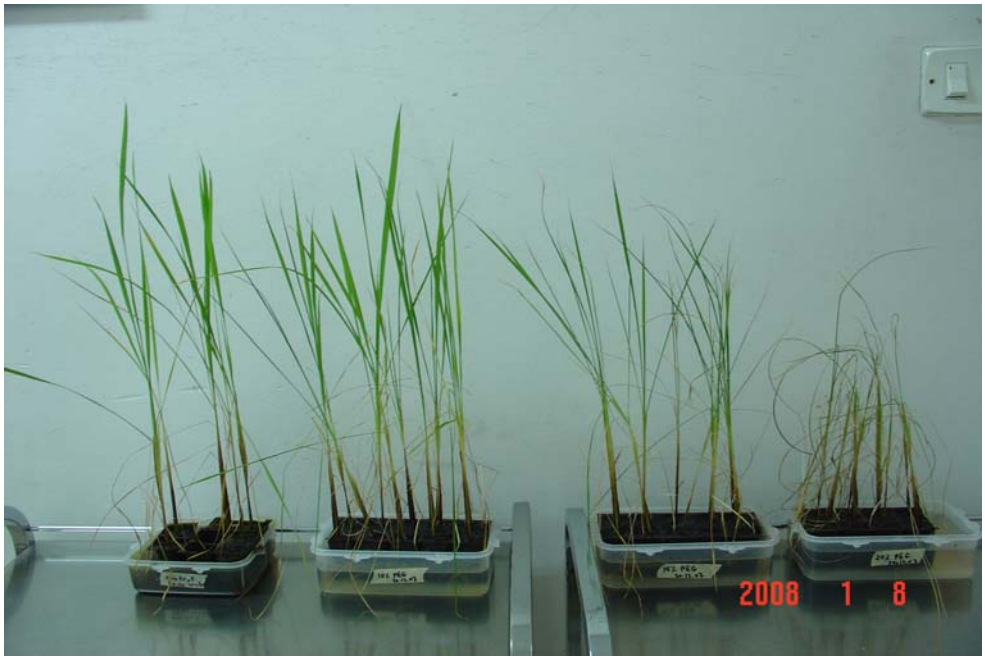

Figure 2.7. Drought stress in short rice plants exposed to different concentrations of PEG for 20 days.

	Big plants				**Small plants**			
day	**control**	**5% PEG**	**10% PEG**	**20% PEG**	**control**	**10% PEG**	**15% PEG**	**20% PEG**
1	+	+	+	+	+	+	+	+
2	+	+	+ -	-	+	+	+	+
8	+	-	-	-	+	+	+	-
14	+	-	- -	- -	+	+	+	- - -
20	+	- -	- - -	- - - -	+	+	-	- - - -

Table 2.1. Comparison of the responses of tall and short rice plants of the same age to drought stress (+ is high water potential and – is low water potential, and the number of "-" indicates severity).

	Drought stress	**Salinity stress**
Rice callus	**400 mM mannitol**	**150 mM NaCl**
Rice plants	**10% PEG**	**100 mM NaCl**

Table 2.2. Standard conditions established for drought and salinity stress assays for rice callus and plants.

2.3.4. Salinity stress assays in rice plants

Six-week-old plants were exposed to 50–200 mM NaCl. After 15 days, the plants exposed to 200 mM NaCl had died, whereas the lower concentrations inhibited growth and caused wilting (**Figure 2.8**). The dry weight of shoots from all treated plants was significantly lower than the dry weight of control shoots (**Figure 2.9**) and shoot growth was inhibited to a greater degree than callus growth under the same conditions (**Figure 2.10**). The roots of plants exposed to 200 mM NaCl were shorter and weaker than the roots of untreated controls (**Figure 2.11**). The total root length measured with *WinRhizo Basic Pro* was significantly shorter in the plants treated with 200 mM NaCl (**Figure 2.12**) but there was no significant difference in dry root weight between treated and untreated plants (data not shown). For the subsequent experiments with bioregulators we selected 100 mM NaCl as a standard condition for salinity stress assay (**Table 2.2**).

control **50mM NaCl** **100mM NaCl** **150mM NaCl** **200mM NaCl**

Figure 2.8. Rice plants exposed to different concentrations of NaCl after 15 days.

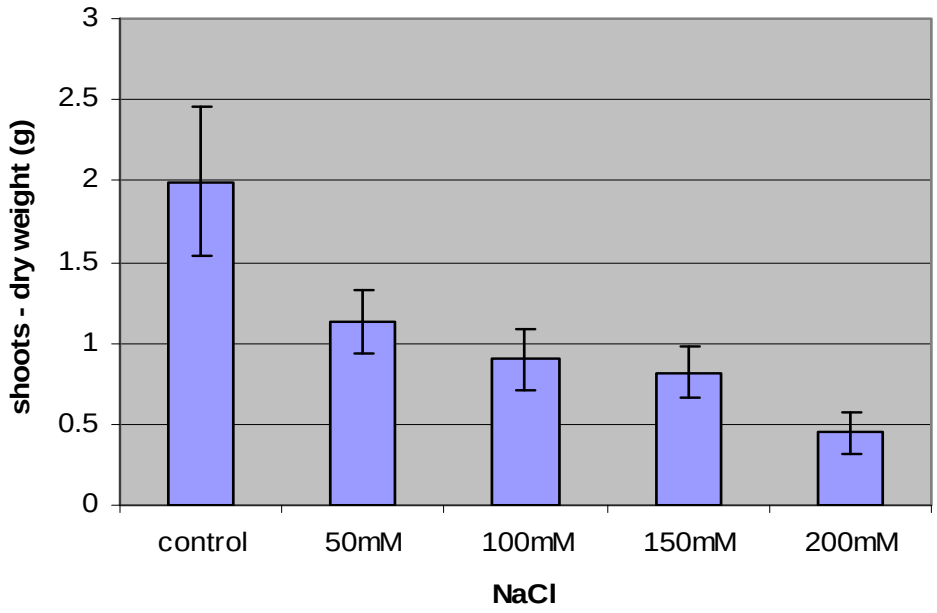

Figure 2.9. Dry shoot weight in rice plant exposed to different concentrations of NaCl for 15 days.

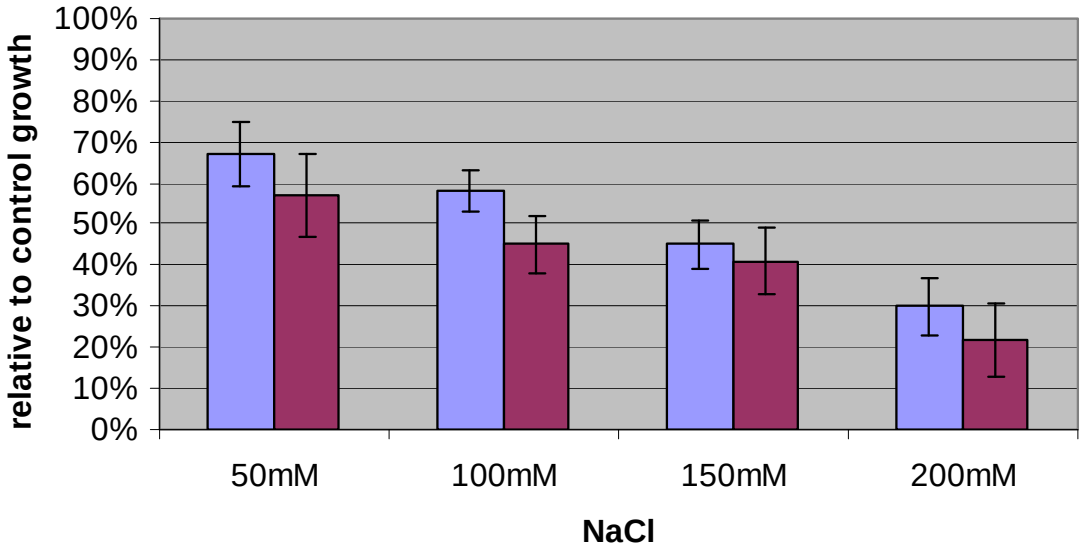

Figure 2.10. Growth rate of rice callus (blue bars) and shoots (purple bars) under salinity stress conditions relative to unstressed controls.

Figure 2.11. Comparison of roots exposed to different concentrations of NaCl for 15 days.

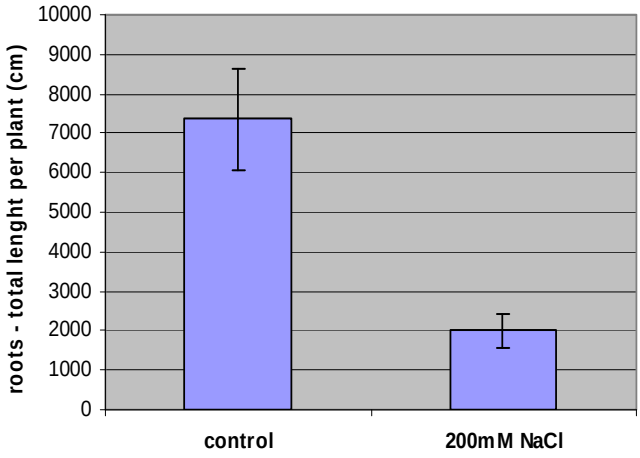

Figure 2.12. Total root length in rice plants growing under normal conditions and exposed to salinity stress.

2.4. Discussion

2.4.1. The use of mannitol and PEG to simulate drought stress

Reduced growth in response to osmotic stress may reflect the loss of cytoplasmic volume and cell turgor as water moves out of the cell (Rhodes and Samaras, 1994). To simulate drought stress in experiments with rice callus, we therefore increased the

osmoticum of the medium. Mannitol was chosen because MS medium supplemented with high concentrations of mannitol can still be solidified, whereas this is not possible with high concentrations of PEG. There was no significant difference between controls and callus grown on media containing 100, 200 and 300 mM mannitol, probably because mannitol can be absorbed and used as a carbon source (Pandey et al., 2004). The standard concentration was therefore chosen as 400 mM mannitol because this induces the appropriate osmotic effect without affecting medium consistency.

For rice plants, the drought stress was established with PEG as previously described (Capell et al., 2004) with some modifications. High-molecular-weight PEG is ideal as a drought-simulation agent because it cannot enter the pores of plant cells and therefore causes cytorrhysis rather than plasmolysis (Verslues et al., 2006). In cytorrhysis, the entire cell collapses (including the cell wall) which is what happens in nature when the soil dries out. However, PEG is preferred to *actual* soil drying as a method to impose drought stress because the severity of the stress can be controlled precisely and different stress levels can be imposed uniformly. We chose 10% PEG as the standard concentration for further experiments with bioregulators because this confers a mild and long-term form of drought stress which is similar to field conditions.

2.4.2. The differences on the performance of rice plants under drought and salinity stress

Our experiments showed that salinity stress has more severe effects on rice callus and plants than drought stress. The osmotic pressure generated by 200 mM mannitol and 100 mM NaCl is very similar (Munns, 2002), but callus growth was reduced by 28% in the presence of 200 mM mannitol but by 42% in the presence of 100 mM NaCl (**Figures 2.2** and **2.4**). In rice plants, Nishimura et al. (2011) also showed that 100 mM NaCl imposed more severe stress than 200 mM mannitol, with fewer seedlings surviving under the saline stress conditions.

Like drought stress, salinity stress affects growth by reducing the intake of water, and has the same effects, i.e. loss of cytoplasmic volume and turgor (Munns, 2002). However, in the case of salinity stress, the almost immediate osmotic impact of the salt solution is followed over a period of days by ion toxicity. Sodium and chloride ions enter the roots and are transferred to shoots where they accumulate. The excess is

sequestered in vacuoles to prevent protein denaturation (Chinnusamy et al., 2005) but once the vacuolar capacity is exceeded, the ions accumulate in the cytoplasm and the resulting electrolyte imbalance is toxic. The leaves are affected first, and the oldest leaves begin to die, while new leaves fail to emerge. Younger leaves fail to reach their full size and the number of emerged tillers is reduced. Flowering and seed setting may also be delayed.

Root growth is also affected by stress albeit to a lesser extent than shoot growth (Munns, 2002). This is probably why we did not observe a significant difference in dry root weight between control plants and roots of plants subjected to either drought or salinity stress. There was a significant difference in root length between untreated plants and those exposed to 200 mM NaCl, suggesting high concentrations of NaCl are toxic to rice roots.

We found that plants were more sensitive than callus to salinity stress even though the plants were exposed for a shorter duration, and this is why the standard salinity stress treatment for plants was set lower than that for the callus assay. Rice is more sensitive to salinity stress during seedling growth and reproductive development than vegetative growth (Heenan et al., 1988). During reproductive development, high salinity reduces the number of spikelets formed per panicle (Pareek et al., 1999).

2.4.3. Plant size

We carried out two drought stress experiments with plants of different sizes, and found that the size of the plants affected the outcome in terms of stress tolerance and susceptibility. Plants differ in their response to stress because of differences in phenological, morphological, physiological, biochemical and molecular adaptive mechanisms (Perez-Morphe-Balch et al., 1996). Therefore, it is critical to select uniform plant populations as the basis for stress experiments.

The salt-specific impact of salinity stress has a profound effect on older leaves, where the salt is imported with the transpiration stream and accumulates to high levels over time (Munns, 2002). It is therefore preferable to work with bigger plants in which the salinity effects are more visible. We chose larger, ten-week-old plants for our subsequent salinity experiments with bioregulators, because not only were these plants

larger, but they also allowed us to evaluate the impact of stress and bioregulators on flowering.

2.5. Conclusions

The intensity and duration of stress conditions should be established empirically because the growth of plants and their response to stress will depend on the available system of growth chambers or greenhouses. It is important to take the significant biological variability among rice plants into account, and we found that the reliability of the stress assays could be improved by pre-selecting a uniform subpopulation from a larger pool of plants, and including as many replicates of each treatment as possible in each experiment. Stress assays must be reproducible to generate reliable molecular data. Even though we grew our plants under controlled conditions, including constant temperature and humidity, seasonal fluctuations in growth were observed and these also must be taken into consideration.

2.6. References

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Chapter 3

An azole derivative induces tolerance to salinity stress and accelerates floral and vegetative development in rice

3.1. Introduction

A number of synthetic compounds such as hormone inhibitors, safeners, herbicides and insecticides can influence plant growth and development. Strobilurines such as azoxystrobine, fluoxastrobine, kresoxim-methyle, picoxystrobine, pyraclostrobine and trifloxystrobine, enhance carbon assimilation and metabolism in general (Gerhard et al., 1998; Grossmann et al., 1999) while inhibiting protein and chlorophyll degradation, thereby delaying senescence (Bryson *et al*., 2000). Azoles can also delay senescence, and often affect hormone biosynthesis. For example, uniconazole promotes winter or early spring flowering in *Globularia sarcophylla* and does so by inhibiting gibberellin biosynthesis (Katz et al., 2003). Although some of the metabolic processes underlying the effects of strobilurines and azoles have been investigated (Clark, 2003), there are no data for many other synthetic compounds and their modes of action in the context of improving crop productivity are poorly understood.

Certain growth regulators can also improve the tolerance of plants to different stresses, including water deficit. Benzyladenine (6-BA) can enhance drought resistance in several species (Shang et al., 2000), including wheat and maize seedlings under water deficit stress, where the application of 10^{-5} M 6-BA significantly increased RuBPCase activity (Dong et al., 1997). Brassinolides can also increase stress tolerance and increase grain yields under stress in wheat and maize (Takematsu et al., 1983; Yokota and Takahashi, 1986). Imidacloprid improves the growth and yield of cotton plants subjected to heat stress in the laboratory and under field conditions (Gonias et al., 2006). Plants sprayed with this chemical grew more vigorously, matured earlier and produced more lint than untreated control plants, reflecting a higher rate of photosynthesis and carbon metabolism, reduced chlorophyll fluorescence and lower membrane leakage. The antioxidant enzyme activity was lower in treated plants than controls, suggesting that imidacloprid reduced the level of oxidative stress perhaps through direct antioxidant activity.

Waterlogging is another abiotic stress that severely reduces the yield of important crops such as wheat and rice. Prohexadione calcium (Pro-Ca) can reduce the impact of waterlogging by inhibiting gibberellin synthesis and thus reducing vegetative growth (Kim et al., 2007). Rice plants treated with Pro-Ca had shorter and stronger stems as well as several improved yield parameters such as ripened grain ratio (percentage of ripened grains in spikelet), 1000 grain weight and milled rice yield. Despite the significant number of studies describing the beneficial effects of growth regulators on stress tolerance in important crops, their precise mechanisms of action remain unknown.

We screened 12 molecules: five fungicides (uniconazol, diniconazol, baronet, tebuconazole and trifloxystrobin), three insecticides (imidacloprid, fipronil and fipronilamid), three safeners (isoxadifen, mefenpyr and cyprosulfamide) and the phytohormone abscisic acid (ABA). Safeners are chemicals that selectively protect crops from herbicide damage without reducing herbicide activity towards target weed species. They can be applied either as a mixture with the herbicide or as a seed treatment prior to sowing (Abu-Qare and Duncan, 2002). Five of the above molecules are azoles (baronet, tebuconazole, uniconazole, diconazole and fipronil) and the combined application of azoles and ABA has been shown to increase the effect of bioregulators on plant growth and productivity under stress conditions (Bayer CropScience, personal communication). ABA plays a role in the adaptation of plants to cold, drought and salinity stress (Zhu, 2002; Seo and Koshiba, 2002; Cowan et al., 1997) and these different stress conditions increase the levels of endogenous ABA. Previous treatment with exogenous ABA can improve the tolerance of rice callus towards osmotic, salinity and freezing stresses (Perales et al., 2005).

Among these 12 molecules, three had a positive effect on rice growth under abiotic stress conditions: fipronil, tebuconazole and cyprosulfamide, with the latter inducing the most consistent responses. Treatment with cyprosulfamide improved the performance of rice plants under salinity stress conditions and promoted the appearance of new tillers and earlier flowering under both normal and stress conditions. Even better performance was achieved when cyprosulfamide was combined with ABA, suggesting the two compounds had a synergic effect.

3.2. Materials and Methods

3.2.1. Preparation and application of bioregulators

The 12 compounds were dissolved in water at appropriate concentrations established during tests on the model plant *Arabidopsis thaliana* (**Table 3.1**), although the total amount applied to rice plants was greater because the aerial parts are much larger. The solutions were homogenized by vortexing and applied by spraying 1 ml of each solution onto the aerial parts of each plant.

3.2.2. Experimental set up and phenotypic analysis of treated plants

Plants were allotted to control and stress treatment cohorts according to the number of treatments applied in each experiment. For example, experiments 17, 19 and 21 involved three control trays and three trays for the stress treatments. Both the control and stress-treatment trays included untreated plants, plants treated with cyprosulfamide, and plants treated with cyprosulfamide plus ABA. Experiment 24 included four control and four stress-treatment trays, each featuring untreated plants, plants treated with ABA, plants treated with cyprosulfamide, and plants treated with cyprosulfamide plus ABA. We collected samples at three time points: the first day of the stress treatment (day 1), the last day of the stress treatment (day x) and the end of the recovery period (day x+y). For experiments 17, 19, 21 and 24, x = 15, 15, 15 and 11, and y = 6, 5, 10 and 11, respectively.

Phenotypic analysis included counting the number of new tillers, recording the flowering time and measuring the chlorophyll content, in each case comparing the various treatments to untreated plants under normal and saline stress conditions. The chlorophyll content was measured using a SPAD-502 Chlorophyll Meter (Minolta Corporation, New Jersey, USA).

3.2.3. Statistical analysis

For experiments 17, 19 and 21, we used 10 plants per treatment (n = 10) and three replicate samples were taken at each time point for each treatment, each replicate comprising one plant. For experiment 24, we used 39 plants per treatment (n = 39) and four replicate samples were taken for each time point in each treatment, each replicate

comprising three plants. For the chlorophyll content measurements one leaf per plant was chosen and three measurements per leaf were done. Measurements were taken from each plant of each treatment and the leaves of the same developmental stage were selected. The data were analyzed by two-way analysis of variance followed by Student's t-test using the residual mean square in the ANOVA as the estimate of variability.

3.3. Results

3.3.1. Screening potential bioregulators

For the first series of experiments, we investigated the effects of all 12 test compounds (**Table 3.1**) on rice plants under drought and salinity stress conditions (**Table 3.2**). A recovery step was introduced to determine whether any of the compounds exerted an effect *after* exposure to stress. Similarly, ABA was introduced as an additional treatment because previous work has shown that this hormone can potentiate many bioregulators (Bayer Crop Sciences; personal communication). Cyprosulfamide, tebucanozole and fipronil were the only three compounds to improve the performance of rice plants under stress.

In experiment 7, cyprosulfamide and tebuconazole (when used independently) had a positive impact on plants subjected to drought stress. On day 5, plants sprayed with cyprosulfamide appeared to be growing more vigorously than control plants, and plants sprayed with cyprosulfamide and ABA appeared better still (**Figure 3.1, Table 3.2**). Differences were still apparent between treated plants and controls on days 7 and 9, but by day 11 all the plants appeared similar. On day 5, plants sprayed with tebuconazole appeared to be growing more vigorously than control plants, and plants sprayed with tebuconazole and ABA appeared better still (**Figure 3.2, Table 3.2**). As with the cyprosulfamide treatment, the phenotypic differences were maintained over the next four days.

Experiment 11 also involved the treatment of plants with tebuconazole, but additional treatment regimens were included in which plants received a double dose of the regulator. Five groups of plants were therefore compared: untreated, tebuconazole, tebuconazole plus ABA, double tebuconazole and double tebuconazole plus ABA. Stress was applied for 11 days and the plants were allowed to recover for 10 days.

Plants subjected to the double-dose treatments appeared marginally healthier than controls on day 10 and these differences remained until recovery (day 11) (**Figure 3.3, Table 3.2**).

Experiment 12 was set up like experiment 11 but the compound in this case was fipronil. Stress was applied for 12 days and the plants were allowed to recover for 10 days. Plants treated with the double-dose of fipronil appeared healthier than controls on day 7 and showed more obvious differences compared to the control group every day thereafter until the day 12, when recovery commenced (**Figure 3.4, Table 3.2**). From day 14 onwards, all the plants appeared similar.

Several positive results were then obtained by treating plants with cyprosulfamide under salinity stress conditions (**Table 3.2**). We therefore focused on the cyprosulfamide/salinity stress model for further investigation.

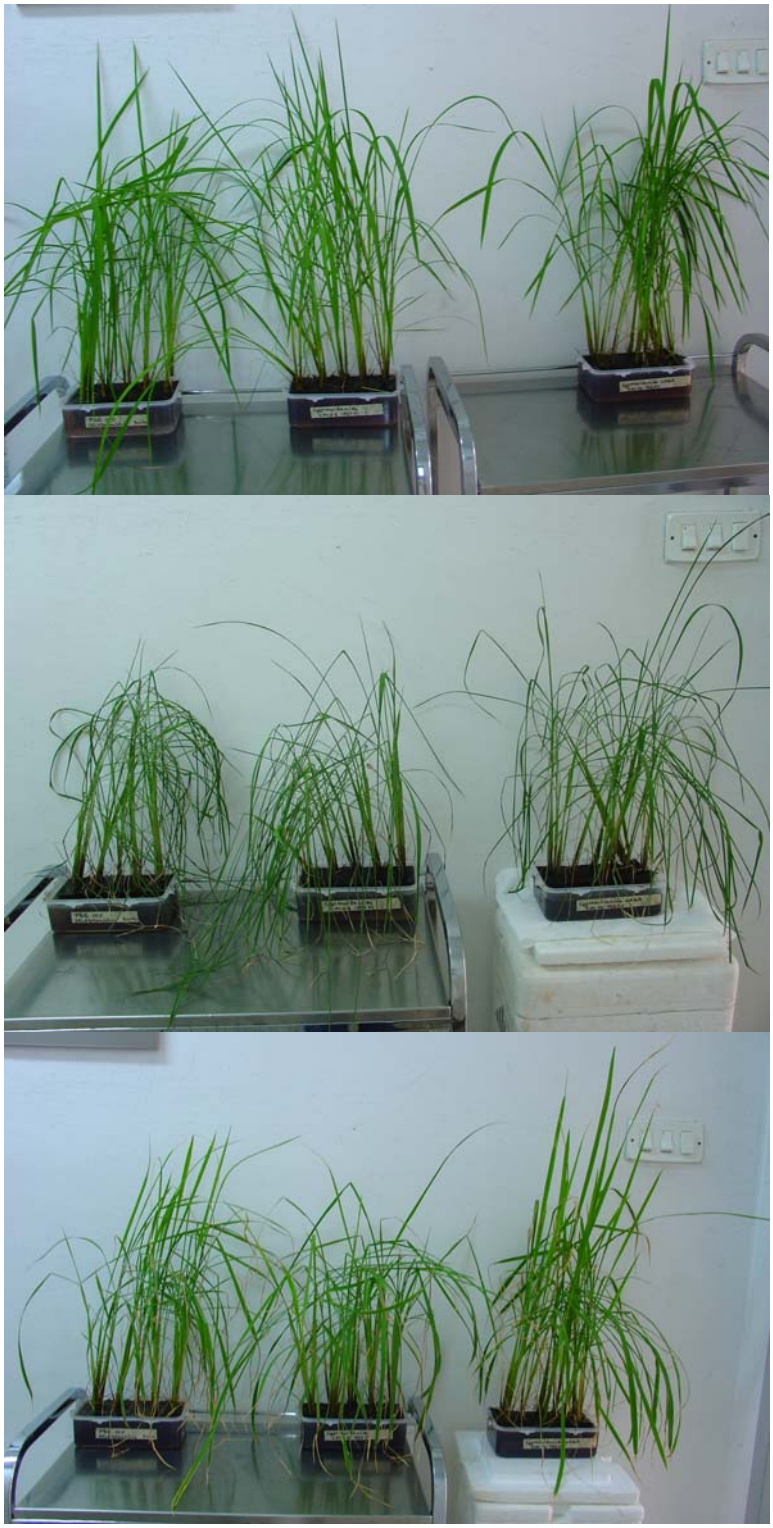
Name	**Mode of Action**	**Concentration (mg/ml)**
Imidacloprid	Insecticide	3.125
Fipronil	Insecticide	0.625
Fipronilamid	Insecticide	0.625
Trifloxystrobin	Fungicide	3.125
Uniconazol	Fungicide	0.625
Diniconazol	Fungicide	0.625
Baronet	Fungicide	0.625
Tebuconazole	Fungicide	1.25
Cyprosulfamide	Safener	3.125
Mefenpyr	Safener	3.125
Isoxadifen	Safener	3.125
Abscisic acid	Hormone	0.125

Table 3.1. Twelve compounds tested for potential bioregulator activity.

Exp. No.	Type of stress	Compound	Duration of stress	Recovery	Effects	Size
1	PEG 10%	Imidachloprid, fipronil, mefenpyr, isoxadifen	21 d	-	Day 21 effect of PEG, no effect of compounds	Short
2	PEG 10%	Imidachloprid, fipronil, mefenpyr, isoxadifen	26 d	-	Day 11 effect of PEG, no effect of compounds	Short
3	PEG 10%	ABA, fipronilamid, trifloxystrobin	18 d	-	Day 6 effect of PEG, no effect of compounds	Tall
4	PEG 10%	Baronet, fipronil, diniconazol, ubiconazol	31 d	-	Day 12 effect of PEG, no effect of compounds	Short
5	50 mM NaCl	ABA, fipronil, fipronilamid	20 d	-	Day 13 effect of NaCl, no effect of compounds	Normal
6	50 mM NaCl	Baronet, baronet + ABA	12 d	9 d	Day 12 effect of NaCl, no effect of compounds	Normal
7	PEG 10%	Tebuconazole, baronet, imidachloprid, cyprosulfamide. All the compounds were sprayed without or with ABA.	6 d	10 d	Day 5 effect of PEG, some effect of tebuconazole and cyprosulfamide + ABA	Tall
8	PEG 10%	Tebuconazole, tebuconazole + ABA, cyprosulfamide cyprosulfamide + ABA	15 d	-	No effect of PEG	Short
9	PEG 10%	Tebuconazole, ABA, tebuconazole + ABA	12 d	14 d	No effect of PEG, no effect of compounds	Short
10	PEG 20%	Tebuconazole, ABA, Tebuconazole + ABA	4 d	5 d	Day 4 effect of PEG, no effect of compounds	Normal
11	PEG 10%	Tebuconazole, Tebuconazole + ABA, Tebuconazole x2, Tebuconazole x2 + ABA	11 d	10 d	Day 6 effect of PEG, some effect of 2x tebuconazole + ABA	Tall
12	PEG 10%	Fipronil, fipronil + ABA, fipronil x2, fipronil x2 + ABA	12 d	10 d	Day 7 effect of PEG, some effect double doze of fipronil	Tall
13	PEG 10%	fipronil x2, fipronil x2 + ABA	14d	6d	No effect of PEG, no effect of compounds	Short

14	PEG 10%	Tebuconazole x2, Tebuconazole x2 + ABA	14 d	6 d	No effect of PEG, no effect of compounds	Short
15	PEG 10%	Tebuconazole x2, Tebuconazole x2 + ABA	20 d	-	Day 12 effect of PEG, no effect of compounds	Tall
16	100 mM NaCl	Tebuconazole x2, Tebuconazole x2 + ABA	12 d	5 d	Day 7 effect of NaCl, no effect of compounds	Tall
17	100 mM NaCl	Cyprosulfamide, cyprosulfamide + ABA	15 d	10 d	Day 6 effect of NaCl, some effect of compounds	Tall
18	100 mM NaCl	Tebuconazole x2, Tebuconazole x2 + ABA	13d	-	Effect of NaCl, no effect of compounds	Tall
19	100 mM NaCl	Cyprosulfamide, cyprosulfamide + ABA	15 d	5 d	Day 5 effect of NaCl, some effect of compounds	Tall
20	100 mM NaCl	Cyprosulfamide, cyprosulfamide + ABA	13 d	-	Effect of NaCl, no effect of compounds	Tall
21	100 mM NaCl	Cyprosulfamide, cyprosulfamide + ABA	15 d	10 d	Effect of NaCl, some effect of compounds	Tall
22	100 mM NaCl	Cyprosulfamide, cyprosulfamide + ABAx10	14 d	6 d	Effect of NaCl, good effect of cyprosulfamide + ABAx10	Tall
23	100 mM NaCl	Cyprosulfamide, cyprosulfamide + ABAx10	15 d	-	Effect of NaCl, no effect of compounds	Tall
24	100 mM NaCl	Cyprosulfamide, ABA cyprosulfamide + ABA	11 d	11 d	Effect of NaCl, some effect of compounds	Tall
25 flag leaf	100 mM NaCl	Cyprosulfamide, ABA, cyprosulfamide + ABA	12 d	-	Effect of NaCl, no effect of compounds	Tall
26	100 mM NaCl	Cyprosulfamide, cyprosulfamide + ABAx10	19 d	9 d	Effect of NaCl, some effect of compounds	Tall

Table 3.2. Summary of experiments to determine potential bioregulator activities during drought or salinity stress and recovery. Experiments where a positive bioregulator effect was observed are boxed in red.



Day1 – stress applied

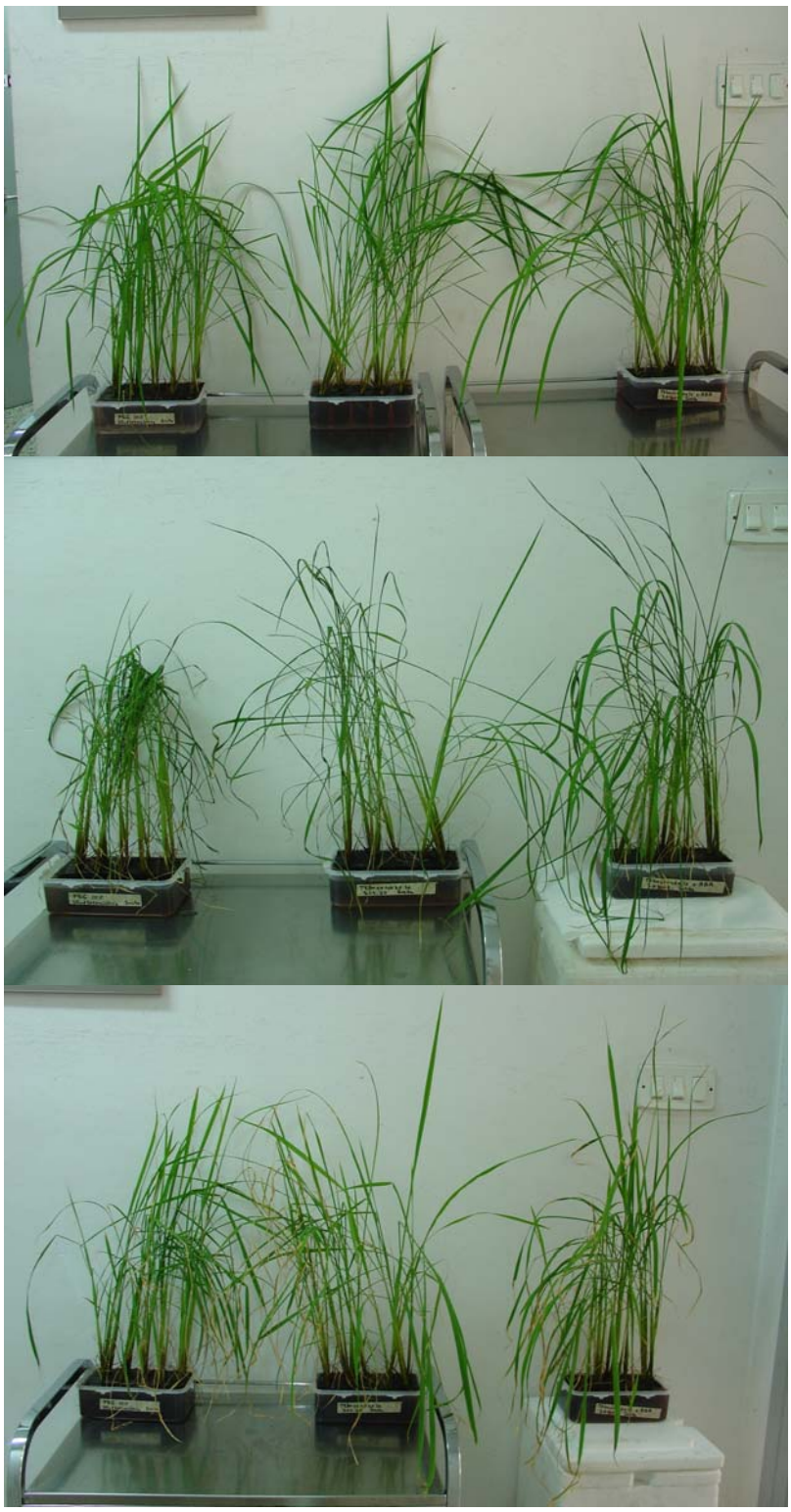
Day5 – recovery applied

Day9 - recovery

control cyprosulfamide cyprosulfamide + ABA

Figure 3.1. Experiment 7: Treatment of drought-stressed rice plants with cyprosulfamide. The application of cyprosulfamide plus ABA improved the growth of plants under stress such that a difference in phenotype was apparent as early as day 5,

one day before the start of the recovery period. Treated plants remained more vigorous until day 11, when the untreated plants recovered fully and regained their former vigor.



Day1 – stress applied

Day5 – recovery applied

Day9 - recovery

control **tebuconazole** **tebuconazole+ABA**

Figure 3.2. Experiment 7: Treatment of drought-stressed rice plants with tebuconazole. The application of tebuconazole (with or without ABA) improved the growth of plants

under stress such that a difference in phenotype was apparent as early as day 5, one day before the start of the recovery period. Treated plants remained more vigorous until day 11, when the untreated plants recovered fully and regained their former vigor.

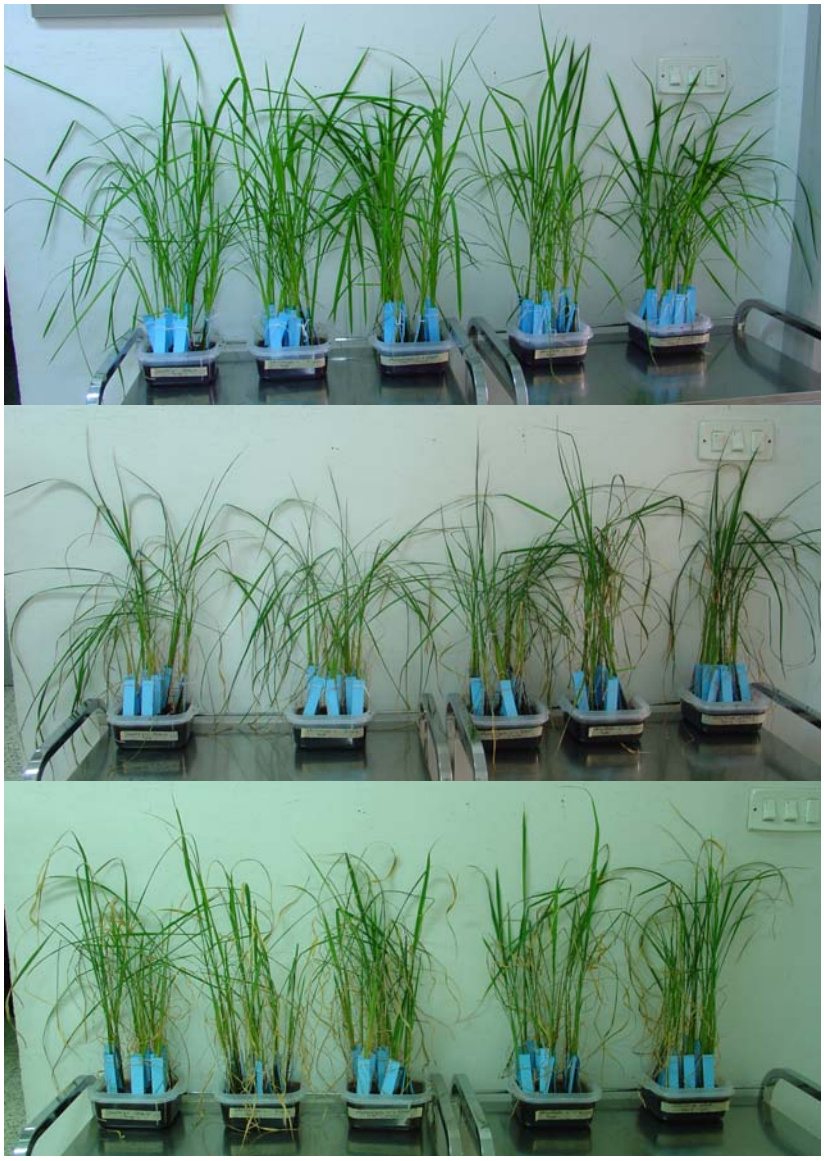


Figure 3.3. Experiment 11: Treatment of drought-stressed rice plants with tebuconazole. The application of a double dose of tebuconazole (tebuconazole x2) and tebuconazole x2 plus ABA improved the growth of plants under stress such that a difference in phenotype was apparent by treatment day 10 and persisted until day 11, the last day of treatent. teb - tebuconazole, teb x2 - tebuconazole x2.

Day1

Day12 –recovery applied

Day14 - recovery

Figure 3.4. Experiment 12: Treatment of drought-stressed rice plants with fipronil. The application of a double dose of fipronil (fipronil x2) and fipronil x2 plus ABA improved the growth of plants under stress such that a difference in phenotype was apparent by treatment day 12 and persisted until day 14, two days into the recovery period.

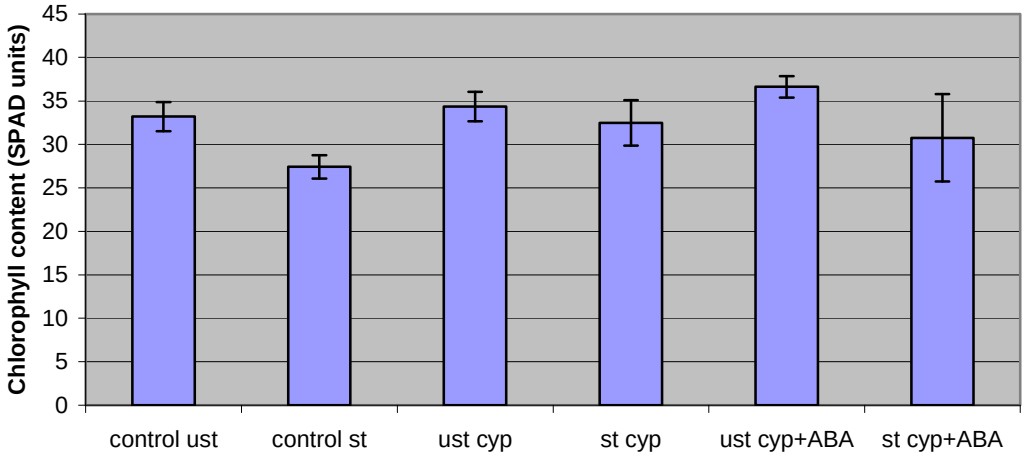
3.3.1.1. Cyprosulfamide improves the growth of rice plants under salinity stress conditions

Experiments 17, 19, 21, 22, 24 and 26 revealed the positive impact of cyprosulfamide on rice plants growing under salinity stress conditions. The combination of

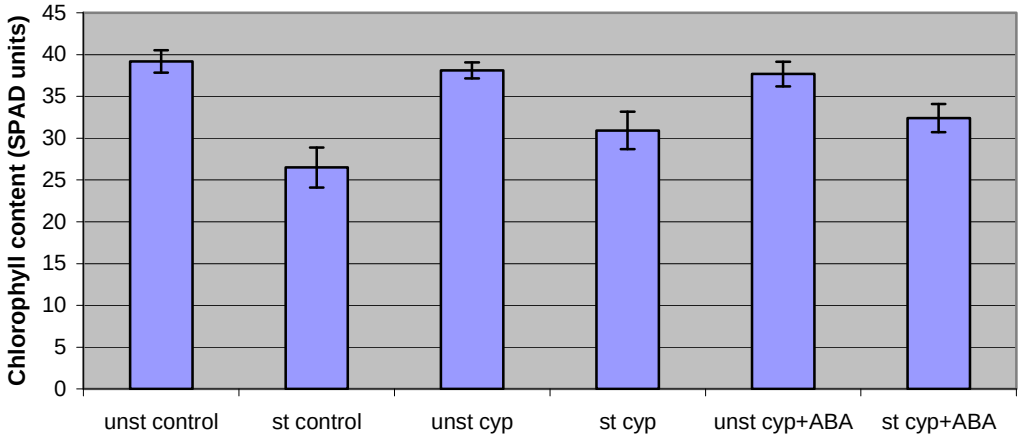
cyprosulfamide plus ABA improved the performance of treated plants further in most of the experiments.

3.3.2. Developmental changes in rice plants induced by cyprosulfamide

The chlorophyll content of rice plants was measured in experiments 17 and 19. In experiment 17, there was a significant difference ($p < 0.05$) between unstressed control plants and unstressed plants treated with cyprosulfamide plus ABA; there was also a significant difference ($p < 0.05$) between stressed control plants and stressed plants treated with cyprosulfamide (**Figure 3.5a**). In experiment 19, there was a significant difference ($p < 0.05$) between stressed control plants and stressed plants treated with cyprosulfamide plus ABA (**Figure 3.5b**). Both experiments indicated that cyprosulfamide has a positive impact on the chlorophyll content of rice plants under salinity stress.



a

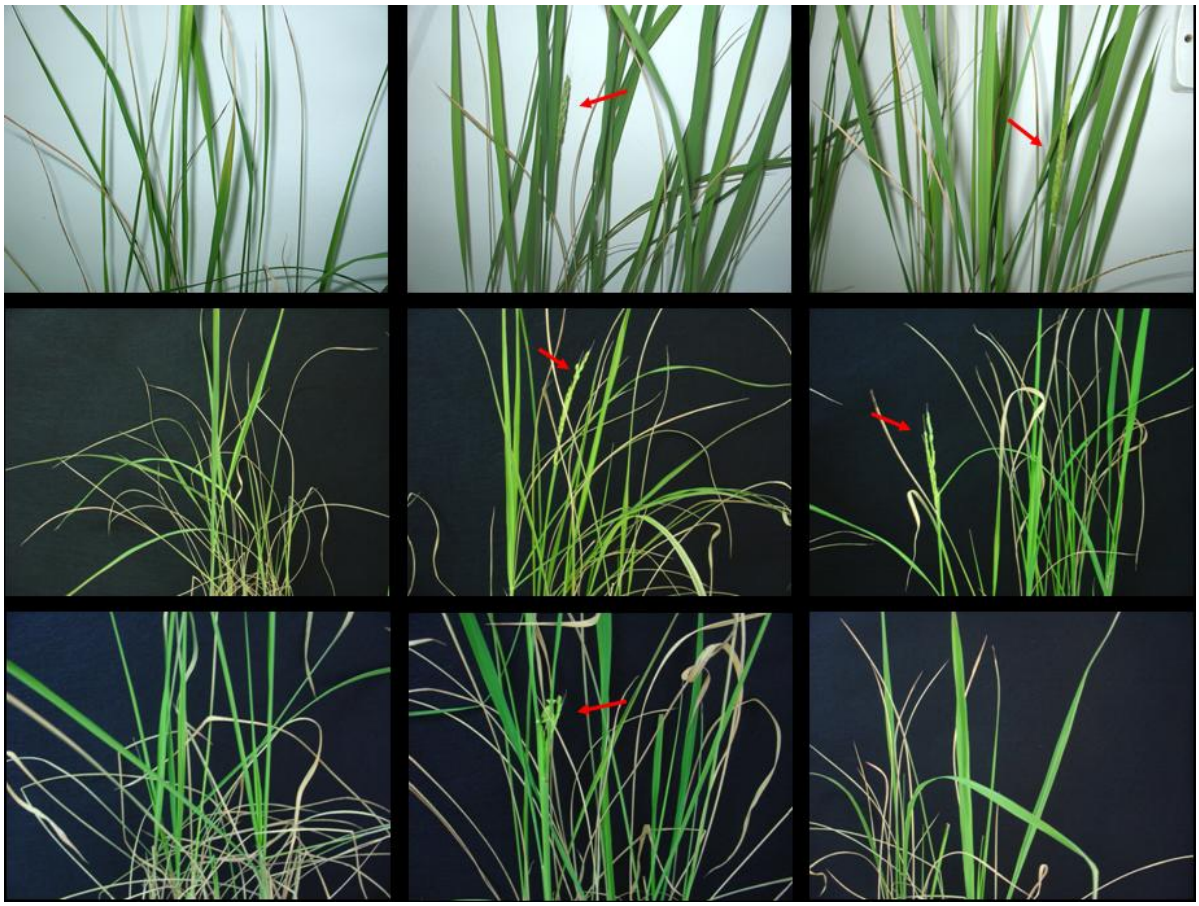


b

Figure 3.5. Chlorophyll content measurements in experiments 17 (**a**) and 19 (**b**). cyp – cyprosulfamide, unst – unstressed, st - stressed.

3.3.2.1. Flowering time in rice plants under salinity stress and treated with cyprosulfamide

In experiments 17, 19 and 21, rice plants treated with cyprosulfamide flowered earlier under salinity stress conditions than untreated plants (**Figure 3.6**). Panicle development was divided into three stages (booting, flowering and seed formation) and plants under all treatment regimens were analyzed at all three developmental stages. Flowering was delayed by stress in all three experiments, but occurred earlier in plants treated with cyprosulfamide plus ABA in experiment 17 and 21 (**Figure 3.7**). In experiment 17, flowering started earlier in stressed plants treated with cyprosulfamide (one panicle at the flowering stage) and with cyprosulfamide plus ABA (one panicle at the flowering stage and one at the booting stage). Control plants had only one panicle at the booting stage (**Figure 3.7a**). In experiment 19, flowering started earlier in stressed plants treated with cyprosulfamide (one panicle at the seed stage) and in those treated with cyprosulfamide plus ABA (one panicle at the flowering stage and one at the booting stage). Control plants had two panicles at the booting stage (**Figure 3.7b**). In experiment 21, flowering started earlier in stressed plants treated with cyprosulfamide (one panicle at the flowering stage) than in control plants with no panicles at all (**Figure 3.7c**).



control **cyprosulfamide** **cyprosulfamide + ABA**

Figure 3.6. Flowering in plants from experiments 17 (upper row), 19 (middle row) and 21 (lower row), subjected to saline stress. The pictures were taken on the last day of stress treatment. Left column shows control plants (no bioregulator treatment). Middle column shows plants treated with cyprosulfamide. Right column shows plants treated with cyprosulfamide plus ABA. Flowering panicles are indicated with red arrows.

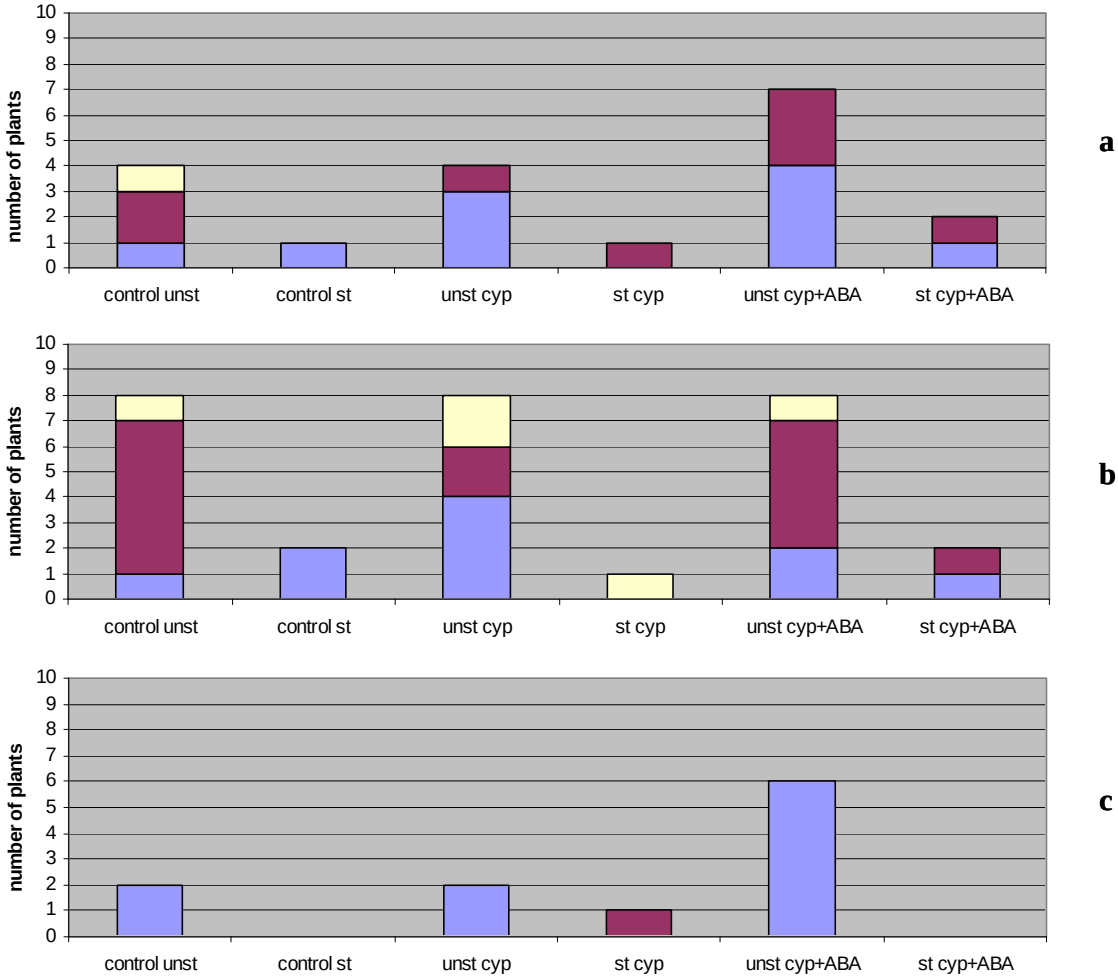
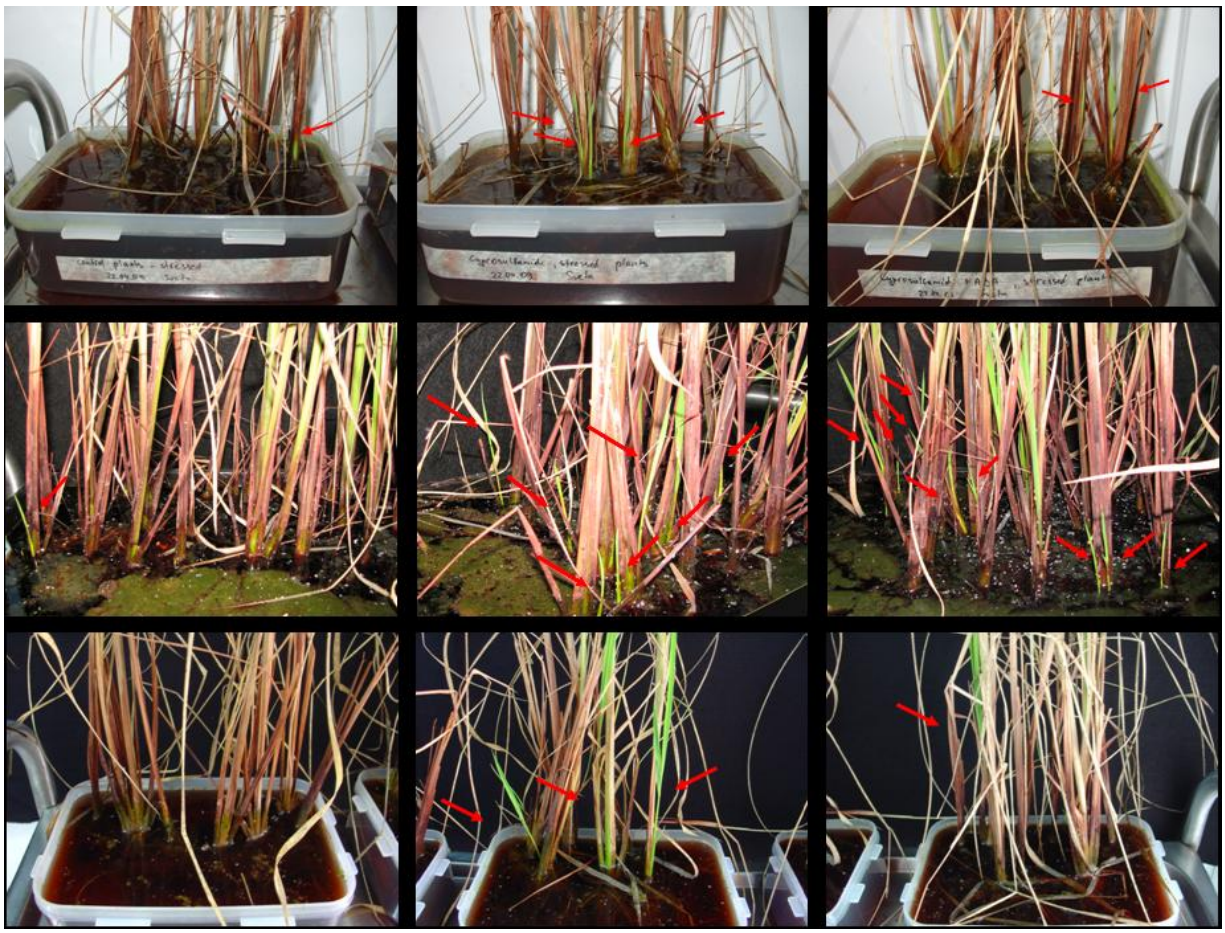


Figure 3.7. Panicle developmental stages in experiments (**a**) 17, (**b**) 19 and (**c**) 21 on the last day of stress. Blue bars represent booting, red bars represent flowering and yellow bars represent seed formation. cyp – cyprosulfamide, unst – unstressed, st – stressed.

3.3.2.2. Accelerated development of rice plants under salinity stress conditions and treated with cyprosulfamide

In experiments 17, 24 and 21, rice plants under salinity-stress conditions that were treated with cyprosulfamide or cyprosulfamide plus ABA produced more tillers than control plants (**Figure 3.8**) although no difference was observed in experiment 19 (data not shown). In experiment 24, the additional treatment with ABA alone had no impact on tillerogenesis, but treatment with cyprosulfamide or cyprosulfamide plus ABA increased the number of tillers produced in both unstressed and stressed plants (**Figure**

3.9a). Treatment with cyprosulfamide plus ABA allowed the treated plants to continue producing more tillers than controls during the recovery period (**Figure 3.9b**).



control **cyprosulfamide** **cyprosulfamide+ABA**

Figure 3.8. Appearance of new tillers in experiments 17 (upper row), 24 (middle row) and 21 (lower row). Pictures were taken on the last day of stress. Left column shows stressed control plants. Middle column shows stressed plants treated with cyprosulfamide. Right column shows stressed plants treated with cyprosulfamide plus ABA. New tillers are indicated with red arrows.

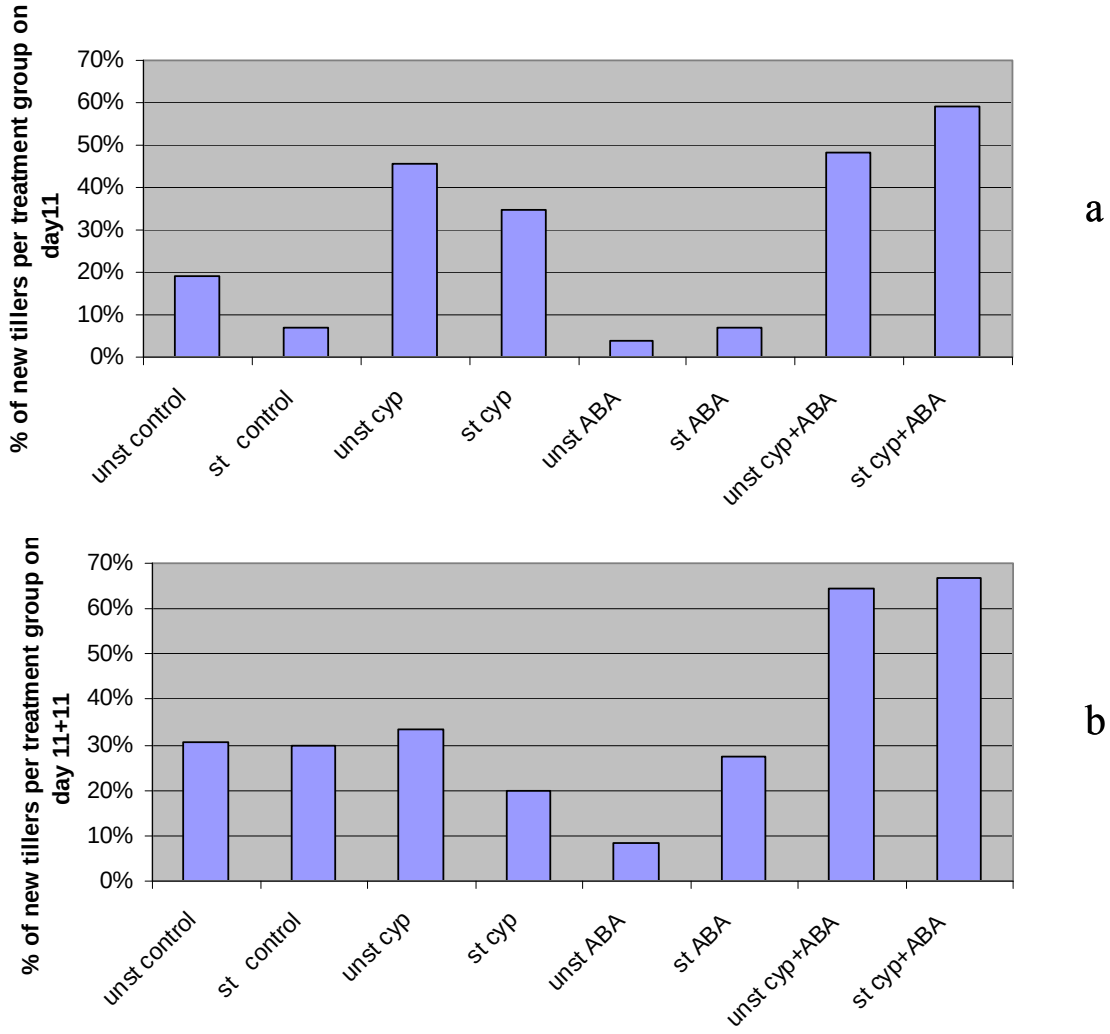


Figure 3.9. Percentage of new tillers formed in plants in experiment 24 under different treatment regimens: (**a**) last day of stress (day 11); (**b**) after recovery (day 11 + 11); unst – unstressed, st – stressed, cyp - cyprosulfamide.

3.4. Discussion

3.4.1. Azole derivatives increase drought stress tolerance in rice

Most of the compounds we tested had no effect on plant growth when applied either alone or in combination with ABA. Tebuconazole, cyprosulfamide and fipronil were the only three chemicals that conferred stress tolerance, and double doses of tebuconazole and fipronil had a more beneficial effect than single doses.

Fipronil is a broad-spectrum phenylpyrazole insecticide that disrupts the central nervous system of insects by blocking the passage of chloride ions through the GABA receptor

and glutamate-gated chloride channels, members of the ligand-gated ion channel superfamily. Tebuconazole is a systemic, triazole-family fungicide that inhibits fungal sterol biosynthesis. Both these azole derivatives improved the growth of rice plants under drought stress conditions, in accordance with previous studies linking azoles to abiotic stress tolerance in plants (Bayer Crop Science, patent WO2010015337). Paclobutrazol and uniconazole (S_{3307}) are triazole plant growth regulators that block gibberellin biosynthesis and act as shoot growth inhibitors. Plants treated with triazole compounds are more resistant to drought, atmospheric pollutants and extreme temperatures (Fletcher and Hofstra, 1988). The plant growth regulators benzyladenine, uniconazole, brassinolide, and ABA minimized yield losses caused by water deficit in soybean (Zhang et al, 2004).

3.4.2. The safener cyprosulfamide increases drought and salinity stress tolerance in rice

Cyprosulfamide is a new synthetic safener that accelerates herbicide detoxification (Pesticide Properties DataBase, 2011). When cyprosulfamide is applied to maize in addition to the herbicide, the selectivity of the herbicide is increased as is the rate at which the herbicide is metabolized into nontoxic products. Cyprosulfamide can also enhance the herbicidal activity of pinoxaden towards weeds and grasses, without harming crop plants (Kotzian and Hall, 2010). Pinoxaden is an active ingredient in the herbicidal mixture Axial® (Syngenta, Switzerland) that controls grass weeds in wheat and barley by inhibiting acetyl-CoA carboxylase (Yu et al., 2010).

We have found that the safener cyprosulfamide improves the performance of rice plants subjected to drought and salinity stress. Under salinity stress conditions, rice plants treated with cyprosulfamide form new tillers and start to flower earlier than untreated plants, the leaves tend not to curl and wilt to the same extent as untreated leaves, and the chlorophyll content is also higher.

Safeners are known to protect plants from herbicide toxicity by coordinately inducing entire herbicide detoxification pathways (Zhang et al., 2007; Riechers et al., 2010). Some of the genes that participate in herbicide detoxification also confer abiotic stress tolerance. Glutathione (GSH) is an antioxidant that provides an important intracellular defense against oxidative damage caused by ROS. GSH is induced when *Anabaena*

doliolum is exposed to salinity stress (Srivastava et al., 2005) and the GSH levels are also higher in the leaves of *Vigna radiata* L. cv. Pusa Bold than *Vigna radiata* L. cv. CO 4 under salinity stress, indicating that cv. Pusa Bold has more efficient antioxidant characteristics (Sumithra et al., 2006). GST is also known to play a role in oxidative stress protection by reducing peroxides with the help of GSH (Gill and Tuteja, 2010). In drought tolerant (M35-1) and drought sensitive (SPV-839) sorghum varieties subjected to salinity stress, M35-1 proved to be more efficient at H_2O_2 scavenging because of its significantly higher GST activity (Jogeswar et al., 2006). Salinity stress and ABA also rapidly induce *osgstu*3 in rice, which encodes a tau-class GST (Moons, 2003). Increased GST activity was also detected in tomato plants under salinity stress (Gapinska et al., 2008).

Forty-nine *A. thaliana* CytP450 genes have been analyzed to determine their potential role in biotic and abiotic stress tolerance, and their response to hormones such as salicylic acid, jasmonic acid, ethylene and ABA (Narusaka et al., 2004). Some of the genes were found to be induced both by abiotic and biotic stresses. The *CYP81D11* and *CYP81D8* promoters contain ABA-response elements, and they are also induced by salinity stress. Safeners therefore induce genes that play a role in abiotic stress adaptation and their application may improve plant stress tolerance through this route.

3.4.3. Synergistic role of ABA

ABA enhanced the effects of tebuconazole and cyprosulfamide but not fipronil. However the synergic effect of ABA and tebuconazole was not consistent because in experiment 11 as there were no differences between applying tebuconazole alone or with ABA. In contrast, most of the experiments involving cyprosulfamide showed that ABA increased its beneficial effects.

ABA is undoubtedly the hormone with the most prominent role in plant stress responses (Swamy and Smith, 1999). ABA is produced under drought, salinity and chilling/freezing stress, and plays an important role in subsequent adaptation (Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2002). Many stress-responsive genes are upregulated by ABA (Xiong et al., 2002) and ABA has been shown to increase the impact of certain bioregulators on plant growth and productivity under stress conditions (Bayer CropScience, personal communication).

3.4.4. Plant size

In our preliminary drought-stress experiments, we selected plants of the same age but one group was tall and the other short. This proved to have a significant impact on the experimental outcome, with the taller plants responding more rapidly to stress and therefore showing greater sensitivity than the shorter plants. In both experiments in which tebuconazole was shown to exhibit a positive effect (experiments 7 and 11), the plants were tall. In the experiments where no effect was observed (experiments 8, 9 and 10), the plants were shorter. The same trend was observed in the experiments utilizing fipronil (tall plants in experiment 13, shorter plants in experiments 1, 2, 4 and 5). These results emphasize once again the crucial effect of the physiological state of the plants used in such experiments.

3.4.5. Recovery

It is not clear whether the bioregulators maintained their positive effect during the recovery period. In experiments 7, 11 and 12, the effect of each compound became evident during the last few days of stress treatment, and one or two days into the recovery period. In experiments 17, 19 and 21, the effect of each compound was also observed one or two days into the recovery period, and then the plants treated with bioregulators recovered in a similar manner to the untreated controls. In experiment 24, cyprosulfamide promoted the formation of new tillers during the stress period and this persisted during recovery, but declined over the recovery period. The bioregulators certainly appear to have a more robust impact during each stress treatment, and the impact diminishes during recover. However, it is necessary to identify and characterize the molecular targets of the bioregulators, using transcriptomics and proteomics, in order to elucidate the process fully.

3.5. Conclusions

Fipronil, tebuconazole and cyprosulfamide are three synthetic bioregulators that appear to improve the growth of rice plants under drought and salinity stress conditions. This study focused on cyprosulfamide, and its potential ability to improve the performance of rice plants exposed to salinity stress. The two major impacts of cyprosulfamide were the

promotion of tiller development and early flowering. ABA appeared to act synergically with cyprosulfamide to enhance these developmental effects.

It is still unclear why plants respond inconsistently to cyprosulfamide. At least one parameter (plant size) was found to play a crucial role in the bioregulator effect. Other factors such as plant age, the stress period and intensity, the number of experimental replicates and highly-controlled growth conditions are also important and have to be optimized in order to achieve reliable results concerning bioregulators activities. The observed developmental impact of cyprosulfamide raises important questions about the underlying molecular mechanism, the relationship between cyprosulfamide and ABA, the genes activated by cyprosulfamide with and without ABA and their relationship to stress responses and adaptation. These questions can be answered by using transcriptomic and proteomic methods to identify the downstream genes, and to find out which are modulated in response to stress, bioregulators or both. These aspects are discussed in some detail in Chapter 4. Field trials are also needed to determine whether cyprosulfamide could be developed into a commercial product.

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Chapter 4

Molecular analysis of rice plants under salinity stress in the presence and absence of bioregulators

4.1. Introduction

Salinity stress has a significant impact on rice growth and productivity, principally by affecting photosynthesis and primary metabolism (Munns et al., 2008; Abbasi and Komatsu, 2004). The responses of plants to salinity and other forms of abiotic stress are mediated by growth regulators such as abscissic acid (ABA), which accumulates when plants encounter stressful environments (Zhu, 2002; Seo and Koshiba, 2002; Cowan et al., 1997). Prior treatment with ABA can improve the tolerance of rice callus towards osmotic, salinity and freezing stress by inducing a spectrum of stress-response genes (Perales et al., 2005).

As discussed in Chapter 3, synthetic bioregulators may also regulate plant growth under abiotic stress conditions, and cyprosulfamide was found to improve the growth of rice plants exposed to salinity stress. To investigate the molecular basis of cyprosulfamide activity and its interactions with endogenous growth and stress-response pathways, we compared the transcriptomes and proteomes of stressed and unstressed rice plants in the presence and absence of cyprosulfamide and/or ABA. This revealed a number of genes/proteins modulated in response to cyprosulfamide alone, to ABA alone and specifically to the combination of both chemicals. Some of these genes were modulated under normal conditions, others only under salinity stress, and many under both conditions although the degree of induction and repression varied. There was an excellent correlation between the expression of *OsLEA3* mRNA and OsLEA3 protein as determined by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) and two-dimensional gel electrophoresis (2DGE), respectively. Similarly, there was good correlation between mRNA and proteins levels for a putative fumarylacetoacetate hydrolase and a mitochondrial import inner membrane translocase. These expression profiles led to a model for the role of cyprosulfamide in the adaptation of rice plants to salinity stress.

4.2. Materials and methods

4.2.1. Sampling

Experiments were carried out in 2009 (no. 19) and 2010 (no. 24). For experiment 19, we used three replicates per treatment per time point, each comprising one plant (the same part of the plant was sampled and analyzed for each replicate). Samples were collected at three time points resulting in nine samples in total per treatment. For experiment 24, we used four replicates per treatment per time point, each comprising three plants. Samples were collected following the careful selection of the same part of the leaf for all the replicates. Samples were collected at three time points resulting in 12 samples per treatment.

4.2.2. Protein extraction

Frozen leaves were ground to a fine powder in liquid N_2. The ground samples were sent in dry ice to Fraunhofer IME (Aachen, Germany) where protein extraction, principal component analysis and proteomics were performed. For protein precipitation aliquots of the ground plant material were resuspended in 1.8 ml of ice cold acetone (100 % with 0.7 % (v/v) mercaptoethanol) and left at -20°C for at least 1 hr. This step was repeated with a centrifugation step in between (4°C, 20 min full speed, bench centrifuge). The protein pellet was then washed two times with ice cold acetone alone, left to dry at room temperature and stored at -20°C until further usage. For solubilisation of the proteins, IEF buffer (7 M urea, 2 M thiourea, 2% (w/v) chaps, 30 mM Tris-HCl (pH 8.8 at 4°C)) was added to the protein pellets. The pellet was resuspended and the protein was left to solubilise over night at room temperature. After centrifugation the supernatant was taken for quantification (2D quant kit, GE Healthcare, München, Germany). Aliquots with 50 µg and a concentration of 5 µg/µl were prepared for each individual sample. Additionally protein from one replicate of each condition was pooled to generate samples for the internal standard (50 µg) and samples for preparative gels (300 µg).

4.2.3. Proteomics

For 2D DIGE (Difference-in-gel-electrophoresis), 50 µg protein (5µg/µl) was labelled with 200 pmol of CyDye (^{TM} GE Healthcare, Freiburg

Germany). Cy2 was used to label the internal standard and Cy3 and Cy5 was used to label the individual samples to be compared. The significance criteria to classify a protein spot as differentially expressed between samples were that the spot must be present on >70% of maps with a fold change of ≥1.5 (Student's t-test, $p < 0.05$). Modulated proteins were then identified by mass spectrometry using a Micromass Q-TOF II Mass Spectrometer. These experiments were performed by colleagues at the Fraunhofer IME (Aachen, Germany) but I participated actively in the analysis (in Aachen) and interpretation of the results.

4.2.4. RNA extraction, reverse transcription and qRT-PCR

Total RNA was extracted from 10-week-old leaves using Trizol® (Invitrogen, Carlsbad, CA, USA). RNA concentrations were measured spectrophotometrically using a NanoDrop® ND-1000 (Thermo Scientific, Wilmington, DE, USA). We reverse transcribed 2 µg of each RNA sample with the Omniscript RT kit (Qiagen, Hilden, Germany) using oligo(dT) primers (Invitrogen, Carlsbad, CA, USA). PCR amplification was carried out in a 25-µl final volume comprising 12.5 µl 2× SYBR Green PCR master mix (BioRad, Hercules, CA, USA), 150 nmol of each of specific forward and reverse primers and 10 ng cDNA as the template. The specific primers for *OsLEA3*, the putative fumarylacetoacetate hydrolase and mitochondrial import translocase, and the rice actin gene as an internal control were designed according to sequences from the EMBL-EBI database (**Table 4.1**). Quantitative real-time PCR was carried out using a Bio-Rad CFX96 sequence detector system (Foster City, California, USA). The amplification program comprised a denaturation step at 95˚C for 3 min followed by 40 cycles of 95˚C for 10 s, 59˚C for 30 s and 72˚C for 20 s. The amplification products were validated by melting curve analysis. The standard curve had an efficiency of 85–100%. Two negative controls were included: (a) no reverse transcriptase in the reaction mixture; and (b) no template in the reaction mixture (water instead of cDNA). As expected, the negative controls did not yield specific amplification fragments.

Name of primer	5´ to 3´ sequence
LEA-qRT-forw	TAGACGCCGTGAATGATTTCC
LEA-qRT-rev	TCACCCACACCCGTCAGAA
Fumar-qRT-forw	AACTGGCACACTAAGCGGACC
Fumar-qRT-rev	TGCAGGCAGAACCTTCCCAG
Mitoc-qRT-forw	GATCGTTCCCGGATCTCAACG
Mitoc-qRT-rev	CCACCACGCTGTTCTTCCA
Actin-qRT-forw	GACTCTGGTGATGGTGTCAGC
Actin-qRT-rev	TCATGTCCCTCACAATTTCC

Table 4.1. The list of primers used for real time PCR analysis.

4.2.5. Real-time RT-PCR statistical analysis

The normalized expression of *OsLEA3* was calculated using CFX Manager Software (BioRad, Hercules, CA, USA) in accordance with the model of Pfaffl (2001). The results of four replicates for each time point were averaged, and the data were analyzed by two-way analysis of variance (ANOVA) followed by a Student's t-test using the residual mean square in the ANOVA as the estimate of variability.

4.3. Results

4.3.1. Identification of proteins induced by stress and/or chemical treatments

Proteins were extracted from the leaves of rice plants from all the different treatment groups. To confirm that the different samples could be clustered into their corresponding groups, we carried out a principal component analysis which showed that clustering was better on day11, when clear separation between salinity stress samples and unstressed samples was most evident (**Figure 4.1**). Protein samples were separated by 2DGE and stained with Coomassie brilliant blue to evaluate the expression levels, resulting in the detection of 134 spots (**Figure 4.2**), 50 of which could be identified by

mass spectrometry. Comparative analysis was carried out to identify proteins modulated by stress, the chemical treatments or combinations of these factors.

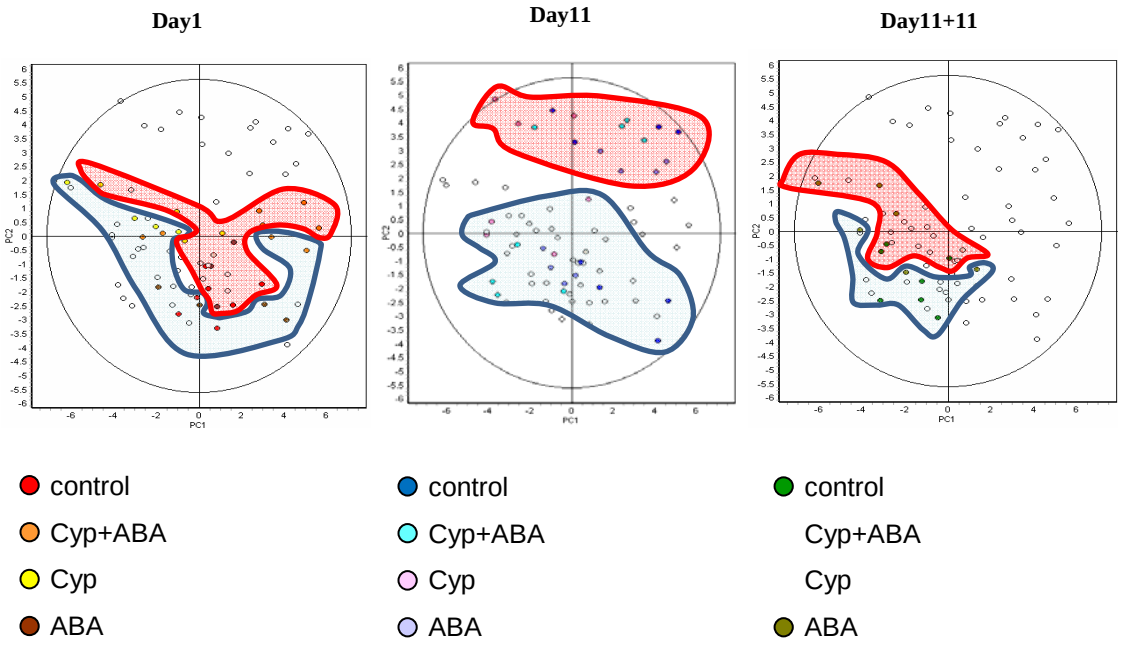

Figure 4.1. Principal component analysis of data from experiment 24 (2010) on days 1, 11 and 11+11. Blue field = unstressed plants, red field = stressed plants. Cyp = cyprosulfamide treatment. On day 11+11, the cyp and cyp + ABA data are missing because of technical problems during protein sample preparation.

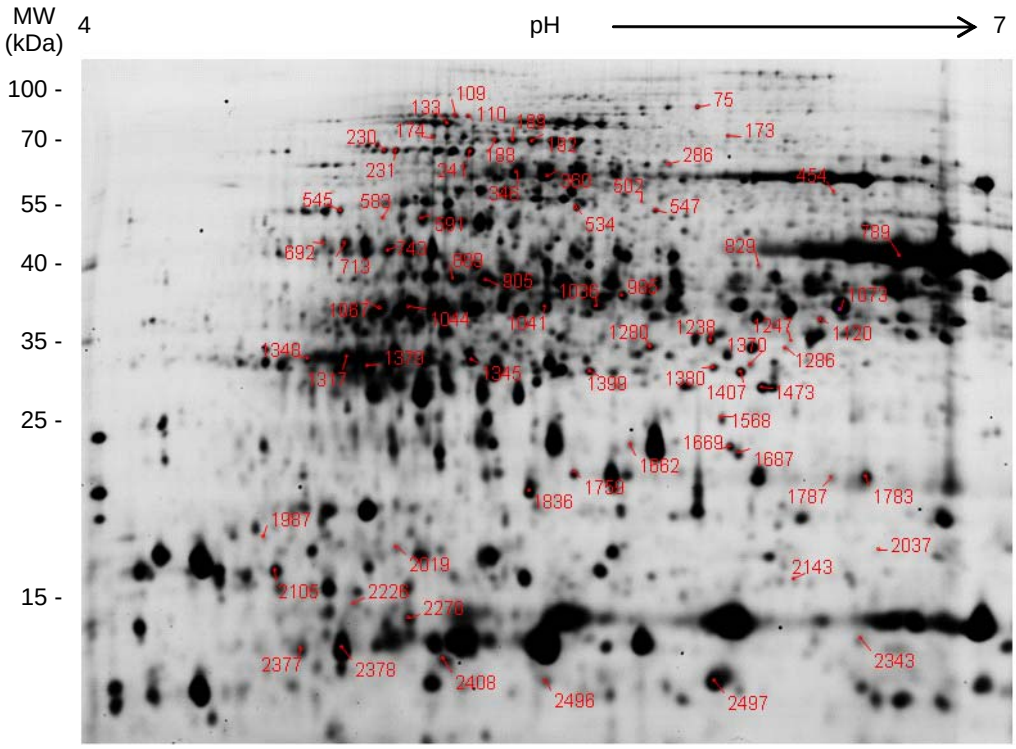

Figure 4.2. 2DGE of proteins from wild type rice plants subjected to salinity stress. Protein spots of interest that changed their expression due to salinity stress are marked with their respective spot numbers.

4.3.1.1. Proteins modulated by salinity stress

We identified 40 proteins with a ≥1.5-fold difference in expression between control rice plants and those subjected to salinity stress ($p < 0.05$). These proteins are listed along with the average expression ratios on days 1, 11 and 11+11 in **Table 4.2**. The 40 modulated proteins include 28 induced by stress and 12 that are repressed. There was strong correlation between the results from experiments 19 (2009) and 24 (2010). Most of the changes in expression were detected on the last day of stress treatment, and 34 of the 40 modulated proteins showed the same expression profile in both experiments. Only three proteins were already modulated on the first day of stress treatment (all three were induced by stress), and 17 proteins were differentially expressed in the recovery period (16 were induced, one repressed). One protein - dehydroascorbate reductase was upregulated on both day 1 and day 11 in both experiments. Two proteins – the DnaK-type molecular chaperone Bip (spot 109) and the 70-kDa heat shock protein – were upregulated on both day 11 and day 11+11 in both experiments.

		day1 (2010)		day15 (2009)		day11 (2010)		day15+10 (2009)		day11+11 (2010)	
Spot #	Upregulated proteins	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test
829	Aspartate aminotransferase	1,35	0,034	**2,25**	0,003	**1,85**	0,001	1,28	0,388	1,21	0,065
454	ATP synthase subunit beta, chloroplastic	1,05	0,469	**2,15**	0,001	**2,26**	0,004	**1,59**	0,030	1,27	0,170
534	ATP synthase subunit beta, chloroplastic	1,24	0,132	**2,51**	0,000	**2,09**	0,008	1,38	0,164	1,41	0,001
2343	Barwin, putative, expressed (chitinase activity)	1,1	0,792	n.i.	n.i.	**3,35**	0,014	n.i.	n.i.	1,2	0,2
1783	Carbonic anhydrase	1,02	0,778	1,05	0,977	**2,07**	0,001	-1,07	0,666	**1,5**	0,004
1787	Carbonic anhydrase	-1,07	0,745	1,32	0,692	1,1	0,360	-1,13	0,542	**1,63**	0,029
1473	Dehydroascorbate reductase	**1,57**	0,037	**3,14**	0,007	**2,6**	0,001	**1,84**	0,016	1,38	0,069
109	Dnak-type molecular chaperone Bip	1,1	0,109	**2,23**	0,000	**2,34**	0,000	**1,73**	0,014	**1,66**	0,002
110	Dnak-type molecular chaperone Bip	1,19	0,109	**3,36**	0,000	**2,86**	0,000	1,5	0,179	**1,64**	0,0042
75	Elongation factor 2	1,3	0,141	**2,11**	0,001	**2,31**	0,024	1,51	0,187	**1,73**	0,001
985	Enolase	1,21	0,052	**1,87**	0,008	**1,97**	0,004	1,37	0,090	**1,51**	0,0049
545	Fructose-1,6-bisphosphatase, chloroplastic	1,18	0,044	**2,53**	0,020	**2,42**	0,000	1,12	0,218	1,11	0,399
133	Heat shock cognate 70 kDa protein, putative, expressed	1,37	0,085	**3,1**	0,001	**2,67**	0,001	**1,64**	0,046	**2,03**	0,001
1041	Lactoylglutathione lyase	1,22	0,092	**1,99**	0,001	**2,36**	0,000	1,35	0,005	1,45	0,005
1399	L-ascorbate peroxidase 1, cytosolic	1,11	0,298	**2,25**	0,001	**2,11**	0,001	1,44	0,074	1,39	0,014
1669	Late embryogenesis abundant protein, group 3	**1,55**	0,013	n.i.	n.i.	**13,3**	0,008	n.i.	n.i.	1,1	0,873
2143	Malate dehydrogenase	1,38	0,110	1,63	0,244	**1,8**	0,004	1,01	0,866	1,26	0,149
173	Malic enzyme	-1,01	n.i.	**2,68**	0,003	**2,51**	0,005	1,1	0,652	1,32	0,144
2037	Mitochondrial import inner membrane translocase subunit Tim1	1,49	0,025	14,03	n.i.	**3,44**	0,006	n.i.	n.i.	1,34	0,158
1662	Probable photosystem II oxygen-evolving complex protein 2	1,39	0,421	**4,43**	0,009	**3,28**	0,001	**2,56**	0,020	-1,24	0,336
231	Protein disulfide isomerase	1,15	0,206	**2,52**	0,000	**1,85**	0,001	1,78	0,058	1,37	0,016
2019	Putative 33kDa oxygen evolving protein of photosystem II (frag	1,28	0,039	**2,78**	0,000	**1,87**	0,001	**1,94**	0,009	1,25	0,111
1238	putative chitinase	1,07	0,715	**2,76**	0,005	**3,75**	0,013	1,63	0,120	**2,41**	0,005
502	Putative fumarylacetoacetate hydrolase	**1,62**	0,008	**3,61**	0,000	**2,84**	0,006	1,82	0,198	1,17	0,156
286	Putative selenium binding protein	1,37	0,147	**2,43**	0,002	**1,9**	0,014	**1,84**	0,041	1,44	0,021
1687	Ribosome-recycling factor, chloroplastic	1,28	0,065	**1,9**	0,000	**2,24**	0,012	1,69	0,477	1,12	0,208
1247	Ribulose bisphosphate carboxylase large chain; (fragment)	1,22	0,252	**1,69**	0,014	**2,62**	0,003	-1,13	0,564	**1,83**	0,013
1073	Ribulose bisphosphate carboxylase large chain; (fragment)	-1,08	0,593	1,21	0,817	**2,52**	0,018	1,01	0,928	1,1	0,44
241	RuBisCo subunit binding-protein beta subunit (60 kDa chaperon	1,37	0,024	**2,53**	0,001	**2,22**	0,000	1,44	0,146	**1,56**	0,006
1568	Superoxide dismutase [Mn], mitochondrial	1,2	0,075	**2,35**	0,000	**2,06**	0,003	1,25	0,123	1,28	0,096
2270	Thioredoxin H-type	1,09	0,223	**2,92**	0,000	**2,32**	0,000	1,65	0,094	1,12	0,168
547	Xylose isomerase	1,3	0,054	**2,56**	0,005	**1,93**	0,002	**1,76**	0,037	1,22	0,235
	Downregulated proteins	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test
889	70 kDa heat shock protein (fragment)	-1,24	0,053	**-1,99**	0,008	**-2,23**	0,001	-1,11	0,591	-1,25	0,050
346	ATP synthase subunit beta, chloroplastic	-1,08	0,470	-1,66	0,213	**-3,38**	0,001	-1,34	0,174	**-1,52**	0,037
360	ATP synthase subunit beta, chloroplastic	1,03	0,655	-1,33	0,362	**-2,04**	0,008	-1,24	0,101	-1,21	0,27
188	Cell division protease ftsH homolog 2, chloroplastic	-1,15	0,140	**-2,32**	0,001	**-2,09**	0,000	-1,24	0,237	0,14	0,020
174	Cell division protease ftsH homolog 2, chloroplastic	1,01	0,884	**-1,95**	0,012	**-2,28**	0,001	-1,08	0,570	1,04	0,49
1317	Chlorophyll A-B binding protein, expressed	-1,24	0,102	**-1,98**	0,017	**-2,23**	0,006	-1,19	0,574	-1,18	0,394
1348	Chlorophyll A-B binding protein, expressed	-1,03	0,711	-1,5	0,087	**-2,16**	0,007	-1,08	0,624	-1,12	0,3
905	Fructose-bisphosphate aldolase	-1,18	0,015	**-2,3**	0,001	**-2,5**	0,000	-1,18	0,408	-1,19	0,113
591	Glutamine synthetase, chloroplastic	-1,13	0,035	**-2,18**	0,005	**-2,44**	0,000	-1,2	0,289	-1,3	0,016
713	Os04g0234600 protein (FBPase class 1 family)	-1,06	0,328	**-1,96**	0,016	**-2,64**	0,000	-1,23	0,195	-1,2	0,042
692	Os04g0234600 protein (FBPase class 1 family)	-1,07	0,658	**-1,98**	0,040	**-2,07**	0,004	-1,29	0,190	-1,21	0,21
1036	Os08g0292600 protein(isomerase, rotamase)	-1,08	0,137	**-2,38**	0,024	**-2,03**	0,001	-1,4	0,177	1,18	0,156
743	Phosphoribulokinase	1,01	0,834	-1,68	0,068	**-2,12**	0,002	-1,17	0,250	-1,24	0,017
1067	Putative 33kDa oxygen evolving protein of photosystem II	-1,19	0,048	**-2,45**	0,011	**-2,59**	0,000	-1,23	0,339	-1,35	0,034
1044	Putative 33kDa oxygen evolving protein of photosystem II	-1,06	0,155	-1,69	0,184	**-2,05**	0,000	-1,2	0,309	-1,32	0,027
189	Putative transketolase	-1,2	0,069	**-2,09**	0,025	**-2,55**	0,000	-1,15	0,435	-1,22	0,094
192	Putative transketolase (shortened sequence from Put transketolas	-1,08	0,244	**-1,97**	0,015	**-2,07**	0,000	-1,19	0,218	-1,21	0,140
789	Ribulose bisphosphate carboxylase large chain	-1,21	0,174	**-3,23**	0,004	**-2,18**	0,001	-1,15	0,608	-1,42	0,012
583	Ribulose bisphosphate carboxylase/oxygenase activase, chlorop	-1,03	0,766	-1,27	0,562	**-2,43**	0,005	-1	0,171	-1,07	0,585
1407	Ribulose-phosphate 3-epimerase, chloroplastic	1,16	0,097	-1,83	0,068	**-2**	0,005	-1,17	0,206	-1,09	0,310
2378	Thioredoxin X, chloroplastic	-1,06	0,198	**-2,8**	0,004	**-2,18**	0,000	-1,41	0,088	-1,1	0,068
2377	Thioredoxin X, chloroplastic	-1,17	0,218	**-2,38**	0,046	**-2,95**	0,000	-1,2	0,350	-1,27	0,025

Table 4.2. Proteins modulated by the exposure of rice plants to 100 mM of NaCl. Data from day 1 in experiment 19 (2009) are not shown because there were no statistically significant ≥1.5-fold changes in expression ($p < 0.05$), which in the other experiments are represented by blue boxes.

4.3.1.2. Proteins modulated by treatment with cyprosulfamide alone

We identified 30 proteins (22 induced and 8 repressed) that were modulated by treatment with cyprosulfamide alone (**Table 4.3**). This included 26 proteins whose expression was already modulated on day 1, ten of which were still modulated on day 11. Nine proteins were upregulated only on day 11. Interestingly, 16 of the modulated proteins were not modulated by stress, whereas the other 14 were also part of the stress-induced protein group, already indicating some overlap between the cyprosulfamide-response and stress-response pathways. Interestingly, some of the proteins that were upregulated on day 1 by cyprosulfamide treatment in the absence of stress were also found to be upregulated by stress in the absence of cyprosulfamide on day 11 [aspartate aminotransferase, chloroplastic ATP synthase subunit beta (spot 534), dehydroascorbate reductase, lactoylglutathione lyase, OsLEA3, malate dehydrogenase, mitochondrial import inner membrane translocase, putative 33kDa oxygen evolving protein of photosystem II, probable photosystem II oxygen-evolving complex protein 2, RuBisCO subunit binding-protein beta subunit and thioredoxin H-type]. All the proteins downregulated by cyprosulfamide without stress on day 1 were also repressed by stress in the absence of chemicals on day 11. These data suggests that cyprosulfamide has the ability to potentiate the plant's normal stress responses.

		day1(2010) unst		day1(2010) st		day11(2010) unst		day11(2010) st	
Spot #	Upregulated proteins	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test
829	Aspartate aminotransferase	**1,82**	0,002	1,39	0,810	1,36	0,069	1,46	0,046
1759	ATP synthase subunit alpha, chloroplastic (fragment)	**2,43**	0,041	2,2	0,140	**3,94**	0,029	2,25	0,160
534	ATP synthase subunit beta	**1,64**	0,009	1,43	0,001	1,46	0,130	-1,04	0,400
1280	ATP synthase subunit beta, chloroplastic (fragment)	1,46	0,042	1,43	0,080	**2**	0,037	1,64	0,063
1345	ATP synthase subunit beta, chloroplastic (fragment)	**2,28**	0,017	1,85	0,100	**2,77**	0,026	1,99	0,120
1370	ATP synthase subunit beta, chloroplastic (fragment)	**3,21**	0,024	2,13	0,140	**2,94**	0,026	2,12	0,190
2343	Barwin, putative, expressed (chitinase activity)	1,68	0,107	**2,29**	0,018	1,43	0,230	1,5	0,205
188	Cell division protease ftsH homolog 2, chloroplastic	-1	0,961	-1,01	0,937	1,03	0,733	**1,6**	0,008
1379	Chlorophyll A-B binding protein, expressed	1,71	0,083	1,43	0,110	**1,74**	0,039	1,31	0,210
1473	Dehydroascorbate reductase	**2,05**	0,003	1,42	0,007	1,19	0,260	-1,19	0,202
110	Dnak-type molecular chaperone Bip	1,49	0,002	**1,52**	0,002	1,29	0,195	-1,21	0,329
1120	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	**2,14**	0,007	**2,13**	0,006	**2**	0,026	1,74	0,090
133	Heat shock cognate 70 kDa protein	**1,73**	0,005	1,53	0,055	1,2	0,520	-1,45	0,086
1041	Lactoylglutathione lyase	**1,55**	0,001	1,38	0,006	1,19	0,260	-1,06	0,290
1987	L-ascorbate peroxidase 2, cytosolic (fragment)	1,29	0,026	1,16	0,400	1,39	0,120	**1,56**	0,002
1669	Late embryogenesis abundant protein, group 3	**8**	0,000	**7,37**	0,000	1,91	0,182	1,41	0,129
2143	Malate dehydrogenase	**2,01**	0,003	1,56	0,052	**1,6**	0,004	1,31	0,090
2037	Mitochondrial import inner membrane translocase subunit Tim	**4,45**	0,000	**3,65**	0,000	**1,77**	0,038	1,33	0,231
1662	Probable photosystem II oxygen-evolving complex protein 2	**2,45**	0,003	**2,1**	0,033	**1,74**	0,038	1,26	0,206
1286	Protochlorophyllide reductase B, chloroplastic	1,08	0,674	-1	0,921	**1,8**	0,008	1,01	0,842
2019	Putative 33kDa oxygen evolving protein of photosystem II	**1,83**	0,004	1,53	0,079	2,72	0,076	**1,61**	0,005
1380	Putative aminotransferase (fragment)	1,72	0,070	1,57	0,190	**2,7**	0,036	1,74	0,210
8408	Ribulose bisphosphate carboxylase large chain	2,46	0,061	1,95	0,192	**3,18**	0,015	2,4	0,118
1073	Ribulose bisphosphate carboxylase large chain; (fragment)	1,49	0,038	1,25	0,120	**2,04**	0,008	1,47	0,210
2105	Ribulose bisphosphate carboxylase large chain; (fragment)	1,05	0,720	-1,03	0,770	**2,15**	0,031	1,58	0,140
2497	Ribulose bisphosphate carboxylase small chain A, chloroplastic	**1,7**	0,046	1,69	0,152	**2,45**	0,023	2,18	0,075
2496	Ribulose bisphosphate carboxylase small chain A, chloroplastic	1,05	0,750	**1,78**	0,036	2,32	0,063	**2,39**	0,039
2226	Ribulose bisphosphate carboxylase small chain, chloroplastic	n.i.	n.i.	**1,54**	0,003	**1,92**	0,001	1,55	0,123
241	RuBisCo subunit binding-protein beta subunit	**1,61**	0,008	1,26	0,017	1,39	0,064	-1,06	0,160
2270	Thioredoxin H-type	**1,67**	0,000	**1,51**	0,000	1,13	0,086	1,05	0,497
	Downregulated proteins	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test
889	70 kDa heat shock protein (fragment)	**-1,83**	0,002	-1,48	0,007	**-1,51**	0,048	-1,32	0,001
346	ATP synthase subunit beta, chloroplastic	**-2,36**	0,023	**-1,8**	0,037	**-2,11**	0,002	-1,36	0,300
360	ATP synthase subunit beta, chloroplastic	-1,96	0,056	-1,59	0,063	**-1,87**	0,005	-1,47	0,250
1783	Carbonic anhydrase	1,3	0,341	-1,22	0,205	1,31	0,203	**-1,81**	0,001
1787	Carbonic anhydrase	-1,25	0,230	-1,45	0,049	1,15	0,720	**-2,02**	0,002
1348	Chlorophyll A-B binding protein, expressed	-1,39	0,002	**-1,55**	0,001	-1,5	0,063	-1,45	0,130
905	Fructose-bisphosphate aldolase	**-1,75**	0,001	-1,46	0,005	-1,38	0,045	-1,21	0,036
591	Glutamine synthetase, chloroplastic	**-1,61**	0,001	**-1,5**	0,004	-1,4	0,015	-1,38	0,030
713	Os04g0234600 protein (FBPase class 1 family)	**-1,69**	0,007	**-1,54**	0,023	-1,47	0,005	-1,21	0,270
743	Phosphoribulokinase	-1,49	0,010	-1,41	0,017	**-1,59**	0,011	-1,29	0,128
1067	Putative 33kDa oxygen evolving protein of photosystem II	**-1,78**	0,001	**-1,57**	0,001	-1,27	0,165	-1,05	0,671
189	Putative transketolase	**-1,89**	0,007	-1,38	0,042	-1,48	0,012	-1,11	0,230
192	Putative transketolase	**-1,57**	0,029	-1,25	0,110	-1,34	0,038	-1,15	0,110
789	Ribulose bisphosphate carboxylase large chain	**-2,14**	0,016	**-1,62**	0,042	**-1,63**	0,014	**-1,56**	0,021
583	Ribulose bisphosphate carboxylase/oxygenase activase, chloro	**-2,04**	0,003	**-1,66**	0,003	**-2,18**	0,009	-1,16	0,400

Table 4.3. Proteins modulated by the exposure of rice plants to cyprosulfamide. Only data from experiment 24 (2010) are shown. Data from recovery day are not shown because of technical difficulties with sample preparation. Statistically significant ≥1.5-fold changes in expression ($p < 0.05$) are represented by blue boxes.

4.3.1.3. Proteins modulated by treatment with ABA alone

Nineteen proteins were modulated by ABA treatment alone, thirteen of which were induced and six repressed (**Table 4.4**). Most of the changes were detected on day 11 and day 11+11 while only two proteins [chlorophyll A-B binding protein (spot 1379) and

putative fumarylacetoacetate hydrolase] were upregulated on day 1. Six of the ABA-modulated proteins in the plants that were not subjected to stress were also modulated in the same way by stress in the absence of ABA during the same time course [carbonic anhydrase (spots 1783, 1787), malate dehydrogenase (spot 2143), putative 33kDa oxygen evolving protein of photosystem II (spot 2019), putative chitinase, FAH and ribulose bisphosphate carboxylase/oxygenase activase].

		day1(2010) unst		day1(2010) st		day11(2010) unst		day11(2010) st		day11+11(2010) unst		day11+11(2010) st	
Spot #	Upregulated proteins	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test
829	Aspartate aminotransferase	-1,15	0,280	1,11	0,560	1,24	0,160	1,03	0,730	1,51	0,120	**1,51**	0,006
346	ATP synthase subunit beta, chloroplastic	1,09	0,720	-1,1	0,500	-1,45	0,034	**1,65**	0,036	-1,37	0,150	-1,62	0,160
1345	ATP synthase subunit beta, chloroplastic (fragment)	-1,04	0,700	1,19	0,390	**1,79**	0,060	-1,16	0,860	1,09	0,940	1,25	0,380
1370	ATP synthase subunit beta, chloroplastic (fragment)	1,14	0,920	1,1	0,660	**1,64**	0,043	-1,75	0,320	1,09	0,950	1,55	0,270
1783	Carbonic anhydrase	-1,21	0,370	1,06	0,850	1,28	0,190	**-1,52**	0,048	**1,58**	0,000	1,03	0,820
1787	Carbonic anhydrase	-1,22	0,210	1	0,950	1,2	0,330	-1,3	0,120	**1,98**	0,010	-1,14	0,480
1379	Chlorophyll A-B binding protein, expressed	-1,01	0,980	**1,56**	0,042	1,16	0,400	-1,1	0,730	1,06	0,940	1,09	0,620
985	Enolase	1,03	0,620	1,09	0,540	1,15	0,380	-1,17	0,042	**1,55**	0,003	1,36	0,018
2143	Malate dehydrogenase (fragment)	1,49	0,130	-1,05	0,780	**1,61**	0,027	-1,21	0,200	1,29	0,190	1,31	0,077
230	Protein disulfide isomerase 1-1	-1,01	0,970	-1,11	0,110	1,07	0,660	1,08	0,420	**1,6**	0,003	-1,12	0,190
231	Protein disulfide isomerase 1-1	1,02	0,790	-1,04	0,680	1,11	0,520	1,02	0,730	**1,62**	0,002	1,06	0,560
2019	Putative 33kDa oxygen evolving protein of photosystem II (fr	1,1	0,650	1,23	0,650	**1,61**	0,003	1,01	0,920	1,14	0,470	**1,79**	0,031
1238	putative chitinase	-1,22	0,540	1,79	0,280	1,26	0,400	-1,93	0,074	**3,29**	0,004	-1,47	0,190
502	Putative fumarylacetoacetate hydrolase	**1,58**	0,027	-1,05	0,680	-1,06	0,840	-1,5	0,160	1,32	0,040	1,2	0,100
2408	Ribulose bisphosphate carboxylase large chain (fragment)	1,03	0,900	1,14	0,510	**1,8**	0,040	-1,88	0,200	1,1	0,920	1,37	0,440
1247	Ribulose bisphosphate carboxylase large chain; (fragment)	1,06	0,720	1,16	0,790	1,25	0,340	**-1,75**	0,042	**2,34**	0,012	-1,29	0,190
2496	Ribulose bisphosphate carboxylase small chain A, chloroplast	-1,47	0,230	-1,24	0,360	**2,12**	0,011	1,09	0,600	-1,09	0,720	1,35	0,360
2497	Ribulose bisphosphate carboxylase small chain A, chloroplast	-1,3	0,330	-1,14	0,570	**2,11**	0,010	-1,41	0,540	-1	0,810	1,48	0,170
2226	Ribulose bisphosphate carboxylase small chain, chloroplastic	-1,17	0,320	1,01	0,960	**1,87**	0,003	-1,03	0,970	1,07	0,680	1,09	0,340
1407	Ribulose-phosphate 3-epimerase, chloroplastic	1,33	0,093	-1,06	0,470	-1,35	0,011	**1,53**	0,042	-1,07	0,560	-1,26	0,210
	Downregulated proteins	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test
889	70 kDa heat shock protein (fragment)	-1,06	0,570	-1,14	0,280	-1,2	0,320	1,22	0,010	-1,35	0,033	**-1,58**	0,006
1669	OsLEA3	1,5	0,050	1,05	0,740	-1,3	0,880	**-2,26**	0,048	1,42	0,320	2,44	0,150
713	Os04g0234600 protein (FBPase class 1 family)	1	0,960	-1,04	0,610	-1,31	0,009	1,49	0,008	-1,18	0,130	**-1,77**	0,030
1036	Os08g0292600 protein(Peptidyl-prolyl cis-trans isomerase fan	1,07	0,690	1,07	0,790	-1,31	0,087	1,18	0,008	-1	0,960	**-1,57**	0,024
789	Ribulose bisphosphate carboxylase large chain	1,11	0,460	-1,02	0,760	-1,31	0,096	1,06	0,510	-1,17	0,280	**-1,73**	0,033
583	Ribulose bisphosphate carboxylase/oxygenase activase	1,22	0,630	1,01	0,960	**-2,07**	0,016	**1,52**	0,012	-1,25	0,210	-1,68	0,062

Table 4.4. Proteins modulated by the exposure of rice plants to ABA. Only data from experiment 24 (2010) are shown. Data from day1 and day11 stress are not shown because of technical difficulties with sample preparation. Statistically significant ≥1.5-fold changes in expression ($p < 0.05$) are represented by blue boxes.

4.3.1.4. Proteins modulated by the application of cyprosulfamide plus ABA

We identified 34 proteins that were modulated by the co-application of both cyprosulfamide and ABA, 26 of which were induced and 8 repressed (**Table 4.5**). None of the proteins were modulated in the same mode at both stress time-points but three proteins were modulated in the opposite mode on day 11 and on day 11. Putative selenium binding protein was upregulated on day 1 but downregulated on day 11, while ribulose bisphosphate carboxylase large chain (spots 2408 and 2105) and small chain (spots 2496 and 2497) were repressed on day 1 but upregulated on day 11. Seventeen proteins were modulated on day 1 in the absence and presence of stress and eight were modulated on day 1 just in the presence of stress. Twelve proteins were modulated only

on day 11. Sixteen proteins that were modulated by cyprosulfamide plus ABA on day 1 independently of stress were also found to be modulated by stress on day 11. Thirteen of these proteins were upregulated [ATP synthase subunit beta (spot 534), dehydroascorbate reductase, dnak-type molecular chaperone Bip (spot 110), elongation factor 2, enolase, heat shock cognate 70 kDa protein, lactoylglutathione lyase, mitochondrial import inner membrane translocase subunit, probable photosystem II oxygen-evolving complex protein 2, putative fumarylacetoacetate hydrolase, putative selenium binding protein, ribosome-recycling factor and RuBisCO subunit binding-protein beta subunit] and three downregulated (putative 33kDa oxygen evolving protein of photosystem II, putative transketolase 1 and chloroplastic thioredoxin X). Five proteins were upregulated due to cyprosulfamide plus ABA under stress on day 1 and also were upregulated due to stress on day 11 (putative barwin protein, malic enzyme, protein disulfide isomerase 1-1, mitochondrial superoxide dismutase [Mn] and xylose isomerase). Five proteins showed identical modulation profiles in response to cyprosulfamide and cyprosulfamide plus ABA [putative barwin protein, chlorophyll A-B binding protein (spot 1379), OsLEA3, putative aminotransferase and putative 33kDa oxygen evolving protein of photosystem II], whereas only two proteins were modulated in the same manner by ABA and by cyprosulfamide plus ABA [ATP synthase subunit beta (spot 1345) and ribulose bisphosphate carboxylase small chain (spot 2226)].

		day1(2010) unst		day1(2010) st		day11(2010) unst	
Spot #	**Upregulated proteins**	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test
1759	ATP synthase subunit alpha, chloroplastic (fragment)	1,03	0,710	-2,17	0,097	**4,3**	0,017
534	ATP synthase subunit beta	**1,67**	0,006	**1,56**	0,001	-1,07	0,914
1370	ATP synthase subunit beta, chloroplastic (fragment)	1,26	0,749	-1,51	0,082	**2,82**	0,006
1280	ATP synthase subunit beta, chloroplastic (fragment)	-1,06	0,670	-1,49	0,018	**2,25**	0,010
1345	ATP synthase subunit beta, chloroplastic (fragment)	1,17	0,980	-1,44	0,100	**2,68**	0,010
2343	Barwin, putative, expressed (chitinase activity)	1,5	0,209	**3,04**	0,002	1,29	0,531
1317	Chlorophyll A-B binding protein, expressed	1,01	0,990	1,14	0,220	**1,53**	0,023
1379	Chlorophyll A-B binding protein, expressed	1,33	0,456	-1,02	0,761	**1,91**	0,015
1473	Dehydroascorbate reductase	**1,76**	0,007	**1,65**	0,001	-1,2	0,389
110	Dnak-type molecular chaperone Bip	**1,58**	0,014	**1,79**	0,000	-1,16	0,120
109	Dnak-type molecular chaperone Bip	1,48	0,009	**1,62**	0,000	-1,02	0,820
75	Elongation factor 2	**1,8**	0,002	**1,61**	0,019	1,09	0,560
985	Enolase	**1,58**	0,000	**1,5**	0,000	-1,04	0,934
133	Heat shock cognate 70 kDa protein, putative, expressed	**2,16**	0,012	**2,36**	0,000	-1,33	0,159
1041	Lactoylglutathione lyase	**1,52**	0,001	**1,55**	0,001	-1,18	0,326
1669	Late embryogenesis abundant protein, group 3	**10**	0,000	**9,22**	0,000	-1,08	0,859
173	Malic enzyme	1,41	n.i.	**1,91**	0,000	-1,14	0,515
2037	Mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family p	**4,17**	0,001	**3,89**	0,000	1,18	0,533
1662	Probable photosystem II oxygen-evolving complex protein 2	**3,08**	0,003	**2,74**	0,005	1,2	0,302
231	Protein disulfide isomerase 1-1	1,37	0,029	**1,52**	0,000	-1,06	0,690
1286	Protochlorophyllide reductase B, chloroplastic	-1,16	0,384	-1,44	0,068	**1,96**	0,007
1380	Putative aminotransferase (fragment)	1,09	0,903	-1,5	0,065	**2,88**	0,015
502	Putative fumarylacetoacetate hydrolase	**2,38**	0,007	**2,36**	0,003	-1,05	0,876
286	Putative selenium binding protein	**1,83**	0,031	**1,69**	0,020	**-1,58**	0,018
1687	Ribosome-recycling factor, chloroplastic	**1,92**	0,003	**1,59**	0,005	1,1	0,585
1073	Ribulose bisphosphate carboxylase large chain	1,19	0,424	-1,04	0,736	**1,77**	0,045
2408	Ribulose bisphosphate carboxylase large chain (fragment)	-1,03	0,710	**-1,76**	0,043	**2,59**	0,023
2105	Ribulose bisphosphate carboxylase large chain; (fragment)	1,08	0,950	**-1,73**	0,005	**2,49**	0,007
2497	Ribulose bisphosphate carboxylase small chain A, chloroplastic	-1,12	0,537	**-1,84**	0,037	**2,62**	0,004
2496	Ribulose bisphosphate carboxylase small chain A, chloroplastic	-1,11	0,630	**-1,73**	0,035	**2,86**	0,030
2226	Ribulose bisphosphate carboxylase small chain, chloroplastic	1,07	0,698	-1,13	0,390	**1,58**	0,007
241	RuBisCo subunit binding-protein beta subunit (60 kDa chaperonin subunit beta)	**1,81**	0,003	**1,73**	0,000	-1,06	0,647
1568	Superoxide dismutase [Mn], mitochondrial	1,48	0,220	**1,72**	0,000	-1,26	0,320
2270	Thioredoxin H-type	1,47	0,002	**1,68**	0,000	1,05	0,380
1836	Triosephosphate isomerase	1,31	0,373	**1,75**	0,017	-1,31	0,122
547	Xylose isomerase	1,57	0,065	**1,67**	0,000	-1,4	0,061
	Downregulated proteins	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test
889	70 kDa heat shock protein (fragment)	**-1,54**	0,008	-1,35	0,010	-1,04	0,870
346	ATP synthase subunit beta, chloroplastic	-1,43	0,100	-1,26	0,071	**-1,68**	0,015
1787	Carbonic anhydrase	-1,21	0,440	**-1,69**	0,007	1,17	0,190
1067	Putative 33kDa oxygen evolving protein of photosystem II	**-1,75**	0,002	**-1,73**	0,000	1,05	0,762
189	Putative transketolase 1	**-1,72**	0,004	**-1,5**	0,001	-1,22	0,110
789	Ribulose bisphosphate carboxylase large chain, putative, expressed (fragment)	**-1,52**	0,039	-1,44	0,005	-1,24	0,130
1407	Ribulose-phosphate 3-epimerase, chloroplastic	1,26	0,250	1,21	0,062	**-1,5**	0,006
2377	Thioredoxin X, chloroplastic	**-1,85**	0,004	**-1,53**	0,010	-1,02	0,910

Table 4.5. Proteins modulated by the exposure of rice plants to cyprosulfamide plus ABA. Only data from experiment 24 (2010) are shown. Data from the recovery period are not shown because of technical difficulties with sample preparation. Data from day 11 in stressed plants are not shown because there were no statistically significant ≥1.5-fold changes in expression ($p < 0.05$), which in the other experiments are represented by blue boxes.

4.3.1.5. Comparison of proteins induced by salinity stress, cyprosulfamide, ABA and cyprosulfamide plus ABA

A total of 50 proteins were modulated by the different treatments, and five of these proteins [carbonic anhydrase (spot 1787), OsLEA3, 70 kDa heat shock protein, ATP synthase subunit beta (spot 346) and ribulose bisphosphate carboxylase large chain (spot 789)] were represented in all four treatment groups (**Table 4.6**). Twenty seven

proteins were modulated in response to three of the four treatments. Twelve of these were modulated by salinity stress, cyprosulfamide and cyprosulfamide plus ABA [ATP synthase subunit beta (spot 534), putative barwin protein, dehydroascorbate reductase, dnak-type molecular chaperone Bip (spot 110), heat shock cognate 70 kDa protein, lactoylglutathione lyase, mitochondrial import inner membrane translocase subunit, probable photosystem II oxygen-evolving complex protein 2, RuBisCO subunit binding-protein beta subunit, thioredoxin H-type, all upregulated, and a putative 33k-Da photosystem II oxygen-evolving protein (spot 1067) and putative transketolase (spot 189), both downregulated]. Six proteins were modulated by salinity stress, cyprosulfamide and by ABA [aspartate aminotransferase, carbonic anhydrase (1783), malate dehydrogenase, putative 33kDa oxygen evolving protein of photosystem II (spot 2019), all upregulated, and Os04g0234600 protein (FBPase class 1 family, spot 713) and ribulose bisphosphate carboxylase/oxygenase activase, both downregulated]. Four proteins were modulated by cyprosulfamide, ABA and by cyprosulfamide plus ABA [ATP synthase subunit beta (spots 1345, 1370), chlorophyll A-B binding protein (spot 1379), ribulose bisphosphate carboxylase large chain (spot 2408) and small chain (spots 2496, 2497, 2226), all upregulated]. Four proteins were modulated by salt stress, ABA and cyprosulfamide plus ABA [enolase, protein disulfide isomerase 1-1 (spot 231), putative fumarylacetoacetate hydrolase, all upregulated, and ribulose-phosphate 3-epimerase, downregulated].

We identified 23 proteins whose expression was modulated by two of the four treatments. These could be divided into four groups, i.e. those modulated by NaCl and cyprosulfamide, NaCl and ABA, NaCl and cyprosulfamide plus ABA, and finally cyprosulfamide and cyprosulfamide plus ABA. The NaCl and cyprosulfamide plus ABA group contained 9 proteins, seven of which were induced [dnak-type molecular chaperone Bip (spot 109), elongation factor 2, malic enzyme, putative selenium binding protein (286) and ribosome-recycling factor] and two [chlorophyll A-B binding protein (spot 1317) and chloroplastic thioredoxin X (spot 2377)], that were repressed. The NaCl and cyprosulfamide group contained seven proteins, all downregulated [ATP synthase subunit beta (spot 360), cell division protease ftsH homolog 2 (spot 188), chlorophyll A-B binding protein (spot 1348), fructose-bisphosphate aldolase, glutamine synthetase, phosphoribulokinase and putative transketolase (spot 192)]. The NaCl and ABA group included three proteins, putative chitinase and ribulose bisphosphate carboxylase large

chain (spot 1247) that were induced and Os08g0292600 protein (peptidyl-prolyl cis-trans isomerase family) that was repressed. The cyprosulfamide and cyprosulfamide plus ABA group comprised four upregulated proteins [ATP synthase subunit alpha, ATP synthase subunit beta (spot 1280), putative aminotransferase and ribulose bisphosphate carboxylase large chain (spot 2105)].

We identified 12 proteins that were modulated in response to one specific treatment. Seven of these proteins were induced solely by salinity stress, including three that were induced [ATP synthase subunit beta (spot 454), enolase and L-ascorbate peroxidase 1 (spot 1399)] and four that were downregulated [chloroplastic cell division protease ftsH homolog 2 (spot 174), Os04g0234600 protein (FBPase class 1 family, spot 692), Putative 33kDa oxygen evolving protein of photosystem II (spot 1044) and chloroplastic thioredoxin X (spot 2378)]. Two proteins were upregulated specifically in response to cyprosulfamide, including a glyceraldehyde-3-phosphate dehydrogenase and L-ascorbate peroxidase 2 (spot 1987). Two proteins were induced specifically in response to cyprosulfamide plus ABA: protochlorophyllide reductase B and triosephosphate isomerase. We found only one protein that was induced solely by ABA [protein disulfide isomerase 1-1 (spot 230)].

Although the total number of proteins that were identified was 50, the total number of spots identified in 2DGE was 70. This is due to the fact, that for a number of proteins (e.g. ATP synthase subunit beta), two or more spots in 2DGE were found (**Table 4.6**). In addition, some of the proteins were found to be differently regulated so that some spots of the same protein were upregulated and some downregulated (e.g. putative 33kDa oxygen evolving protein of photosystem II). This finding can be attributed to a different regulation within a multiple gene family. It was also found that for some proteins the same spot was differently regulated during the course of experiment (e.g. carbonic anhydrase).

Most of the similarities between the different treatment groups were found between the NaCl and cyprosulfamide treatments (31 spots in common) (**Figure 4.3c, d**) and the NaCl and cyprosulfamide plus ABA treatments (30 spots in common) (**Figure 4.3a, d**). All the proteins modulated by ABA, except of one, were also modulated by salinity stress (19 spots), or cyprosulfamide (19 spots), or cyprosulfamide plus ABA treatment (16 spots) (**Figure 4.3a, b**). The cyprosulfamide and cyprosulfamide plus ABA

treatments shared 28 common spots (**Figure 4.3b**), and the NaCl, cyprosulfamide and cyprosulfamide plus ABA groups shared 17 spots (**Figure 4.3d**).

		NaCl	Cyp		ABA		ABA+Cyp	
Spot #	**Upregulated proteins**	**S**	**C**	**S**	**C**	**S**	**C**	**S**
829	Aspartate aminotransferase	11+11	1			11+11		
1759	ATP synthase subunit alpha, chloroplastic (fragment)		1,11				11	
534	ATP synthase subunit beta	11	1				1	1
454	ATP synthase subunit beta, chloroplastic	11						
1280	ATP synthase subunit beta, chloroplastic (fragment)		11				11	
1345	ATP synthase subunit beta, chloroplastic (fragment)		1,11		11		11	
1370	ATP synthase subunit beta, chloroplastic (fragment)		1,11		11		11	
2343	Barwin, putative, expressed (chitinase activity)	11		1				1
1783	Carbonic anhydrase	11, 11+11		11	11+11	11		
1787	Carbonic anhydrase	11+11		11	11+11			1
1379	Chlorophyll A-B binding protein, expressed		11			1	11	
1473	Dehydroascorbate reductase	1, 11	1				1	1
109	Dnak-type molecular chaperone Bip	11, 11+11						1
110	Dnak-type molecular chaperone Bip	11, 11+11		1			1	1
75	Elongation factor 2	11, 11+11					1	1
985	Enolase	11, 11+11			11+11		1	1
545	Fructose-1,6-bisphosphatase, chloroplastic	11						
1120	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic		1, 11	1				
133	Heat shock cognate 70 kDa protein	11, 11+11	1				1	1
1041	Lactoylglutathione lyase	11	1				1	1
1399	L-ascorbate peroxidase 1, cytosolic	11						
1987	L-ascorbate peroxidase 2, cytosolic (fragment)			11				
2143	Malate dehydrogenase (fragment)	11	1,11		11			
173	Malic enzyme	11						1
2037	Mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein, putative, expressed	11	1, 11	1			1	1
1669	OsLEA3	1, 11	1	1		11	1	1
1662	Probable photosystem II oxygen-evolving complex protein 2	11	1, 11	1			1	1
230	Protein disulfide isomerase 1-1				11+11			
231	Protein disulfide isomerase 1-1	11			11+11			1
1286	Protochlorophyllide reductase B, chloroplastic						11	
2019	Putative 33kDa oxygen evolving protein of photosystem II (fragment)	11	1	11	11	11+11		
1380	Putative aminotransferase (fragment)		11				11	
1238	putative chitinase	11, 11+11			11+11			

502	Putative fumarylacetoacetate hydrolase	1, 11			1		1	1
286	Putative selenium binding protein	11					1, 11	1
1687	Ribosome-recycling factor, chloroplastic	11					1	1
2408	Ribulose bisphosphate carboxylase large chain (fragment)		11		11		11	1
1073	Ribulose bisphosphate carboxylase large chain; (fragment)	11	11				11	
1247	Ribulose bisphosphate carboxylase large chain; (fragment)	11,11+11			11+11	11		
2105	Ribulose bisphosphate carboxylase large chain; (fragment)		11				11	1
2496	Ribulose bisphosphate carboxylase small chain A, chloroplastic			1,11	11		11	1
2497	Ribulose bisphosphate carboxylase small chain A, chloroplastic		1, 11		11		11	1
2226	Ribulose bisphosphate carboxylase small chain, chloroplastic		11	1	11		11	
241	RuBisCo subunit binding-protein beta subunit	11, 11+11	1				1	1
1568	Superoxide dismutase [Mn], mitochondrial	11						1
2270	Thioredoxin H-type	11	1	1				1
1836	Triosephosphate isomerase							1
547	Xylose isomerase	11						1
		NaCl	**Cyp**		**ABA**		**ABA+Cyp**	
	Downregulated proteins	**S**	**C**	**S**	**C**	**S**	**C**	**S**
889	70 kDa heat shock protein (fragment)	11	1,11			11+11	1	
360	ATP synthase subunit beta, chloroplastic	11	11					
346	ATP synthase subunit beta, chloroplastic	11, 11+11	1, 11	1		11	11	
188	Cell division protease ftsH homolog 2, chloroplastic	11		11				
174	Cell division protease ftsH homolog 2, chloroplastic	11						
1348	Chlorophyll A-B binding protein, expressed	11		1				
1317	Chlorophyll A-B binding protein, expressed	11					11	
905	Fructose-bisphosphate aldolase	11	1					
591	Glutamine synthetase, chloroplastic	11	1	1				
692	Os04g0234600 protein (FBPase class 1 family)	11						
713	Os04g0234600 protein (FBPase class 1 family)	11	1	1		11+11		
1036	Os08g0292600 protein(Peptidyl-prolyl cis-trans isomerase family)	11				11+11		
743	Phosphoribulokinase	11	11					
1044	Putative 33kDa oxygen evolving protein of photosystem II	11						
1067	Putative 33kDa oxygen evolving protein of photosystem II	11	1	1			1	1
192	Putative transketolase	11	1					
189	Putative transketolase	11	1				1	1
789	Ribulose bisphosphate carboxylase	11	1,11	1,11		11+11	1	

	large chain							
583	Ribulose bisphosphate carboxylase/oxygenase activase	11	1, 11	1	11	11		
1407	Ribulose-phosphate 3-epimerase, chloroplastic	11				11	11	
2378	Thioredoxin X, chloroplastic	11						
2377	Thioredoxin X, chloroplastic	11					1	1

Table 4.6. List of all proteins modulated by salinity stress and/or chemical treatments at three different time points, i.e. the beginning of stress treatment (day 1), the end of stress treatment (day 11), and the end of the recovery period (day11+11). Up-regulation of protein spot at the indicated day of the experiment is shown in red, down-regulation of the protein spot is shown in black. . Cyp – cyprosulfamide, S – stressed, C- control unstressed.

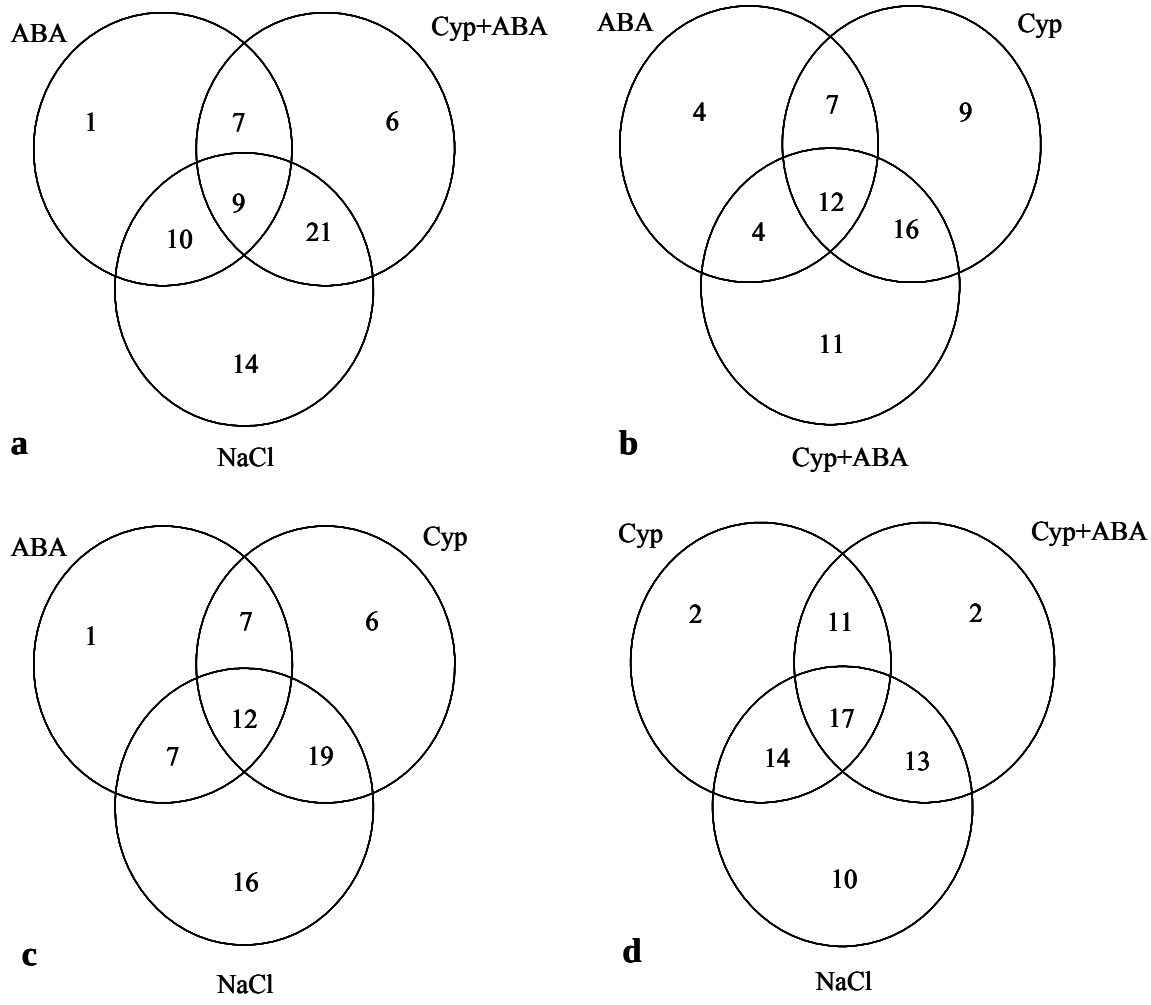

Figure 4.3. Venn diagram showing a number of modulated spots identified for each treatment category, and the overlaps between categories. (a) Comparison of spots induced by ABA, cyprosulfamide plus ABA and NaCl. (b) Comparison of spots

induced by ABA, cyprosulfamide and cyprosulfamide plus ABA. (c) Comparison of spots induced by ABA, cyprosulfamide and NaCl. (d) Comparison of spots induced by cyprosulfamide, cyprosulfamide plus ABA and NaCl. Here the term "spot" is used instead of "protein" as in some cases for one protein several spots were identified.

4.3.1.6. OsLEA3 – the rice group 3 late embryogenesis abundant protein

Several proteins were induced by salinity stress throughout the treatment period, but OsLEA3 showed the strongest modulation (**Table 4.2**). This protein was marginally induced on day 1 (1.55-fold) but induction increased to 13.3-fold by day 11 then returned to basal levels during the recovery period (**Table 4.2**). Interestingly, OsLEA3 was also induced strongly by cyprosulfamide (7–8-fold induction on day 1 in both unstressed and stressed plants), returning to basal levels by day 11 (**Figure 4.4a and 4.4b**). Over the same treatment course, there was no evidence of significant induction by ABA, but the combination of cyprosulfamide and ABA induced OsLEA3 to an even greater extent than cyprosulfamide alone before falling to basal levels by day 11 (**Figure 4.4a and 4.4b**).

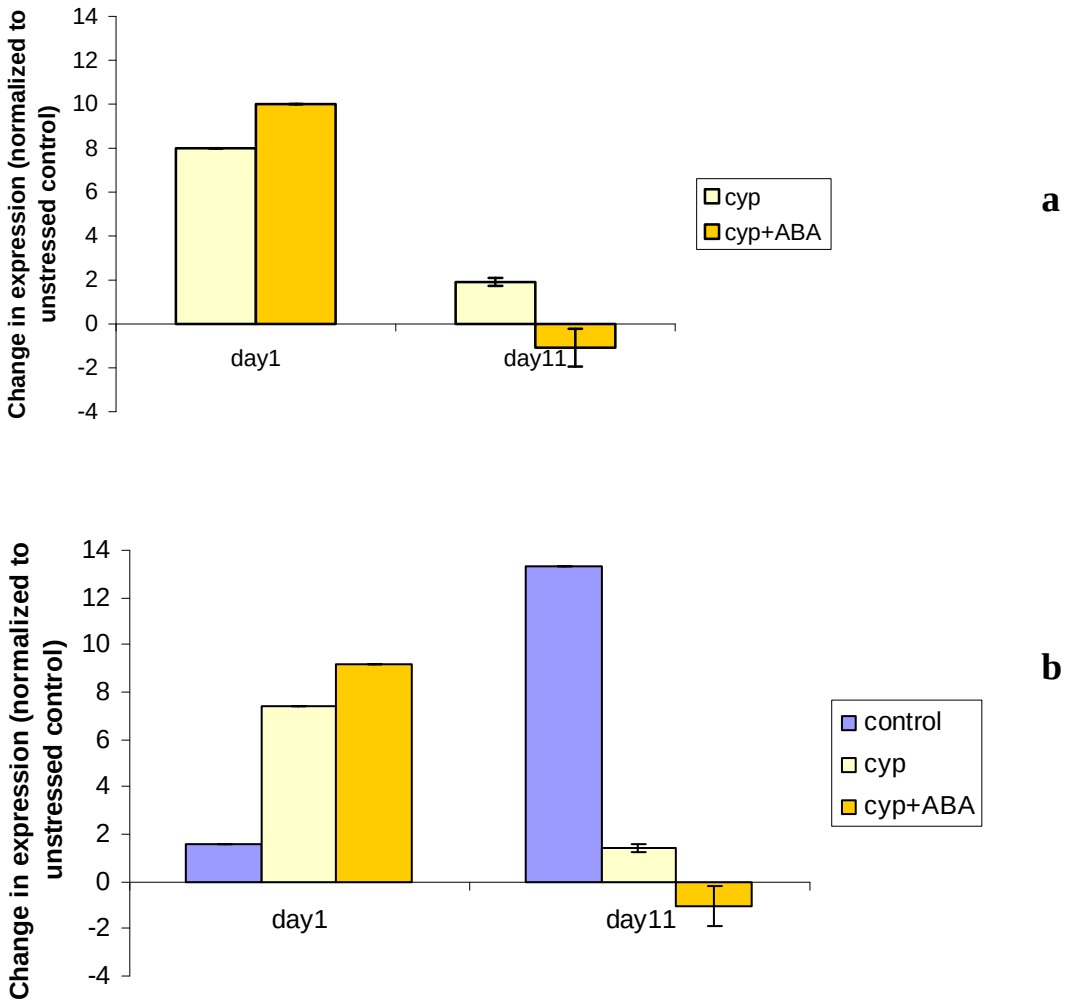

Figure 4.4. 2DGE showing the modulation of OsLEA3 in response to salinity stress and to cyprosulfamide and cyprosulfamide plus ABA treatment without stress (**a**) and with stress (**b**).

4.3.1.7. Putative fumaryl acetoacetate hydrolase

A putative fumaryl acetoacetate hydrolase was also induced by salinity stress (1.62-fold on day 1, and 2.84-fold on day 11, returning to basal levels during recovery) and the same protein was induced by cyprosulfamide plus ABA treatment (2.36–2.38-fold induction on day 1 in both unstressed and stressed plants, returning to basal levels by day 11). The data are shown in **Figure 4.5a and 4.5b**. Over the same treatment course, there was no evidence of significant induction by ABA or cyprosulfamide alone.

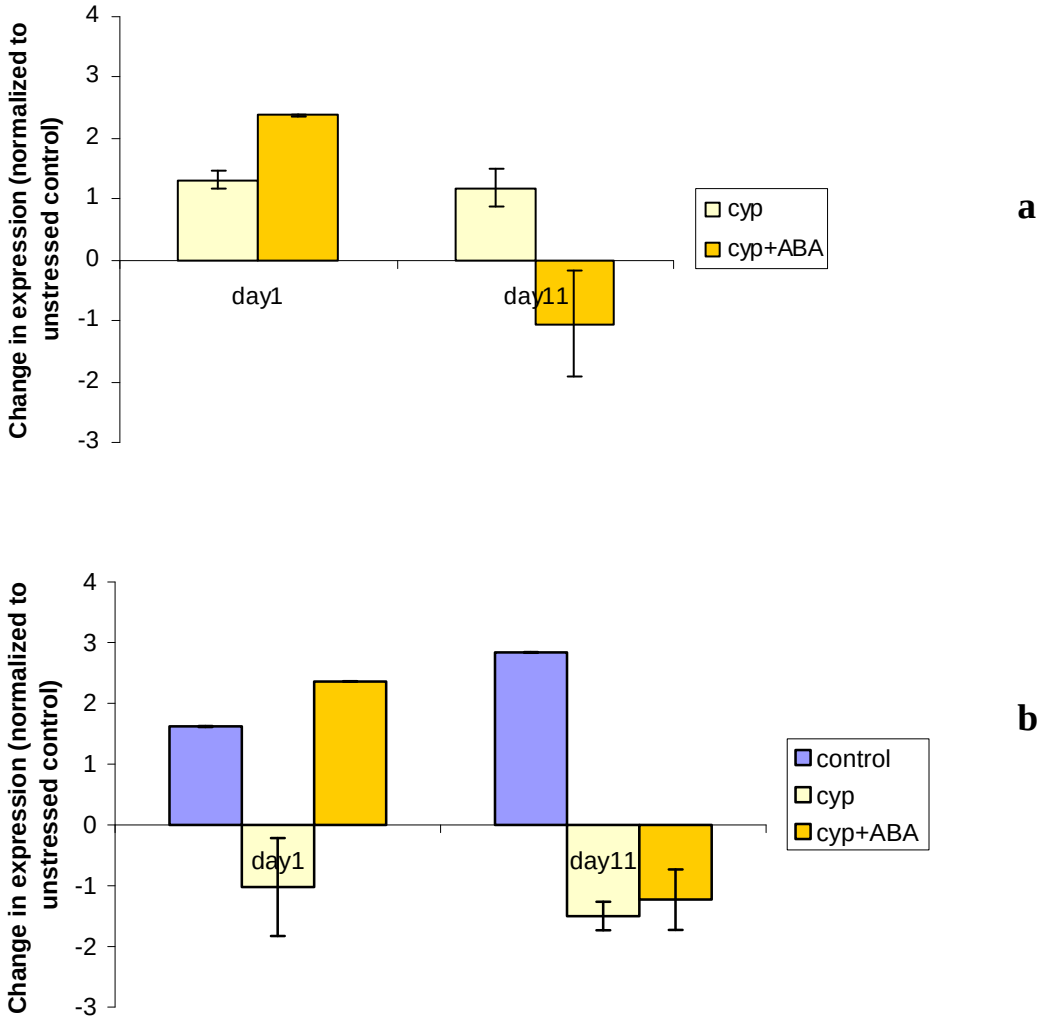

Figure 4.5. 2DGE showing the modulation of a putative fumarylacetoacetate hydrolase in response to salinity stress and to cyprosulfamide and cyprosulfamide plus ABA treatment without stress (**a**) and with stress (**b**).

4.3.1.8. Mitochondrial import inner membrane translocase

A mitochondrial import inner membrane translocase was also induced by salinity stress (3.44-fold after 11 days of stress, returning to basal levels during the recovery period). The same protein was also induced strongly by cyprosulfamide treatment (3.65–4.45-fold induction on day 1 in both stressed and unstressed plants, and 1.77-fold induction on day11 in unstressed plants while in stressed plants the protein levels fell back to basal levels). The data are shown in **Figure 4.6a and 4.6b**. Over the same treatment

course, there was no evidence of significant induction by ABA, but the combination of cyprosulfamide plus ABA induced this protein 3.89–4.17-fold on day 1 before falling to basal levels by day 11 (**Figure 4.6a and 4.6b**).

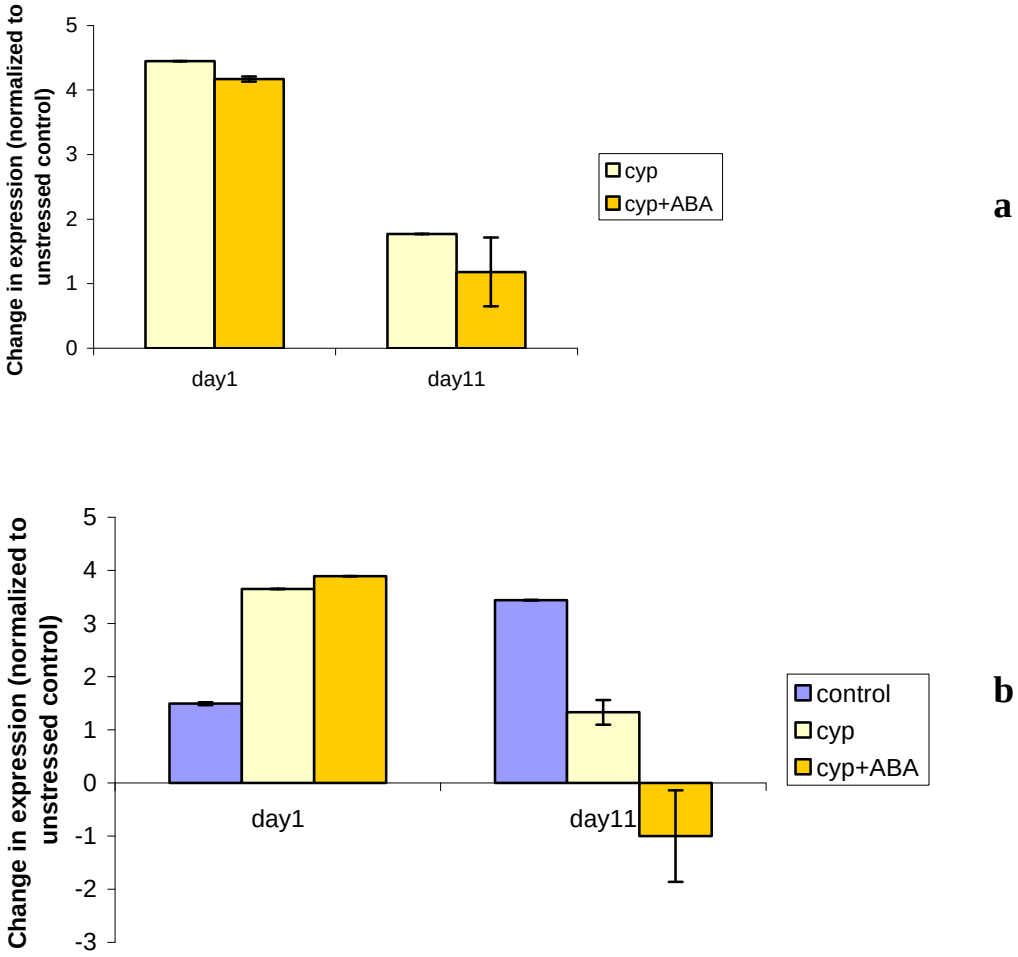

Figure 4.6. 2DGE showing the modulation of a mitochondrial translocase in response to salinity stress and to cyprosulfamide and cyprosulfamide plus ABA treatment without stress (**a**) and with stress (**b**).

4.3.2. Real time RT-PCR analysis

Real-time RT-PCRs was carried out using specific primers for *OsLEA3*, and the putative fumarylacetoacetate hydrolase and mitochondrial translocase subunit to confirm the proteomic data.

4.3.2.1. RT-PCR analysis of *OsLEA3* expression

We carried out real-time RT-PCR experiments to determine the level of *OsLEA3* mRNA in the same leaf sample replicates used for protein extraction. On day 1 without stress *OsLEA3* was strongly upregulated by cyprosulfamide or cyprosulfamide plus ABA but not by ABA alone (**Figure 4.7a**). However, ABA had the strongest impact on *OsLEA3* expression when stress was also applied (**Figure 4.7b**). The cyprosulfamide and cyprosulfamide plus ABA treatments caused a significant induction, but the induction ratio was higher in stressed plants. On day 11, neither cyprosulfamide nor cyprosulfamide plus ABA appeared to affect OsLEA3 expression in the presence or absence of stress, although ABA treatment resulted in a slight induction of *OsLEA3* in unstressed plants (**Figure 4.7a**). In contrast, the application of ABA to stressed plants resulted in the downregulation of *OsLEA3* on day 11 (**Figure 4.7b**). On day 11+11 without stress none of the compounds appeared to have any effect on *OsLEA3* (**Figure 4.7a**). However, in plants that had been subject to salinity stress, the opposite results were observed: the cyprosulfamide, ABA and cyprosulfamide plus ABA treatments all induced *OsLEA3* expression with cyprosulfamide plus ABA showing the strongest effects (**Figure 4.7b**).

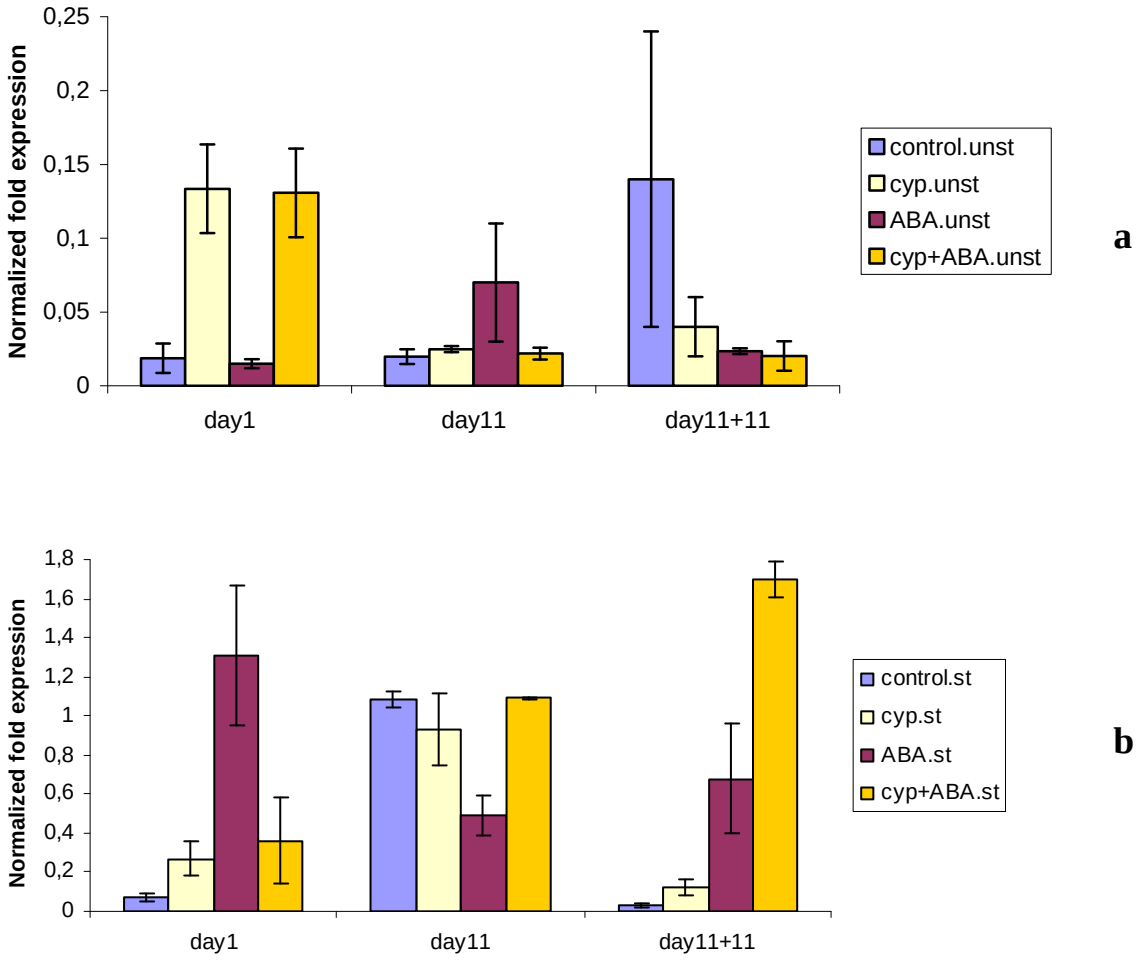

Figure 4.7. Relative expression levels of *OsLEA* mRNA (normalized to actin) after rice plants were exposed to different chemical treatments (**a**) in the absence of salinity stress and (**b**) with salinity stress applied for 11 days. Each bar represents the average of four replicates.

4.3.2.2. Putative fumarylacetoacetate hydrolase

Real-time RT-PCR analysis was performed on all four leaf sample replicates at three time points. On day1 without stress putative fumarylacetoacetate hydrolase was upregulated by cyprosulfamide or cyprosulfamide plus ABA but not by ABA alone (**Figure 4.8a**). However when stress was applied all three treatments significantly upregulated the expression of putative fumarylacetoacetate hydrolase (**Figure 4.8b**). On day 11 without stress none of the compounds induced the gene (**Figure 4.8a**). On day11 with stress, only cyprosulfamide plus ABA treatment upregulated the expression of

putative fumarylacetoacetate hydrolase while ABA downregulated the expression of the gene (**Figure 4.8b**). On day11+11 without stress none of the compounds modulated the expression of putative fumarylacetoacetate hydrolase (**Figure 4.8a**). Under stress conditions ABA or cyprosulfamide plus ABA induced expression of the gene (**Figure 4.8b**).

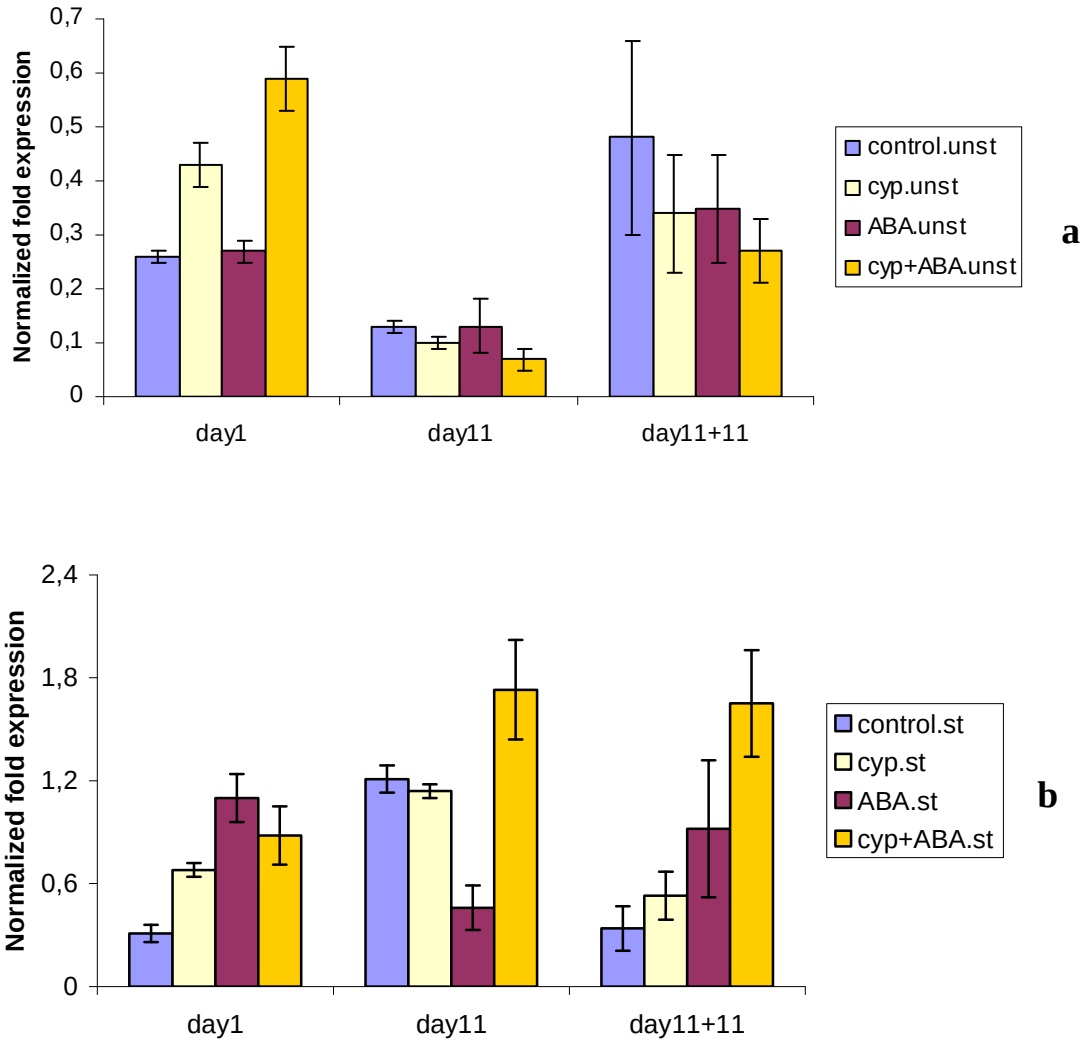

Figure 4.8. Relative expression levels of *FAH* mRNA (normalized to actin) after rice plants were exposed to different chemical treatments (**a**) in the absence of salinity stress and (**b**) with salinity stress applied for 11 days. Each bar represents the average of four replicates.

4.3.2.3. Mitochondrial import inner membrane translocase

Real-time RT-PCR experiments were also conducted to investigate the expression profile of the mitochondrial membrane translocase. On day 1 without stress, the translocase gene was strongly upregulated by cyprosulfamide and by cyprosulfamide plus ABA but not by ABA alone (**Figure 4.9a**). A similar pattern was observed under salinity stress conditions (**Figure 4.9b**). On day 11, the translocase gene was unaffected by any of the chemical treatments in both stressed and unstressed plants (**Figure 4.9a and 4.9b**). On day 11+11 the translocase genes was unaffected by any of the treatments in unstressed plants (**Figure 4.9a**) but in plants recovering from stress, the gene was induced by ABA and by cyprosulfamide plus ABA (**Figure 4.9b**).

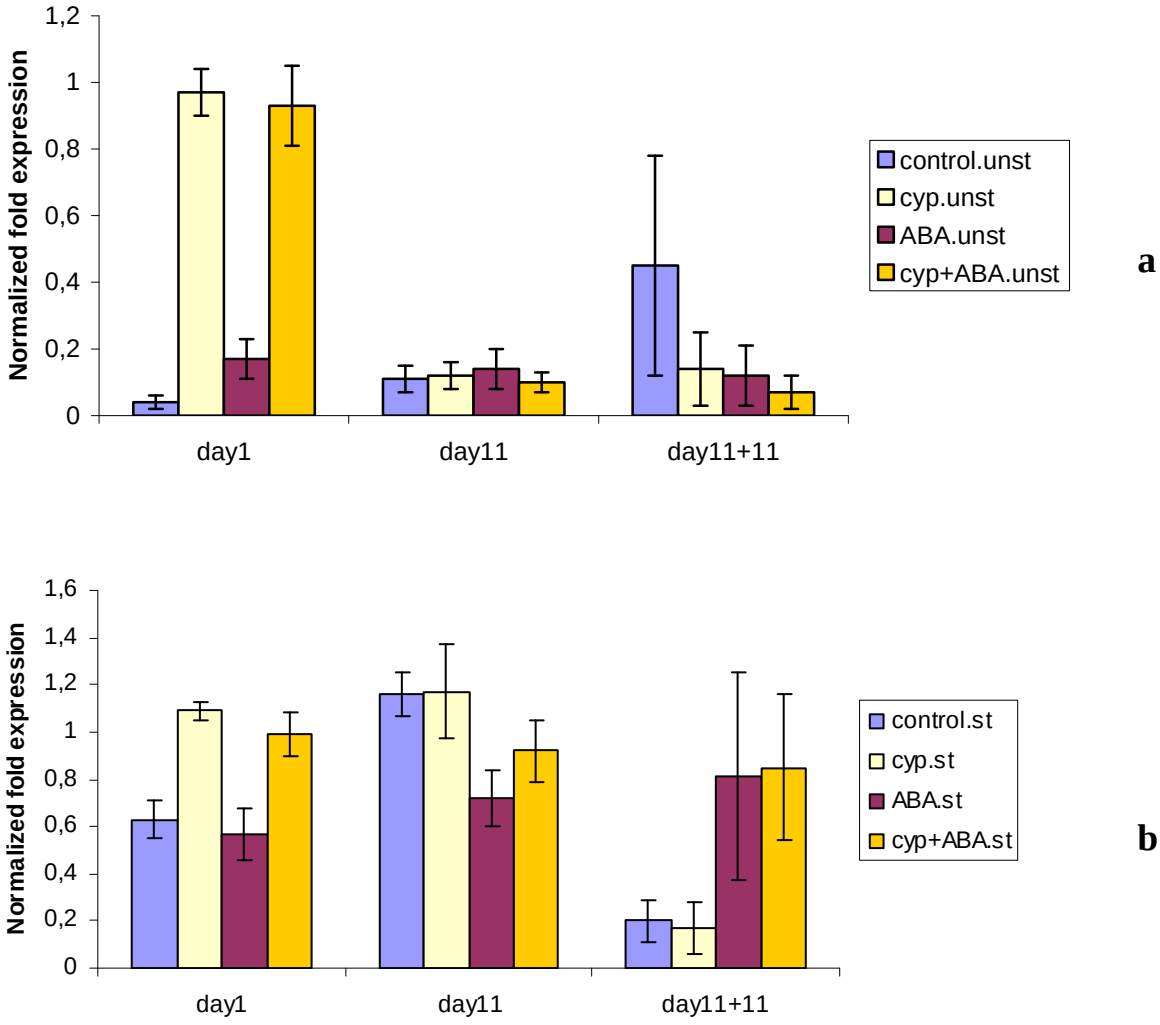

Figure 4.9. Relative expression levels of *TIM* mRNA (normalized to actin) after rice plants were exposed to different chemical treatments (**a**) in the absence of salinity stress

and (**b**) with salinity stress applied for 11 days. Each bar represents the average of four replicates.

4.3.3. Overview of OsLEA3, FAH and TIM mRNA and protein expression profiles in the different treatment groups

The mRNA and protein expression profiles of the three key target genes we identified were compared under the four treatment regimens in the stressed and unstressed plants to identify common factors responsible for their behavior throughout the experiment. OsLEA3 mRNA and protein levels increased in response to salinity stress on day 11, and also in response to cyprosulfamide or cyprosulfamide plus ABA on day 1 in both the unstressed and stressed plants. In unstressed plants treated as above, OsLEA3 mRNA and protein fell back to basal levels by day 11, but in stressed plants treated as above the protein fell to basal levels by day 11 but the mRNA level remained high.

FAH protein was induced on day 1 of the salinity stress treatment, but we did not observe induction of the corresponding gene. However, by day 11, induction was evident at both the mRNA and protein levels. Treatment with cyprosulfamide plus ABA induced FAH mRNA and protein on day 1 in both stressed and unstressed plants, and they largely returned to basal levels by day 11, with just the FAH transcript showing a marginal induction in stressed plants. Treatment with cyprosulfamide alone did not induce the FAH protein, but the transcript was induced in both stressed and unstressed plants on day 1, falling back to basal levels by day 11.

The translocase gene was induced by salinity stress on day 1 while the protein remained at basal levels, but induction at both the transcript and protein levels was apparent by day 11. Both the transcript and the protein were induced by cyprosulfamide or cyprosulfamide plus ABA treatment on day 1 (in both unstressed and stressed plants) and the protein was also induced by cyprosulfamide on day 11 in unstressed plants.

4.4. Discussion

4.4.1. Proteomics is a powerful tool for identification of stress-induced proteins

Plant cells modulate the expression of a large number of genes in response to osmotic stress (Zhu et al., 1997). This has an impact on the composition of the proteome, so the

comparison of the stressed and unstressed proteomes by 2DGE can identify the proteins whose abundance change in response to stress, and these potentially play a role in stress tolerance. For example, when three cultivars of indica rice plants (Sinaloa, IR10120 and Chiapas) were grown in soil supplemented with PEG, the comparison of 2D protein gels with those prepared from unstressed plants revealed a number of stress-induced proteins, including dehydrin and osmotin (Perez-Morphe-Balch et al., 1996). Most of these stress-induced proteins were also regulated by ABA.

More than 35 proteins with roles in salinity tolerance have been identified by 2DGE (Claes et al., 1990; Moons et al., 1995, 1997; Ramani et al., 1997; Singla et al., 1998). Prominent proteins induced by salinity stress in rice include members of the SalT, LEA2 and LEA3, HSP100 and OSR40 families, active oxygen scavengers such as SOD and ascorbate peroxidase, betaine aldehyde dehydrogenase, and several other novel proteins with unknown functions. Although the identification of stress-response proteins is fairly straightforward, unraveling the regulatory mechanisms and corresponding signaling pathways remains a challenge. The most important salinity stress pathways identified thus far are those controlling energy metabolism, ROS scavenging, photorespiration (in C3 species), the synthesis of compatible solutes, water uptake, sodium/potassium discrimination and the synthesis of chaperones (Kosová et al., 2011).

4.4.2. Defense-related proteins

Several of the stress-response proteins identified in the proteomics analysis are defense-related proteins. These include putative barwin protein, OsLEA3, and putative chitinase (**Table 4.7**). The exact role of LEA proteins in dehydration is not clear, but they may prevent the formation of protein aggregates during water stress (Zhu, 2001; Xiong and Zhu, 2002; Goyal et al., 2005). LEA proteins can protect proteins and lipids in stressed plants (Shinozaki and Yamaguchi-Shinozaki, 1999), and in this context group 3 LEA proteins have been shown to protect enzymes such as lactate dehydrogenase, malate dehydrogenase, citrate synthase, fumarase, and rhodanase against partial dehydration (Battaglia et al., 2008). LEA proteins may also provide a water-rich local environment for client enzymes, preventing their inactivation by possibly maintaining protein integrity under water-limiting conditions. LEA proteins are also known to protect membrane structures from damage (Bravo et al., 2003; Hara et al., 2001).

Chitinase class III *AtChiA* gene from Arabidopsis was found to be upregulated due to environmental stresses, especially to salt and wound stresses (Takenaka et al., 2009). A class II chitinase *AtCTL1* gene is responsible for salt and drought stresses (Kwon et al., 2007).

Spot #	Upregulated proteins	Function	Treatment
2343	Barwin, putative, expressed (chitinase activity)	Defense	1,2,4
1669	OsLEA3	Defense	1,2,3,4
1238	putative chitinase	Defense	1,3
1473	Dehydroascorbate reductase	Detoxification	1,2,4
1399	L-ascorbate peroxidase 1, cytosolic	Detoxification	1
1987	L-ascorbate peroxidase 2, cytosolic (fragment)	Detoxification	2
286	Putative selenium binding protein	Detoxification	1,4
1568	Superoxide dismutase [Mn], mitochondrial	Detoxification	1,4
2270	Thioredoxin H-type	Detoxification	1,2,4
534	ATP synthase subunit beta	Energy	1,2,4
1280	ATP synthase subunit beta, chloroplastic (fragment)	Energy	2,4
1345	ATP synthase subunit beta, chloroplastic (fragment)	Energy	2,3,4
1370	ATP synthase subunit beta, chloroplastic (fragment)	Energy	2,3,4
1759	ATP synthase subunit alpha, chloroplastic (fragment)	Energy	2,4
454	ATP synthase subunit beta, chloroplastic	Energy	1
173	Malic enzyme	Energy	1,4
2143	Malate dehydrogenase (fragment)	Energy	1,2,3
829	Aspartate aminotransferase	Metabolism	1,2,3
985	Enolase	Metabolism	1,3,4
1120	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	Metabolism	2
1286	Protochlorophyllide reductase B, chloroplastic	Metabolism	4
502	Putative fumarylacetoacetate hydrolase	Metabolism	1,3,4
547	Xylose isomerase	Metabolism	1,4
1380	Putative aminotransferase (fragment)	Metabolism	2,4
1836	Triosephosphate isomerase	Metabolism	4
1783	Carbonic anhydrase	Photosynthesis	1,2,3
1787	Carbonic anhydrase	Photosynthesis	1,2,3,4
1379	Chlorophyll A-B binding protein, expressed	Photosynthesis	2,3,4
545	Fructose-1,6-bisphosphatase, chloroplastic	Photosynthesis	1
1662	Probable photosystem II oxygen-evolving complex protein 2	Photosynthesis	1,2,4
2497	Ribulose bisphosphate carboxylase small chain A, chloroplastic	Photosynthesis	2,3,4
2496	Ribulose bisphosphate carboxylase small chain A, chloroplastic	Photosynthesis	2,3,4
2226	Ribulose bisphosphate carboxylase small chain, chloroplastic	Photosynthesis	2,3,4
1073	Ribulose bisphosphate carboxylase large chain; (fragment)	Photosynthesis	1,2,3
1247	Ribulose bisphosphate carboxylase large chain; (fragment)	Photosynthesis	1,3
2105	Ribulose bisphosphate carboxylase large chain;	Photosynthesis	2,4

	(fragment)		
2408	Ribulose bisphosphate carboxylase large chain (fragment)	Photosynthesis	2,3,4
2019	Putative 33kDa oxygen evolving protein of photosystem II (fragment)	Photosynthesis	1,2,3
110	Dnak-type molecular chaperone Bip	Protein folding, targeting, degradation	1,2,4
109	Dnak-type molecular chaperone Bip	Protein folding, targeting, degradation	1,4
133	Heat shock cognate 70 kDa protein	Protein folding, targeting, degradation	1,2,4
231	Protein disulfide isomerase 1-1	Protein folding, targeting, degradation	1,3,4
230	Protein disulfide isomerase 1-1	Protein folding, targeting, degradation	3
241	RuBisCo subunit binding-protein beta subunit	Protein folding, targeting, degradation	1,2,4
75	Elongation factor 2	Protein synthesis	1,4
1687	Ribosome-recycling factor, chloroplastic	Protein synthesis	1,4
2037	Mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein, putative, expressed	Protein transport	1,2,4
1041	Lactoylglutathione lyase	Secondary metabolism	1,2,4
	Downregulated proteins		
2378	Thioredoxin X, chloroplastic	Detoxification	1
2377	Thioredoxin X, chloroplastic	Detoxification	1,4
360	ATP synthase subunit beta, chloroplastic	Energy	1,2
346	ATP synthase subunit beta, chloroplastic	Energy	1,2,3,4
591	Glutamine synthetase, chloroplastic	Metabolism	1,2
1348	Chlorophyll A-B binding protein, expressed	Photosynthesis	1,2
1317	Chlorophyll A-B binding protein, expressed	Photosynthesis	1,4
905	Fructose-bisphosphate aldolase	Photosynthesis	1,2
692	Os04g0234600 protein (FBPase class 1 family)	Photosynthesis	1
713	Os04g0234600 protein (FBPase class 1 family)	Photosynthesis	1,2,3
743	Phosphoribulokinase	Photosynthesis	1,2
1044	Putative 33kDa oxygen evolving protein of photosystem II	Photosynthesis	1
1067	Putative 33kDa oxygen evolving protein of photosystem II	Photosynthesis	1,2,4
192	Putative transketolase	Photosynthesis	1,2
189	Putative transketolase	Photosynthesis	1,2,4
789	Ribulose bisphosphate carboxylase large chain	Photosynthesis	1,2,3,4
583	Ribulose bisphosphate carboxylase/oxygenase activase	Photosynthesis	1,2,3
1407	Ribulose-phosphate 3-epimerase, chloroplastic	Photosynthesis	1,3,4
188	Cell division protease ftsH homolog 2, chloroplastic	Protein folding, targeting, degradation	1,2
174	Cell division protease ftsH homolog 2, chloroplastic	Protein folding, targeting, degradation	1
889	70 kDa heat shock protein (fragment)	Protein folding, targeting, degradation	1,2,3,4
1036	Os08g0292600 protein(Peptidyl-prolyl cis-trans isomerase family)	Protein folding, targeting, degradation	1,3

Table 4.7. The biological role of all the proteins identified by comparing the proteomes of plants treated with (1) NaCl (salinity stress), (2) cyprosulfamide, (3) ABA, and (4) cyprosulfamide plus ABA.

4.4.3. Detoxification

Many forms of abiotic stress, including salinity stress, can increase the production of ROS such as O_2^-, H_2O_2, $^1O_2$, HO_2^-, ROOH and ROO^-, which can cause significant damage to cellular structures (Gill and Tuteja, 2010). To avoid such damage, plants detoxify excess ROS by activating a variety of scavengers, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), gluthatione peroxidase (GPX), glutathione-S-transferase (GST) and catalase (CAT), as well as non-enzymatic low-molecular-weight metabolites such as glutathione (GSH), α-tocopherol, carotenoids and flavonoids. SOD acts as the first line of defense converting highly-toxic superoxide into hydrogen peroxide. In turn, H_2O_2 is detoxified by APX, GPX, CAT and 2-cys peroxiredoxin. The most important reducing substrate for H_2O_2 detoxification in plant cells is ascorbate (Mehlhorn et al., 1996). APX reduces H_2O_2 to water, with the concomitant generation of monodehydroascorbate (MDHA), a short-lived radical that disproportionates to ascorbate and dehydroascorbate (DHA). In turn, DHA is reduced to ascorbate by DHA reductase (DHAR), using glutathione as the reducing substrate. Peroxiredoxin has a similar role to peroxidases, reducing hydrogen peroxide, lipid hydroperoxide and peroxynitrite; it is also involved in redox signaling (Dietz, 2003).

We identified several proteins that participate in detoxification reactions among the proteins induced by salinity stress, cyprosulfamide and/or cyprosulfamide plus ABA. These included L-ascorbate peroxidase 1, DHAR, a mitochondrial SOD, a putative selenium-binding protein and thioredoxin H-type (**Tables 4.6 and 4.7**). In previous studies, some of these proteins have been identified in rice plants subjected to salinity stress. For example, SOD was induced 1.7-fold in rice leaf lamina after 7 days exposure to salinity stress, but there was no change after 1 day (Parker et al., 2006). In our experiments we also observed that the induction of SOD was upregulated after 11 or 15 days under stress but not on day 1. Dooki et al. (2006) reported that ascorbate peroxidase 1 and DHAR were induced in the young panicles of rice plants exposed to salinity stress, and Eltelib et al. (2011) more recently reported the induction of MDHAR

and DHAR transcripts and enzyme activities in the leaves of acerola plants subjected to salinity and chilling stress.

4.4.4. Energy

We observed the induction of several proteins related to energy metabolism, including malate dehydrogenase, malic enzyme and ATP synthase subunit beta (**Table 4.7**). ATP synthases are membrane-bound enzyme complexes/ion transporters that combine ATP synthesis and/or hydrolysis with the transport of protons across membranes. ATP is required for many biosynthesis pathways in plant cells, and energy requirements may increase considerably during stress meaning that larger amounts of ATP are required (Tezara et al., 1999). Several energy-metabolism proteins were induced in *Suaeda salsa* plants subjected to salinity stress, including choline monooxygenase, plastid ATP synthase subunit beta, and a V-type proton ATPase catalytic subunit A (Li et al., 2011).

Cytosolic malate dehydrogenase and malic enzyme have a central role in carbon metabolism and energy transduction. They are induced under stress conditions to cope with the changes in carbon metabolism that occur in response to reduced photosynthesis and the diversion of carbon into the synthesis of compatible solutes required for osmotic adjustment. Salinity stress induces malic enzyme gene expression and also increases the enzyme activity (Liao et al., 2007). Cytosolic malate dehydrogenase was identified as one of the highly abundant proteins in young rice panicles, reflecting the increased demand for ATP in the rapidly-dividing cells (Dooki et al., 2006).

4.4.5. Metabolism

4.4.5.1. Primary metabolism

We identified several proteins that participate in metabolism among the proteins induced by salinity stress, cyprosulfamide, ABA and/or cyprosulfamide plus ABA. These included aspartate aminotransferase, enolase, glyceraldehydes-3-phosphate dehydrogenase, protochlorophillide reductase B, FAH, xylose isomerase, putative aminotransferase and triosephosphate isomerase (**Table 4.7**). Aspartate aminotransferase is an important enzyme in amino acid metabolism as it catalyzes the reversible transfer of an α-amino group between aspartate and glutamate. In rice seedlings it was found to be upregulated due to salt stress and pretreatment with ABA

(Li et al., 2010). In our experiments we also found to be induced due to salt stress and due to ABA under salinity stress as well as due to cyprosulfamide regardless of stress (**Table 4.7**).

FAH is the last enzyme in the tyrosine degradation pathway in animals, where it converts the tyrosine byproduct fumaryl acetoacetate into fumarate and acetoacetate for excretion or further metabolic processing. Less is known about the role of FAH in plants, but Dixon and Edwards (2006) showed that FAH is also expressed in Arabidopsis, and that immediately upstream is a zeta-glutathione-S-transferase (ζ-GST) that converts maleylacetoacetate to fumaryl acetoacetate, and also participates in the detoxification of xenobiotics such as herbicides. GSTs are induced by safeners in cereals (Davies and Caseley, 1999), and the overexpression of ζ-GST can enhance the germination and growth of rice seedlings at low temperatures (Takesawa et al., 2002). This provides a potential mechanistic link between the application of cyprosulfamide, the induction of FAH and the developmental phenotype we observed in plants treated with cyprosulfamide and with cyprosulfamide plus ABA.

4.4.5.2. Secondary metabolism

Lactoylglutathione lyase or glyoxalase I (gly I) catalyzes the detoxification of methylglyoxal (MG) to S-D lactoyl-glutathione using reduced GSH as the cofactor. S-D lactoyl is further catalysed to D-lactate by glyoxalase II (gly II) and in the process GSH is recycled back. Transgenic tobacco plants overexpressing gly I and gly II were more resistant to salinity stress than control plants demonstrating a potential role of the glyoxalase pathway in salinity tolerance (Singla-Pareek et al., 2003). Recently gly I was also found to be upregulated due to safener treatment in Arabidopsis (Behringer et al., 2011). These authors suggested its possible participation in phase IV of the detoxification of xenobiotic compounds. Our findings are consistent with these two studies as lactoylglutathione lyase was induced by the safener cyprosulfamide and by cyprosulfamide plus ABA as well as by salinity stress (**Table 4.7**).

4.4.6. Photosynthesis

Photosynthesis is highly sensitive to many environmental stresses including salinity and drought (Chaves et al., 2009). The effects of salinity are more severe than drought

stress, modulating a larger number of genes to cope not only with osmotic stress but also ion toxicity. The same stress can have different qualitative and quantitative effects on genes in the same multi-gene family (Chaves et al., 2009). For example, the impact of salinity stress on photosynthesis in *A. thaliana* has been investigated using an Affymetrix ATH1 chip, showing that both genes encoding fructose-bisphosphatase were similarly repressed but others involved in the Calvin cycle and photorespiration were modulated in different directions (Kilian et al., 2007). It was found that while both genes of fructose-bisphosphatase were similarly down-regulated by salt stress, genes encoding further enzymes of the Calvin cycle and photorespiration (fructose-bisphosphate aldolase, phosphoribulokinase, transketolase, ribose-5-phosphate isomerase, phosphoglycolate phosphatase and glycine hydroxymethyltransferase) were differently affected (Chaves et al., 2009). We found that fructose-bisphosphate aldolase, phosphoribulokinase, a putative transketolase 1, ribulose bisphosphate carboxylase/oxygenase activase and a ribulose-phosphate 3-epimerase were downregulated by salinity stress whereas putative 33-kDa oxygen-evolving proteins from photosystem II, ribulose bisphosphate carboxylase large chain and small chain and chlorophyll A-B binding protein were modulated in different directions (**Table 4.7**) potentially indicating that different isoforms of each protein are regulated separately. This last finding may be related to the fact that these proteins were identified in different spots of 2-D gel and may belong to a multigene family with some of them up-regulated and some down-regulated during the same treatment condition. Fragmentation of some of the identified proteins especially RuBisCO large subunit and ATP synthase subunit beta probably is a result of degradation. However, we do not know if it happened *in vivo* or *in vitro* during sample preparation. In other proteomics studies on rice RuBisCO degradation was also encountered (Rakwal and Komatsu, 2004; Zhao et al., 2005).

The putative photosystem II oxygen-evolving complex protein 2 we identified is induced by salinity stress on days 11, 15, 15+10 and also by cyprosulfamide on day 1 and day 11 or cyprosulfamide plus ABA on day 1, in the presence or absence of stress. This protein may be important for rice plants to adapt to salinity stress because it was previously shown to be induced by salinity stress in the leaf sheath and blade regardless of whether or not ABA was also present (Abbasi and Komatsu, 2004).

4.4.7. Protein folding, targeting and degradation

We identified a number of proteins that were induced by salt, cyprosulfamide, or ABA, and/or cyprosulfamide plus ABA. These included dnak-type molecular chaperone Bip, heat shock cognate 70 kDa protein, protein disulfide isomerase 1-1 and RuBisCO subunit binding-protein beta subunit.

Heat-shock proteins and chaperones are responsible for protein folding, assembly, translocation and degradation as part of many normal cellular processes, and they also stabilize proteins and membranes and can assist in protein refolding under stress conditions (Wang et al., 2004). In transgenic tobacco plants, the overexpression of a DnaK-type molecular chaperone from the halotolerant cyanobacterium *Aphanothece halophytice* improved resistance to salinity stress (Sugino et al., 1999). Plant chaperonins facilitate the post-translational assembly of plastid proteins such as Rubisco (Boston et al., 1996). A mutant form of the *A. thaliana* chloroplast chaperonin 60a has defective chloroplast development, which prevents normal embryonic development and seedling growth (Apuya et al., 2001). Hsp70 prevents protein aggregation and refolds denatured proteins under both normal and stress conditions (Frydman, 2001). The overexpression of Hsp70 has been shown to enhance salinity tolerance in plants (Sugino et al., 1999).

4.4.8. Protein transporters

Two translocases on the inner mitochondrial membrane, known as TIM17:23 and TIM22, are required for the general and carrier import pathways, respectively (Pfanner and Geissler, 2001). The TIM17:23 complex is responsible for the import of precursor proteins that contain N-terminal targeting signals, which are removed after import by the mitochondrial processing peptidase. TIM22 is responsible for the import of carrier proteins into mitochondria (Rehling et al., 2003). Tim17/Tim22/Tim23 translocases are induced by water stress in rice embryos (Farinha et al., 2011) and we similarly found that a putative Tim17/Tim22/Tim23 translocase was strongly induced by salinity stress and by treatment with cyprosulfamide and cyprosulfamide plus ABA. This translocase could potentially be overexpressed to increase the abiotic stress tolerance of rice plants, but it also represents a useful molecular marker for screening new bioregulators.

4.4.9. Induction of OsLEA3 by stress, cyprosulfamide and ABA

We found that OsLEA3 was induced by salinity stress, ABA, cyprosulfamide and cyprosulfamide plus ABA. Xiao et al. (2007) previously demonstrated the induction of *OsLEA3-1* in rice cv. IRAT109 in response to treatment with 200 mM NaCl, the response commencing within 18 h with peak induction at 72 h. This compares well with our RT-PCR data from rice plants treated with 100 mM NaCl, which showed that *OsLEA3* transcripts were induced within 24 h with peak induction after 11 days falling to basal levels during recovery.

Several LEA genes are regulated by ABA, and ABA-response elements are found in the promoters of rice LEA genes (Bray 1997; Zhu et al. 1997; Ono et al., 1996; Rabbani et al., 2003; Wang et al., 2007). LEA2 and LEA3 are expressed in rice and finger millet seedlings in response to ABA treatment (Jayaprakash et al., 1998). An ABA-inducible dehydrin gene is also strongly expressed in rice varieties adapted for salinity tolerance (Moons et al., 1995). Wang et al. (2007) used microarray analysis and semi-quantitative RT-PCR to show that 16 LEA genes were induced by ABA in japonica rice cultivar Nipponbare. LEA3 is also induced by ABA in wheat seedlings (Ried and Walker-Simmons, 1993). The application of exogenous ABA mimics the effect of salinity stress (150 mM NaCl) in the indica cultivar Taichung N1, as both treatments rapidly induce *OsLEA3* expression in seedling roots (Moons et al., 1997). This agrees well with our finding that exogenous ABA applied to rice plants treated with 100 mM NaCl induces *OsLEA3* mRNA (and OsLEA3 protein) expression after 1 day. No proteomic data are available for ABA treatment alone due to technical problems affecting sample preparation, but the effects of ABA can be inferred by comparing the impact of cyprosulfamide alone (7–8-fold induction) to those of cyprosulfamide plus ABA (9–10-fold induction). Treatment with cyprosulfamide plus ABA also increased the expression of *OsLEA3* during recovery to a greater extent than cyprosulfamide alone, revealing a synergic effect between cyprosulfamide and ABA that directly affects the regulation of OsLEA3. One potential mechanism is that cyprosulfamide in some way promotes the accumulation of ABA, which in turn induces the expression of *OsLEA3*.

4.4.10. Induction of FAH and translocase by stress, cyprosulfamide and ABA

We identified a putative fumarylacetoacetate hydrolase and a mitochondrial import inner membrane translocase that were induced by salinity stress, ABA, cyprosulfamide and cyprosulfamide plus ABA. Although it is unclear precisely how these proteins are related to the salinity stress response in rice plants, both the corresponding genes could be useful markers for salinity stress and for the protective activity of potential new bioregulators. Transgenic plants overexpressing these genes could also be generated to investigate their abiotic stress tolerance and adaptation.

4.4.11. Cyprosulfamide may pre-adapt rice plants to tolerate salinity stress

Many proteins induced by stress after 11 days were also upregulated by cyprosulfamide or cyprosulfamide plus ABA on day 1 and/or day 11 whether or not the plants were subjected to stress. The induced proteins included those required for the detoxification of ROS (e.g. DHAR), stress adaptation (e.g. OsLEA3), energy (e.g. malate dehydrogenase and ATP synthase subunit beta), protein transport (e.g. the mitochondrial import inner membrane translocase), photosynthesis (e.g. photosystem II oxygen-evolving complex protein 2), protein biosynthesis (e.g. elongation factor 2 and ribosome-recycling factor), primary metabolism (e.g. the putative fumarylacetoacetate hydrolase) and secondary metabolism (e.g. lactoylglutathione lyase). We therefore propose that cyprosulfamide may pre-adapt the plant to tolerate salinity stress by inducing genes required for stress acclimatization well before exposure to stress alone would promote their expression.

4.4.12. Hypothetical model for the interplay between stress, ABA and cyprosulfamide

Safeners are known to protect plants from herbicide toxicity by coordinately inducing entire herbicide detoxification pathways (Zhang et al., 2007; Riechers et al., 2010). Some of the genes that participate in herbicide detoxification also confer abiotic stress tolerance. GSH is an antioxidant that provides an important intracellular defense against oxidative damage caused by ROS. GSH is induced when *Anabaena doliolum* is exposed to salinity stress (Srivastava et al., 2005) and the GSH levels are also higher in the leaves of *Vigna radiata* L. cv. Pusa Bold than *Vigna radiata* L. cv. CO 4 under salinity

stress, indicating that cv. Pusa Bold has more efficient antioxidant characteristics (Sumithra et al., 2006). GST is also known to play a role in oxidative stress protection by reducing peroxides with the help of GSH (Gill and Tuteja, 2010). In drought tolerant (M35-1) and drought sensitive (SPV-839) sorghum varieties subjected to salinity stress, M35-1 proved to be more efficient at H_2O_2 scavenging because of its significantly higher GST activity (Jogeswar et al., 2006). Salinity stress and ABA also rapidly induce *osgstu*3 in rice, which encodes a tau-class GST (Moons, 2003). Increased GST activity was also detected in five-week-old tomato plants (*Lycopersicon esculentum* Mill. cv "Perkoz") exposed to salinity stress (Gapinska et al., 2008).

CytP450 enzymes also facilitate the detoxification of herbicides induced by safeners (Persans et al., 2001; Zhang et al., 2007). These enzymes participate in detoxification phase I by catalyzing the oxidation of xenobiotic substances, thus introducing or exposing a functional group suitable for subsequent conjugation with GSH by GST. Forty-nine *A. thaliana* CytP450 genes have been analyzed to determine their potential role in biotic and abiotic stress tolerance, and their response to hormones such as salicylic acid, jasmonic acid, ethylene and ABA (Narusaka et al., 2004). Some of the genes were found to be induced both by abiotic and biotic stresses. The *CYP81D11* and *CYP81D8* promoters contain ABA-response elements, and they are also induced by salinity stress. Safeners therefore induce genes that play role in abiotic stress adaptation and their application may improve plant stress tolerance through this route.

GST and CytP450 enzymes were not identified in our proteomics analysis, but other proteins that participate in detoxification reactions were shown to be induced by cyprosulfamide or cyprosulfamide plus ABA. These included L-ascorbate peroxidase 1, DHAR, a mitochondrial SOD, a putative selenium-binding protein and thioredoxin H-type. Like CytP450 enzymes, peroxidases also are known to participate in phase I of the detoxification process induced by safeners (Behringer et al., 2011). The application of isoxadifen-ethyl or mefenpyr-diethyl to *A. thaliana* leaves resulted in the induction of many detoxification genes, incuding peroxidase and DHAR (Behringer et al., 2011). MDHR was also induced in the wheat species *Triticum tauschii* by the safener cloquintocet-mexyl (Zhang et al., 2007).

Cyprosulfamide improved the performance of rice plants subjected to salinity stress and also increased the expression of LEA genes, which are ABA-dependent. Cyprosulfamide may therefore work by promoting the accumulation of ABA, but it is

also likely that the two regulators have synergic effects since more proteins are induced by the combined treatment than the sum of the individual treatments. CytP450, GST and GSH may therefore be induced by cyprosulfamide and by higher levels of ABA to increase the tolerance of rice plants towards salinity stress, as shown in our proposed model (**Figure 4.10**).

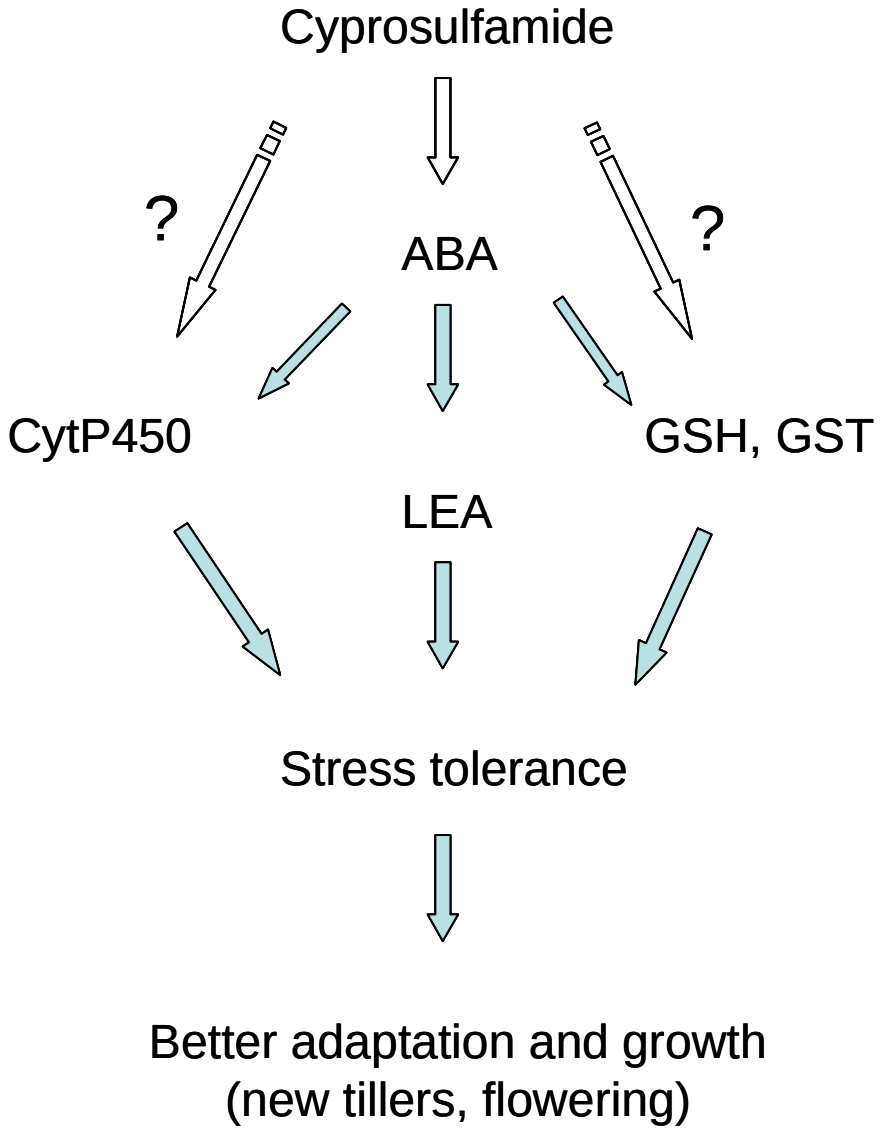

Figure 4.10. Theoretical model for the role of cyprosulfamide in rice salinity stress tolerance. Cyprosulfamide induces the expression of LEA genes probably by promoting the accumulation of endogenous ABA. This achieves better stress tolerance and therefore better adaptation and growth, resulting in earlier flowering and the appearance of new tillers. In addition, cyprosulfamide may achieve detoxification in a manner similar to other safeners by inducing CytP450, GSH and GST genes. Relationships

already described in the literature are shown in blue, whereas those indicated by our experimental results are shown in white.

4.5. Conclusions

Novel genes with potential roles in abiotic stress adaptation can be identified by applying high-throughput "omics" technologies, and the data gathered from such experiments allows us to link genes into functional networks and therefore to develop models for the mechanisms involved in stress tolerance and adaptation. These experiments can also identify candidate genes that could be used to generate stress-tolerant plants. The bioregulator cyprosulfamide induces stress-related genes, preparing plants to withstand salinity stress. The precise interplay between cyprosulfamide and ABA is not clear yet, but cyprosulfamide may promote the accumulation of ABA, which in turn activates a spectrum of downstream genes that reduce the impact of salinity stress. It is important to confirm that cyprosulfamide as well as other safeners can induce proteins that participate in herbicide detoxification as well the relationship with ABA and/or other hormones. Further investigations are needed to determine the precise role and mechanism of action of cyprosulfamide and similar compounds in the development of tolerance to abiotic stresses in crop plants.

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Chapter 5

Towards the development of a novel cell based assay to screen candidate bioregulators

5.1. Introduction

Genes that are modulated by bioregulators are potentially useful as markers to screen for novel bioregulator compounds. One way in which this could be achieved is to isolate the corresponding promoters from such genes from plant genomic DNA and link them to fluorescent reporter genes. A suitable report gene might be DsRED encoding a red fluorescent protein. This gene was cloned from a Discosoma coral (Matz et al., 1999). Callus cultures transformed with such reporter constructs could then be tested against a panel of known bioregulators to determine whether the reporter construct is induced. Once the principle of the assay has been validated with known bioregulators, the same assay could be used to test for novel bioregulator molecules. The quantitative detection of reporter fluorescence could also allow the potency of new bioregulators to be determined.

The same genes could also be used as markers for the early selection of improved plant lines with sustainable high yields under stress conditions (marker assisted selection). They could also be overexpressed in transgenic plants to generate plant lines with improved stress tolerance. The identification of markers in one species could lead to the discovery of orthologs in other species, allowing potential chemical regulators to be tested in diverse crops, both in the laboratory and under field conditions.

Rab16A is a rice gene encoding a group 2 LEA protein (dehydrin) which could be valuable as a marker of abiotic stress and bioregulator activity. It has been identified as a stress-response gene induced by salinity, drought and the application of exogenous ABA (Mundy and Chua, 1988; Ono et al., 1996). Homologs of *Rab16A* have been identified in maize, sorghum (Buchanan et al., 2004) and cotton (Baker et al., 1988).

We cloned the 5′ proximal 2 kb of the *Rab16A* promoter and created a reporter construct that was introduced into rice callus to develop such a bioregulator assay. This was tested by subjecting callus tissue to salinity stress, cyprosulfamide, ABA and combinations of these treatments.

5.2. Materials and Methods

5.2.1. DNA extraction

DNA was isolated from rice leaves using a modification of the procedure described by Edwards et al. (1991). Samples were ground to powder under liquid nitrogen and dissolved in 4 ml extraction buffer containing 300 µl 20% SDS. After vortexing for 10 min, the samples were heated to 65ºC for 10 min and protein was extracted with phenol. Nucleic acids were isolated by phenol:chloroform:iso-amylalcohol extraction (25:24:1) and the aqueous phase was treated with 15 µl RNase A (10mg/ml) at 37ºC for 1 h to remove the RNA. DNA was precipitated with one volume of isopropanol, washed with 1 ml 70% ethanol if necessary and centrifuged at 5000 rpm for 15 min. The pellet was washed with 1 ml 70% ethanol, centrifuged at 5000 rpm for 10 min and then the supernatant was discarded allow the pellet to air dry. The pellet was dissolved in 50 µl distilled water. The DNA concentration was determined by nanodrop spectrophotometry and the quality checked by agarose gel electrophoresis. Aliquots were diluted to a concentration of 50 ng/µl for subsequent reactions.

5.2.2. Cloning, vector construction, transformation and recovery of transgenic rice callus

Genomic DNA from wild type rice plants *Oryza Sativa* L. subsp. Japonica cv EYI105 was used as the source of the *Rab16A* promoter. The promoter sequence was identified in GenBank (accession number Os.12633.1.s1_s_at) and primers were designed to flank the 2 kb of sequence immediately upstream of the open reading frame, so that relevant *cis*-acting regulatory elements were likely to be included. All *cis*-acting regulatory elements were predicted using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The *Rab16A* promoter was amplified in two steps from the genomic DNA with two sets of primers: set1 (Forw-Deh-NcoI and Rev-Deh-NcoI) amplifying 2057 bp upstream of the ATG and set2 (Forw-Deh-BclI and Rev-Deh-BclI) amplifying 2038 bp upstream of the ATG. The PCR was carried out using a 50-µl final volume containing 5µl of cDNA template (50 ng/µl), 2.5µl each of the forward and reverse primers (20 µM each primer), 1 µl dNTP mix (2.5 mM each nucleotide), 10 µl Taq buffer x5, 1.25 µl Taq polymerase (5 units/µl) and 27.75 µl water. Each of the 30 amplification cycles comprised a denaturation step at

94°C for 45 s, annealing at 60°C for 30 s and extension at 72°C for 135 s. The two PCR products, in both cases 2 kb, were cloned into the pGEM-T-easy vector (Promega, Spain) for sequencing and analysis with DANMA software (UAB, Barcelona, Spain).

The set1 fragment (NcoI-dehydrin-NcoI) was released from the pGEM-T-easy vector and ligated to the 5′ end of the DsRed gene in pTRAK at the NcoI enzyme site, forming the cytoplasmic dehydrin-DsRed cassette (Figure 1a). The set2 fragment (BclI-dehydrin-BclI) was released from the pGEM-T-easy vector and ligated to 5′ end of the transit peptide (TP) in pTRAK at the BclI enzyme site, forming the plastid-targeted dehydrin-TP-DsRed cassette (Figure 1b). The orientation of the dehydrin promoter within the plasmid was verified by diagnostic restriction digests. The cassette from each plasmid was then amplified with a common primer set (Forw-Deh-KpnI and Rev-DsRed-KpnI) designed to amplify 2736 bp of the dehydrin-DsRed fragment and 2892 bp of the dehydrin-TP-DsRed fragment using the reaction conditions described above but with a longer extension step in each cycle (3 min). The resulting PCR products were transferred to pGEM-T-easy for sequencing as above.

The KpnI-dehydrin-DsRed-KpnI and KpnI-dehydrin-TP-DsRed-KpnI fragments were released from pGEM-T-easy and transferred to the KpnI site of *phpt-gusA*, which contains the hygromycin phosphotransferase selectable marker *hpt* (the cytoplasmic construct is shown in Figure 1c). The orientation of the cassette was verified by diagnostic restriction digests. The cytoplasmic DsRed construct was named *phpt-gusA* (N) and the plastid-targeted construct was named *phpt-gusA* (B).

Rice callus was transformed by particle bombardment (Christou et al., 1991). Seeds from the japonica cultivar EYI105 were dehusked and sterilized in 70% ethanol for 3 min and in 5% sodium hypochlorite for 20 min then rinsed three times in sterile distilled water. The callus induction medium (MSP) was based on MS basal medium supplemented with 2.5 mg/l 2,4-D and 3% (w/v) sucrose (pH 5.8). About 10 seeds were placed on each MSP plate and were grown in the dark in a controlled environment chamber (26 ± 2ºC, 80% relative humidity). Expanded mature embryos were separated from the endosperm 7 days after incubation (the day before bombardment) and then transferred to MSP medium supplemented with 0.4 M mannitol as an osmoticum 4 h before and 16 h after bombardment.

Gold particles were coated with DNA as described (Christou et al., 1991). Two bombardments were carried out at 15 kV for higher efficiency with a 4-h interval

between the two bombardments. The following day bombarded tissues were transferred to MSP medium, and then to selection medium (MSP supplemented with 50 mg/ml hygromycin). Callus was transferred to fresh selection plates every 2 weeks.

Name of the primer	**5´ to 3´ sequence**
Forw-Deh-BclI	GTGATCAGACTTGATGTTCATTCCTTGTGACG
Rev-Deh-BclI	GAGATCGAGGTGTTCTTCTGATCAG
Forw-Deh-KpnI	GGTACCGACTTGATGTTCATTCCTTGTGACG
Rev-DsRed-KpnI	GGTACCTAAAGGAACAGATGGTGGCGTC
Forw-Deh-NcoI	CCATGGACTTGATGTTCATTCCTTGTGACG
Rev-Deh-NcoI	CCATGGAGATCGAGGTGTTCTTCTGATCAG
Forw DsRed RT-PCR	GTCTTCCAAGAATGTTATCAAGGAG
Rev DsRed RT-PCR	CTGCTCAACGATTGTATAGTCTTCG

Table 5.1. Sequence of the primers used to clone the dehydrin *Rab16A* promoter and for the RT-PCR experiments.

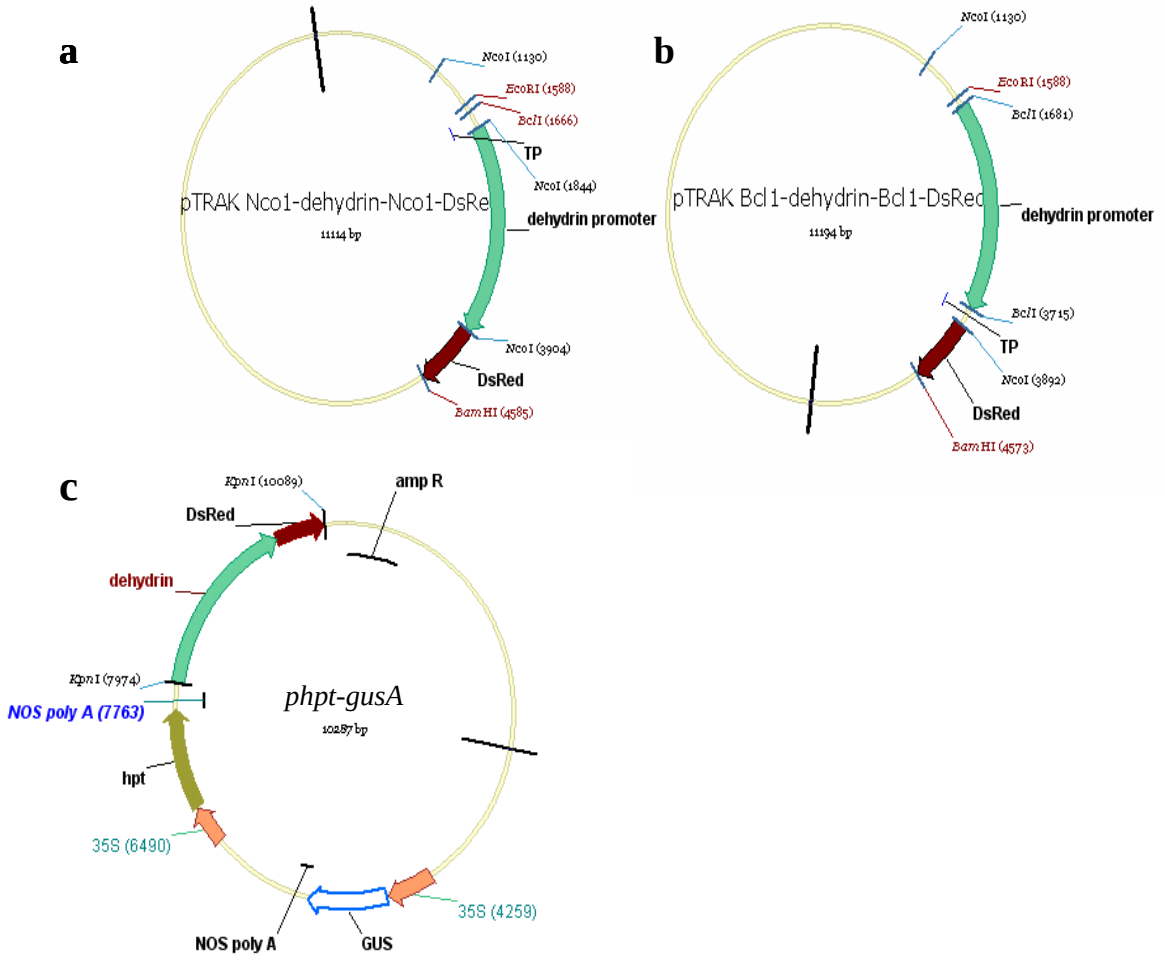

Figure 5.1. Plasmids used for dehydrin promoter cloning. **a** – pTRAK containing the dehydrin promoter fused to cytoplasmic DsRed, **b** - pTRAK containing the dehydrin promoter fused to plastid-targeted DsRed, **c** – final construct for transformation, phpt-gusA containing the dehydrin-DsRed cassette

5.2.3. RNA extraction, reverse transcription and RT-PCR

Total RNA was extracted from callus tissue using Trizol® (Invitrogen, Carlsbad, CA, USA). RNA concentrations were measured spectrophotometrically using a NanoDrop® ND-1000 (Thermo Scientific, Wilmington, DE, USA). We reverse transcribed 2 µg of each RNA sample with the Omniscript RT kit (Qiagen, Hilden, Germany) using oligo(dT) primers (Invitrogen, Carlsbad, CA, USA). A set of primers (Forw DsRed RT-PCR and Rev DsRed RT-PCR) was designed to cover 632 bp of the DsRed coding sequence (**Table 5.1**). RT-PCR amplification was carried out in a 20-µl final volume containing 2 µl of the cDNA template (50 ng/µl), 1 µl each of the forward and reverse primers (20 µM each), 0.4 µl of the dNTP mix (2.5 mM each dNTP), 4 µl Taq buffer x5, 0.6 µl $MgCl_2$ (25 mM), 0.5 µl Taq polymerase (5 units/µl) and 11.1 µl of sterile water. We carried out 35 cycles of denaturation (94°C, 45 s), annealing (55°C, 30 s) and extension (72°C, 30 s).

5.2.4. Assays in solid and liquid media

Small, uniform pieces of callus were chosen from the fresh transgenic callus and were transferred to solid selection medium supplemented with 150 mM NaCl to impose salinity stress. Each plate contained five uniform callus pieces and three plates were used per treatment. The callus pieces were transferred to fresh medium every two weeks.

Liquid MSS medium with or without NaCl was prepared as above without agar. Four flasks were prepared for control (untreated), cyprosulfamide (C9), ABA (C10) and cyprosulfamide plus ABA (C9+C10). ABA was prepared in DMSO (final concentration 10 µg/µl) and cyprosulfamide was prepared in NaOH (final concentration 10 µg/µl). Five to ten pieces of transgenic callus were placed in each flask, which were incubated in an orbital shaker at 100 rpm for 5-7 days and then analyzed for DsRed expression using a fluorescence microscope.

5.2.5. Microscopy

Each callus was cut into small thin pieces and observed under a fluorescence microscope (Leica, Wetzlar, Germany). The expression of DsRed in callus pieces subjected to salinity stress (150mM for 5-7 days) was compared to unstressed callus and to control non-transgenic callus under a fluorescent microscope equipped with a filter set for excitation at 530-560 nm and emission at 590-650 nm.

5.3. Results

5.3.1. Cloning, plasmid construction and generation of the transgenic lines

The 2-kb dehydrin promoter was amplified with two sets of primers and the products were transferred to the pTRAK vector containing the sequence for DsRed (**Figure 5.1**). The dehydrin promoter fused to cytosolic and plastid-targeted dehydrin was then amplified and transferred to phpt-gusA to generate the cytosolic (N) and plastid-targeted (B) versions. The constructs were introduced into rice by bombarding 200 embryos, resulting in the recovery of 24 callus lines, 13 carrying the plastid-targeted construct and 11 carrying the cytosolic construct.

5.3.2. Molecular analysis of transgenic lines

We evaluated DsRed expression in seven lines by non-quantitative RT-PCR to provide a rough estimate the relative levels, and we also tested two lines (8N and 12N) under both normal and salinity-stress conditions. All lines expressed *DsRed* mRNA under both stressed and unstressed conditions (**Figure 5.2**).

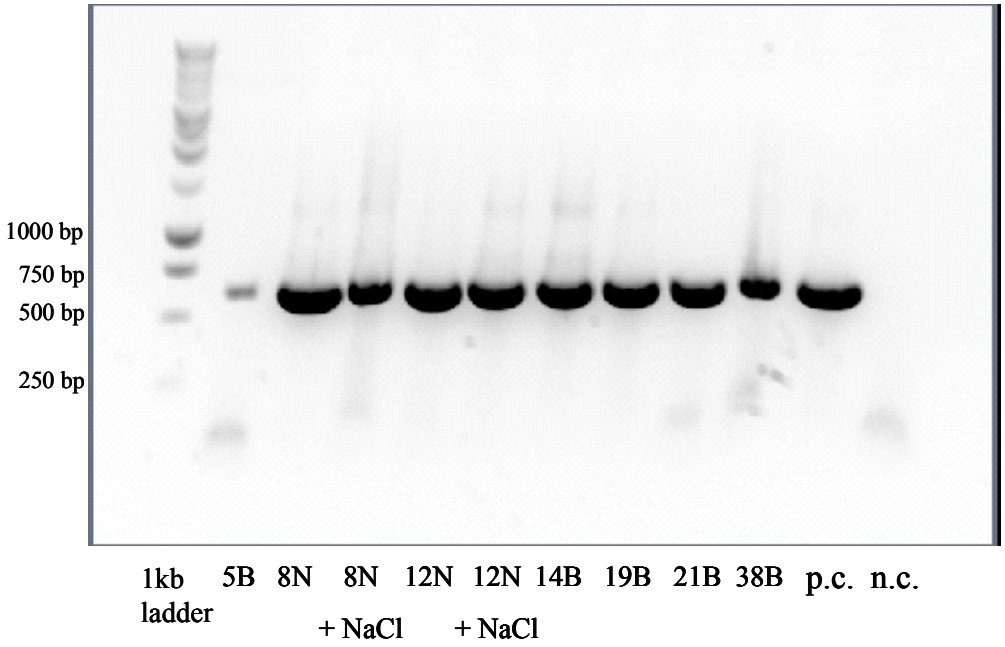

Figure 5.2. RT-PCR to detect the expression of the *DsRed* gene in seven different transgenic lines. Abbreviations: p.c. - positive control, n.c. - negative control.

5.3.3. DsRed expression in transgenic rice cell lines

DsRed expression was evaluated by fluorescence microscopy in the different transgenic cell lines. There was no overt difference in expression between callus samples expressing the cytoplasmic and plastid-targeted DsRed. There was no overt difference in expression between callus samples cultured in the presence or absence of NaCl. Finally, there were no overt differences in fluorescence in callus cultures from line 38B (**Figure 5.3**) or 12N (**Figure 5.4**) subjected to different chemical treatments. There was a marginal difference between the experimental callus cultures and the negative control, but the negative control itself generated red fluorescence which made the results more difficult to interpret. Overall we found that neither stress nor treatment with bioregulators induced significant changes in DsRed fluorescence in any of 11 experiments with any of the 18 callus lines we analyzed.

In order to improve these results and reduce background fluorescence in the negative control samples, the callus pieces were sectioned by microtome to yield 14-µm sections. Although this succeeded in eliminating the background fluorescence in negative control samples, the fluorescence in the transgenic lines was reduced to near imperceptible

levels too. Root tissue from transgenic line 3B that was grown to full maturity was compared to wild type roots after five days in 100 mM NaCl but there was no difference in DsRed fluorescence between the samples.

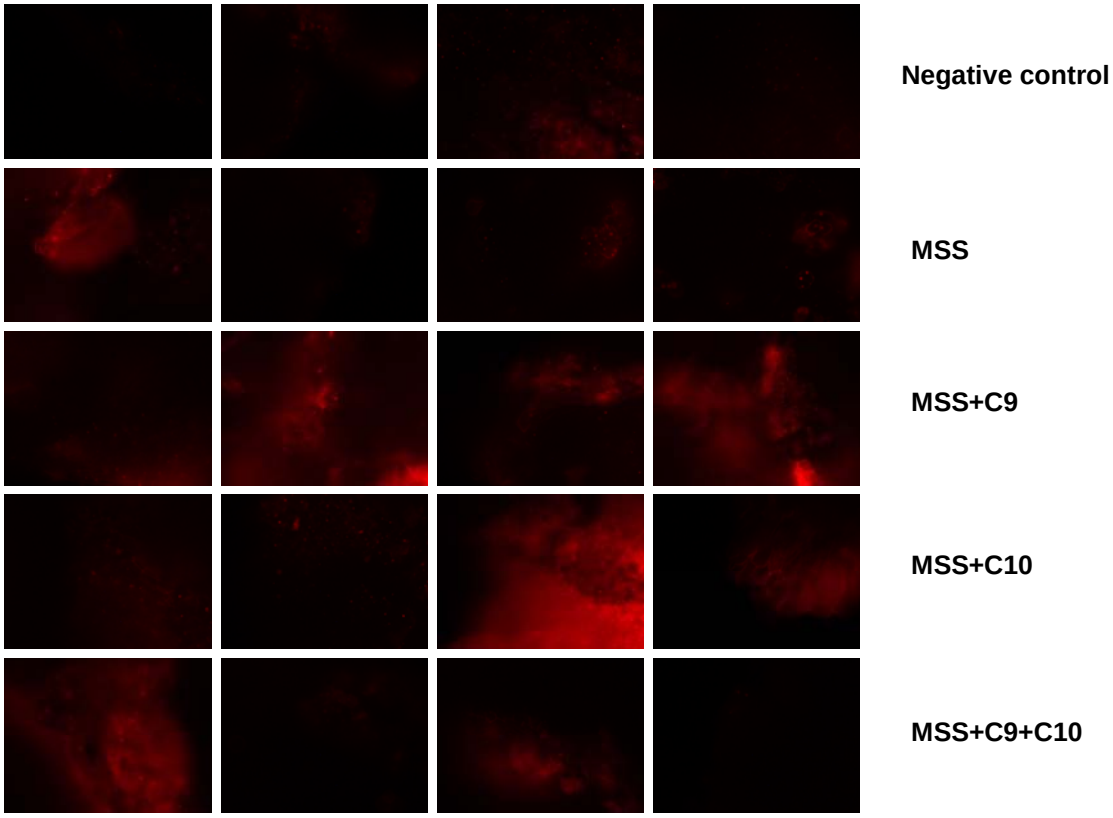

Figure 5.3. DsRed expression of line 38B subjected to different treatments, magnified x 40.

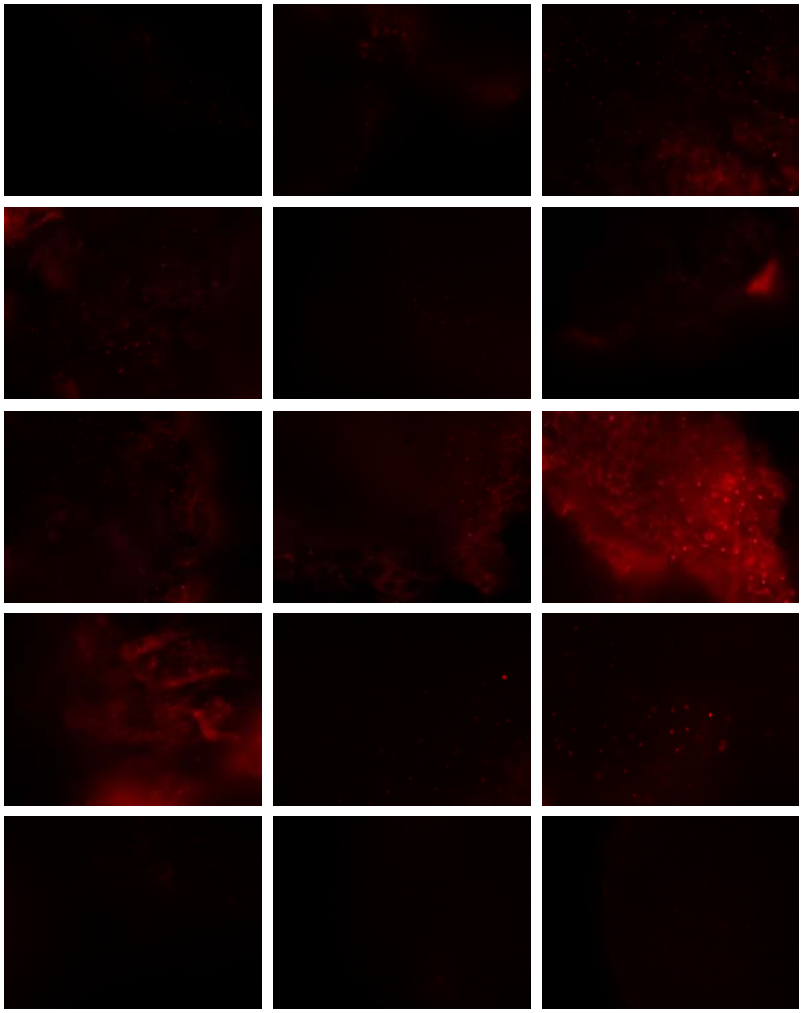

Figure 5.4. DsRed expression of line 12N subjected to different treatments, magnified x 40.

5.4. Discussion

Rab16A is a rice gene encoding a group 2 LEA protein (dehydrin). These are typically hydrophilic proteins that bind water strongly and therefore retain water to prevent the crystallization of important proteins and other macromolecules during desiccation (Baker et al., 1988). The rice *Rab16A* gene is expressed strongly in embryos during the late stage of grain development (Yamaguchi-Shinozaki et al., 1989) as well as in response to ABA and osmotic stress in vegetative tissues (Ono et al., 1996). An ABA-inducible dehydrin was shown to be strongly expressed in rice varieties that tolerate high-salinity (Moons et al., 1995). Transgenic tobacco plants overexpressing *Rab16A* cDNA isolated from the salt tolerant rice cultivar Pokkali, were also found to be tolerant towards salinity stress (RoyChoudhury et al., 2007).

The *Rab16A* promoter had been cloned and tested previously. The region between positions -294 and +27 was sufficient to confer ABA-responsive transient expression of the *cat* reporter gene in transfected rice protoplasts (Mundy et al., 1990). The promoter has also been used to drive *gusA* expression in tobacco (Yamaguchi-Shinozaki et al., 1990) and rice (Ono et al., 1996) in each case showing that the promoter responds to ABA. Ono et al. (1996) further showed that the *Rab16A* promoter contained ABA-response elements and that the first 800 bp of the promoter was sufficient for reporter gene induction by 10^{-4} M ABA, 1% NaCl or desiccation (Ono et al., 1996). A 358-bp fragment spanning the -480 to -60 bp region of the *Rab16A* promoter was shown to contain 12 stress response elements (Rai et al., 2009). This segment was fused to the *gusA* gene resulting in constitutive GUS activity in the leaves and roots of 15-day-old rice seedlings, and a 2–3-fold induction after incubation for 20 h with 100 μM ABA. The construct was also induced in leaves and roots by prolonged exposure to 100 mM NaCl salinity stress (Rai et al., 2009).

We found that 2 kb of upstream sequence containing all the important *cis*-acting regulatory elements was sufficient to achieve *DsRed* gene expression in rice callus (demonstrated by RT-PCR) but we were unable to detect significant DsRed fluorescence. It is possible that the mRNA is not translated, or that the protein is non-functional because it is not processed or modified correctly. Western blots will be carried out to determine whether the protein is present but not active, or whether it is unstable and subject to degradation or not synthesized at all. Interestingly, exposure of transgenic callus lines to 150mM NaCl did not even induce *DsRed* expression at the mRNA level.

5.5. Conclusions

The *Rab16A* promoter contains ABA-response elements and is known to be inducible by abiotic stress. Therefore it should be a good candidate for the development of assays for stress-response regulators and for the development of stress-tolerant transgenic plants. Unfortunately, we were unable to induce reporter constructs containing this promoter in rice callus either by imposing salinity stress or by exposing the callus cultures to ABA or cyprosulfamide. More studies will be carried out to determine

whether our results reflect a peculiarity of the rice callus system or can be traced to a technical problem with the reporter construct.

5.6. References

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General conclusions

1. Tebuconazole, cyprosulfamide and fipronil improved rice drought stress tolerance and cyprosulfamide also improved rice salinity stress tolerance.

2. ABA in combination with cyprosulfamide improved the positive effect of cyprosulfamide on rice stress tolerance.

3. The two main impacts of cyprosulfamide on rice plants were to induce the appearance of new tillers and to promote earlier flowering.

4. OsLEA3, a putative fumarylacetoacetate hydrolase and mitochondrial import inner membrane translocase were induced by salinity stress, ABA, cyprosulfamide and cyprosulfamide plus ABA.

5. Cyprosulfamide probably elevates in some way the endogenous levels of ABA, and this in turn induces the LEA genes.

6. *OsLEA3*, fumarylacetoacetate hydrolase and mitochondrial import inner membrane translocase genes could serve as marker genes for further screening and studies of new potential bioregulators.

7. Many proteins that were upregulated by stress after 11 days also were upregulated by cyprosulfamide or cyprosulfamide plus ABA on day1 or day 11 whether or not stress was applied.

8. Cyprosulfamide may pre-adapt rice plants to cope with salinity stress by inducing stress-response genes that are important in abiotic stress acclimatization.

9. More stress induced proteins are upregulated following treatment with cyprosulfamide plus ABA than due to cyprosulfamide alone indicating that ABA has a synergistic effect.

10. LEA proteins are induced through an ABA-dependent pathway and they are also induced by cyprosulfamide, suggesting that cyprosulfamide may enhance endogenous ABA levels.

Part B

Towards the engineering of nitrogen fixation in rice

Abbreviations

AM symbiosis	Arbuscular mycorrhizal symbiosis
bp	Base pair(s)
BSA	Bovine serum albumin
CaMV 35S	Cauliflower mosaic virus 35S
dNTP mix	Mix of deoxynucleotide 5`-triphosphate
EDTA	Ethylene diamino tetra acetic acid
e.g.	For example
h	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hpt	Hygromycin phosphotransferase gene
IRRI	International Rice Research Institut
IT	Infection thread
kDa	Kilodalton(s)
kg	Kilogram(s)
LB	Luria Burtoni medium
mg	Milligrams
MgATP	Magnesium adenosine triphosphate
min	Minutes
ml	Milliliter(s)
mM	Millimolar
mm	Millimeter(s)
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
ng	Nanogram(s)
Os	Oryza sativa (rice)
PAGE	Polyacrylamide gel
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
RNS	Root nodule simbiosis
RT-PCR	Reverse transcription polymerase chain reaction
s	Second(s)
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
SSC	Saline sodium citrate
SYM	Symbiotic component
T0	Primary transformants
T1	First transgenic generation
UV	Ultra violet
µg	Microgram(s)
µl	Microliter(s)

Abstract

Nitrogen is the most abundant gas in the atmosphere and it is also an important organic component of living organisms together with carbon, hydrogen and oxygen, forming the basis of both nucleic acids and amino acids. Animals get their nitrogen from plants and other animals, whereas plants get nitrogen from inorganic compounds in the soil, mostly nitrate and ammonium. Nitrogen gas is very stable and only diazotrophic bacteria and some algae can convert it to ammonia, a reaction catalyzed by the enzyme nitrogenase. This is a complex enzyme comprising two oxygen-sensitive proteins: Fe protein acting as a dinitrogenase reductase and MoFe protein acting as a dinitrogenase. Genes required for nitrogenase biosynthesis and activity are grouped in the *nif* gene cluster in bacteria. In 1972 the *Klebsiella pneumoniae nif* gene cluster was introduced into *Escherichia coli* creating for the first time a transgenic diazotroph that could fix atmospheric nitrogen.

The success of this experiment was the first step towards nitrogenase expression in plants, but because the enzyme is so complex only the Fe protein has been transferred thus far. The Fe protein was expressed in tobacco but it degraded rapidly because of its sensitivity towards oxygen. The discovery of an oxygen-insensitive nitrogenase in *Streptomyces thermoautotrophicus* suggests that this nitrogenase could be suitable for expression in plants.

Rice is the staple crop for much of the world's population, therefore the demand is increasing as the population grows, but production is being constrained by the loss of agricultural land to urbanization, pollution and other forms of abiotic stress. New approaches to increase rice productivity are required to guarantee food security and increasing nitrogen availability by introducing nitrogenase into rice plants could greatly enhance grain yields.

Particle bombardment is an efficient method for multiple gene engineering and it has been optimized for staple cereal crops including rice, maize and wheat. Using this method I generated transgenic rice plants stably expressing dinitrogenase reductase and dinitrogenase. Expression in transgenic plants was verified at the mRNA level, but only one subunit could be detected at the protein level and nitrogenase activity was not observed. Further studies are needed to address these challenges.

Resum

El nitrogen és el gas més abundant a l'atmosfera i també és un component orgànic important dels organismes vius juntament amb el carboni, l'hidrogen i l'oxigen, formant la base dels àcids nucleics i aminoàcids. Els animals obtenen el seu nitrogen de les plantes i altres animals, mentre que les plantes obtenen el nitrogen a partir de compostos inorgànics del sól, principalment de nitrats i amoni. El gas nitrogen és molt estable i només els bacteris diazotròfics i algunes algues poden convertir-lo en amoni, una reacció catalitzada per l'enzim nitrogenasa. Es tracta d'un complex enzimàtic format per dues proteïnes sensibles a l'oxigen: la proteïna Fe que actua com una dinitrogenasa reductasa i la proteïna MoFe que actua com a dinitrogenasa. Els gens necessaris per a la biosíntesi i l'activitat de la nitrogenasa s'agrupen en els bacteris en el clúster de gens *nif*. El 1972, el cluster *nif* de *Klebsiella pneumoniae* va ser introduït en *Escherichia coli* creant per primera vegada un diazòtrof transgènic que podria fixar el nitrogen atmosfèric. L'èxit d'aquest experiment va ser el primer pas cap a l'expressió de la nitrogenasa en plantes, però com que l'enzim és tan complex, fins al moment només s'ha pogut transferir la proteïna Fe. Aquesta proteïna es va expressar en tabac, però es va degradar ràpidament a causa de la seva sensibilitat cap a l'oxigen. El descobriment d'una nitrogenasa insensible a l'oxigen en *Streptomyces thermoautotrophicus* suggereix que aquesta nitrogenasa podria ser adequada per a l'expressió en plantes.

L'arròs és l'aliment bàsic per a gran part de la població mundial, per tant, la demanda està augmentant a mesura que creix la població, però la producció està limitada per la pèrdua de terres agrícoles augmentant la urbanització, la contaminació i altres formes d'estrès abiòtic. Nous enfocaments per augmentar la productivitat de l'arròs es requereixen per tal de garantir la seguretat alimentària i en aquest cas, poder augmentar la disponibilitat de nitrogen mitjançant la introducció de la nitrogenasa en plantes d'arròs podria augmentar la producció de gra en gran mesura.

El bombardeig de partícules és un mètode eficaç per a l'enginyeria de múltiples gens i aquest ha estat optimitzat per als cultius de gra bàsics com l'arròs, el blat de moro i el blat. Amb aquest mètode he generat plantes d'arròs transgèniques que expressen de manera estable la dinitrogenasa reductasa i la dinitrogenasa. L'expressió en plantes transgèniques es va verificar a nivell d'ARNm, però només una subunitat va poder ser detectada a nivell de proteïna i l'activitat de la nitrogenasa no es va observar. En

conclusió, podem dir que es necessiten estudis addicionals per adreçar aquests nous reptes.

Resumen

El nitrógeno es el gas más abundante en la atmósfera y es también un importante componente orgánico de los organismos vivos junto con el carbono, el hidrógeno y el oxígeno, formando la base de los ácidos nucleicos y aminoácidos. Los animales obtienen su nitrógeno de las plantas y otros animales, mientras que las plantas obtienen el nitrógeno a partir de compuestos inorgánicos en el suelo, principalmente nitratos y amonio. El gas nitrógeno es muy estable y sólo las bacterias diazotróficas y algunas algas pueden convertirlo en amoníaco, una reacción catalizada por la enzima nitrogenasa. Se trata de un complejo enzimático formado por dos proteínas sensibles al oxígeno: la proteína Fe que actúa como una dinitrogenasa reductasa y la proteína MoFe que actúa como dinitrogenasa. Los genes necesarios para la biosíntesis y la actividad de la nitrogenasa se agrupan en las bacterias en el clúster de genes *nif*. En 1972, el clúster *nif* de *Klebsiella pneumoniae* fue introducido en *Escherichia coli* creando por primera vez un diazótrofo transgénico que podría fijar el nitrógeno atmosférico.

El éxito de este experimento fue el primer paso hacia la expresión de la nitrogenasa en plantas, pero como la enzima es tan compleja, sólo la proteína Fe se ha transferido hasta el momento. La proteína Fe se ha expresado en tabaco, pero se degrada rápidamente debido a su sensibilidad hacia el oxígeno. El descubrimiento de una nitrogenasa insensible al oxígeno en *Streptomyces thermoautotrophicus* sugiere que esta nitrogenasa podría ser adecuada para la expresión en plantas.

El arroz es el alimento básico para gran parte de la población mundial, por lo tanto, la demanda está aumentando a medida que crece la población, pero la producción está limitada por la pérdida de tierras agrícolas a favor de la urbanización, por la contaminación y otras formas de estrés abiótico. Nuevos enfoques para aumentar la productividad del arroz se requieren para garantizar la seguridad alimentaria y en este caso, el aumento de la disponibilidad de nitrógeno mediante la introducción de la nitrogenasa en plantas de arroz podría aumentar la producción de grano en gran medida.

El bombardeo de partículas es un método eficaz para la ingeniería genética de múltiples genes y ha sido optimizado para los cultivos de grano básicos como el arroz, el maíz y el trigo. Utilizando este método he generado plantas de arroz transgénico que expresan de manera estable la dinitrogenasa reductasa y la dinitrogenasa. La expresión en plantas transgénicas se verificó a nivel de ARNm, pero sólo una subunidad pudo ser detectada a nivel de proteína y la actividad de la nitrogenasa no se observó. En conclusión se necesitan estudios adicionales para hacer frente a estos nuevos desafíos.

Chapter 1

General introduction

Fixed nitrogen is the most critical factor for plant growth and crop productivity after water. Nitrogen gas (N_2) is converted into a number of complex organic molecules including amino acids, proteins and nucleic acids. Approximately 78% of the atmosphere is N_2 but this is unusable because of the strong triple bond between the nitrogen atoms. N_2 must therefore be converted to chemically available forms of nitrogen such as ammonium and nitrate.

Three different nitrogen fixation processes are known: atmospheric, biological and chemical. Atmospheric fixation occurs when lightning provides the energy to break nitrogen triple bonds, allowing nitrogen and oxygen atoms to form nitrogen oxides that dissolve in atmospheric water and fall to the ground as rain. About 5–8% of all fixed nitrogen is created in this manner. Biological fixation is carried out by certain bacteria and archaea and will be discussed below. Chemical fixation is a human industrial process that yields up to 85 million tonnes of fixed nitrogen a year (Vitousek et al., 1997). Atmospheric nitrogen is combined with hydrogen from fossil fuels at high temperatures and pressures and in the presence of a metal catalyst to form ammonia, which is used directly as a fertilizer, or further converted to urea and ammonium nitrate (NH_4NO_3). The production of these fertilizers is expensive because non-renewable fossil fuels are required and they are also sources of environmental pollution (Peters et al., 1995).

1.1. Nitrogen cycle

In nature, nitrogen exists in multiple organic and inorganic forms that are interconverted by the nitrogen cycle (**Figure 1.1**). Organic forms of nitrogen such as proteins and nucleic acids are synthesized by living organisms and enter the soil in the form of decomposing organic matter, such as animal carcasses, plant roots and leaves, manure/sewerage and compost. This is eventually converted to humus by the activity of soil organisms, and is a rich source of inorganic forms of nitrogen such as ammonium (NH_4^+), ammonia (NH_3), nitrate (NO_3^-), and nitrite (NO_2^-). Inorganic forms of nitrogen are available to plants and soil microorganisms, and they are also mobile in the soil as

water moves through it, but most soil nitrogen is organic and unavailable. The decomposition of organic matter eventually produces nitrogen gas which enters the atmosphere but this occurs in stages, first yielding ammonia and ammonium ions that are rapidly oxidized to nitrite and nitrate. Ammonia is only stable under anaerobic conditions (such as waterlogged soils). Nitrifying bacteria oxidize ammonia to nitrites and nitrates, whereas *Nitrosomonas* (nitrite) and *Nitrobacter* (nitrate) oxidize ammonium to generate energy for carbon fixation, a process known as nitrification:

$NH_4^+ + 1½O_2 \rightarrow NO_2^- + H_2O + 2H^+$

$NO_2^- + ½O_2 \rightarrow NO_3^-$

Denitrification involves the conversion of nitrate to nitrogen. Some bacteria, such as *Pseudomonas*, are able to use nitrate as an electron acceptor in respiration (Carlson and Ingraham, 1983):

$2NO_3^- + 12H^+ + 10e^- \rightarrow N_2 + 6H_2O$

Plants assimilate nitrogen in the form of nitrate and (more rarely) ammonium ions. Plant nitrogen assimilation is described in detail below. Animals generally assimilate nitrogen by breaking protein down into amino acids and reusing them. When plants and animals decay, putrefying bacteria produce ammonia from the proteins they contain. Animals also produce breakdown products such as ammonia, urea, allantoin and uric acid from excess dietary nitrogen. These compounds are also targets for ammonification by bacteria.

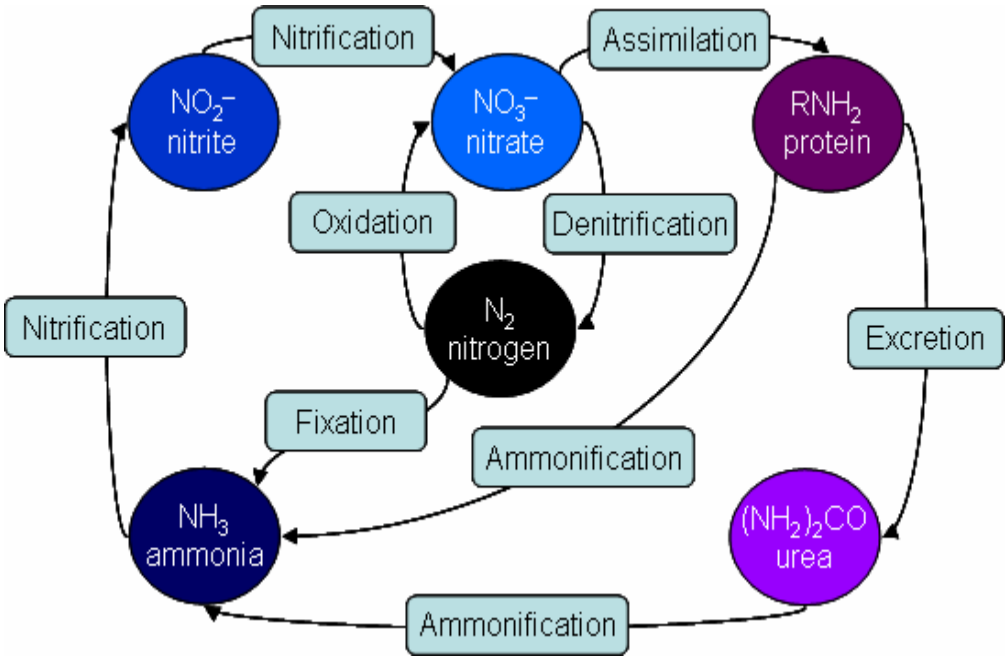

Figure 1.1. The nitrogen cycle (Sprent, 1987).

1.2. Nitrogen fixation by microorganisms

Bacteria that can fix nitrogen form three groups: free-living nitrogen fixers, symbiotic fixers and associative fixers. The free-living nitrogen fixers include anaerobic bacteria such as *Clostridium pasteurianum* and aerobic bacteria such as *Azotobacter chroococcum*. They fix 1–5 kg of nitrogen per hectare annually (Beringer and Hirsch, 1984). Symbiotic bacteria from the genus *Rhizobium* grow within a plant host and form specific structures called nodules. This group is further classified according to the host species (e.g. *Rhizobium trifolii* for clovers and *Rhizobium japonicum* for soybean). Symbiotic microorganisms are much more efficient than free-living ones, fixing 100–200 kg of nitrogen per hectare annually. Finally, the associative fixers also live within a host plant but do not form nodules. One example is *Acetobacter diazotrophicus*, which is associated with sugar cane; it donates some of its fixed nitrogen to the plant in exchange for sugar. *A. diazotrophicus* can fix up to 150 kg nitrogen per hectare in this crop.

Each of the nitrogen-fixing bacteria employs the same enzyme (nitrogenase), which converts N_2 into two molecules of NH_3 as shown below. This is known as biological nitrogen fixation.

$N_2 + 8e^- + 8H^+ + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16Pi$

Nitrogenase requires a large amount of energy to convert N_2 to NH_3. In some bacteria, this energy can be preserved if they contain ammonia. For example, the addition of ammonia to *Rhodospirillum rubrum* cultures inactivates nitrogenase through a regulatory system that covalently modifies the enzyme, rendering it inactive (Pope et al., 1985).

1.2.1. *Nif* genes encoding nitrogenase

Nitrogen fixation in bacteria such as *Klebsiella pneumoniae* requires the expression of 16 *nif* genes (**Figure 1.2**). The comparison of *nif* genes from different diazotroph organisms has revealed a number of genes that are probably essential for nitrogenase activity, i.e. *nif* H, D, K, Y, T, E, N, X, U, S, V, Z, W, M, B and Q (Dean and Jacobson,1992; Merrick,1993). Some of them form structural components of the enzyme and others are required for enzyme activation and full activity.

The nitrogenase structural genes are *nif*H (encoding an iron-binding protein that contains a single Fe_4S_4 cluster), and *nif*D and *nif*K which encode a tetrameric MoFe-binding protein with the conformation $\alpha_2\beta_2$. The primary translation products of the nitrogenase structural genes (*nif*HDK) are not active, and additional *nif* gene products are required to process the immature nitrogenase structural components to yield active forms (Dean et al., 1993).

The biochemical and genetic analysis of *nif* genes has helped to determine their functions (particularly complementation analysis using extracts prepared from different nitrogenase-defective mutants). The *nif*M product processes the immature form of the Fe protein NifH, and the *nif*S and *nif*U products are needed for the synthesis of the Fe_4S_4 cluster, helping to mobilize sulfur and iron respectively. The *nif*H, *nif*E, *nif*N and *nif*B gene products are absolutely required for FeMo-cofactor biosynthesis because, in their absence, only the apo form of the MoFe protein is produced (Dean et al 1993). The *nif*E and *nif*N gene products form a complex upon which FeMo-cofactor is preassembled. The *nif*V gene product is required for formation of homocitrate, the organic constituent of the FeMo cofactor. The *nif*Q and *nif*B gene products are required to form the metal core of the FeMo-cofactor. The *nif*Y gene product stabilizes a conformation of the apo-MoFe protein allowing the FeMo cofactor to be inserted.

Mutations in some of the genes (particularly *nif*T, *nif*Y, *nif*X, *nif*W, *nif*U, *nif*S, and *nif*Z) reduce but do not eliminate nitrogenase activity (Dixon et al 1997). There are other regulatory genes such as *nif*A, which encodes an enhancer binding protein for the *nif* cluster, and additional genes outside the *nif* operon encode molecular chaperones that are required for full nitrogenase activity (Dixon et al 1997).

The first *Rhizobium* genes for nitrogen fixation (*nif*) and for nodulation (*nod*) were cloned in the early 1980s (Meade et al., 1982) and soon many more *nif*, *nod* and *fix* (symbiotic fixation) genes were identified (Spaink et al., 1998).

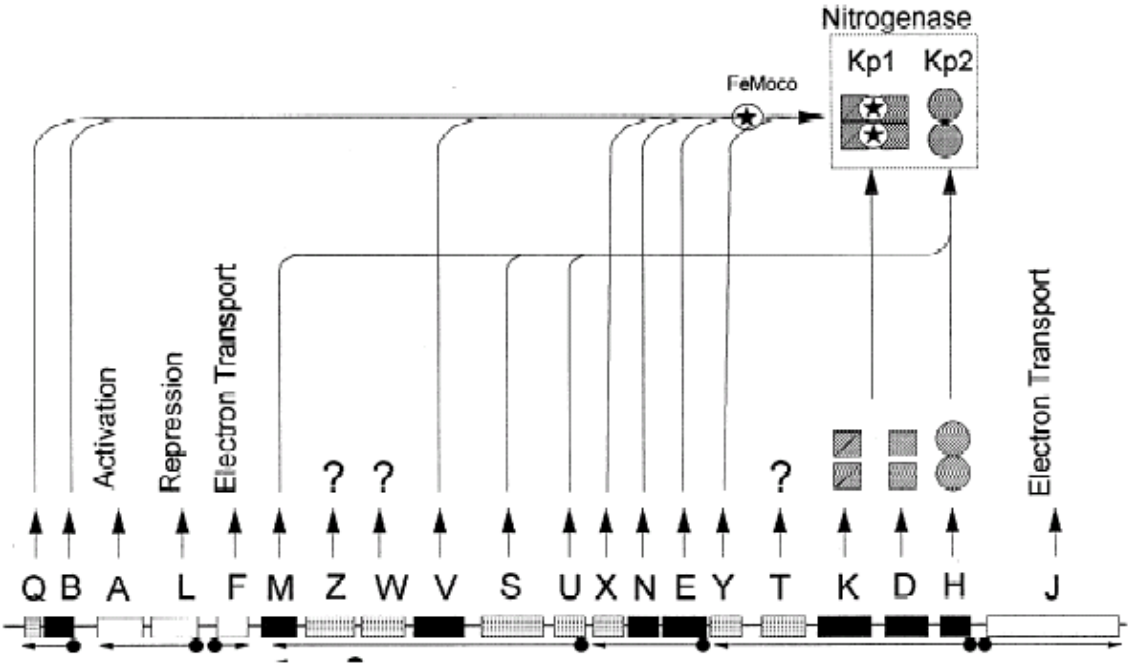

Figure 1.2. Map of the *nif* gene cluster in *Klebsiella pneumoniae* (Dixon et al., 1997). Functions of the genes if known are indicated above the arrows. Black boxes show essential "core" genes. Grey boxes indicate genes that can be potentially substituted by homologs from plants. White boxes indicate non-essential genes that probably are not required for the expression of nitrogenase in chloroplast.

1.2.2. Transferring the *nif* gene cluster from *Klebsiella pneumoniae* to *Escherichia coli*

The *nif* gene cluster was transferred from *Klebsiella pneumoniae* to *E. coli* by Dixon

and Postgate (1972) thus creating the first engineered diazotroph. Nitrogenase activity in the transformed *E.coli* was confirmed anaerobically by acetylene reduction in NH_4^+-free medium. In addition, nitrogen fixation in *E. coli* was demonstrated directly using $^{15}N_2$. When ammonium was added to the medium, acetylene reduction was completely repressed indicating that the control genes as well as structural *nif* genes were functional in the *E. coli* background.

1.2.3. Nitrogenase diversity

MoFe nitrogenase is highly conserved among aerobes, anaerobes, free-living and symbiotic nitrogen fixers (Burris 1991). This was shown by producing active enzyme complexes by combining the dinitrogenase and dinitrogenase reductase enzymes from different species. It was initially thought that MoFe was the only form of nitrogenase and that molybdenum was a prerequisite for dinitrogenase activity. However bacteria such as *Azotobacter uinelandii* were found to grow in media lacking molybdenum (Bishop et al., 1980) leading to the discovery of enzymes with vanadium or iron in the metal cluster instead of molybdenum. The vanadium and iron dinitrogenases have three subunits, rather than the two subunits characteristic of MoFe dinitrogenases (Burris 1991). In 1990 a fourth class of nitrogenase was found in the bacterium *Streptomyces thermoautotrophicus* (Gadkari et al., 1990). This nitrogenase consists of three components: molybdenum-CO dehydrogenase, molybdenum-dinitrogenase and a manganese-superoxide oxidoreductase. The striking difference of *S. thermoautotrophicus* nitrogenase from other three classes of nitrogenases is that all three components of the enzyme complex are insensitive to oxygen.

1.2.3.1. Components of nitrogenase and their roles in nitrogen fixation

Nitrogenase consists of the Fe protein (or component II, dinitrogenase reductase) and the MoFe protein (or component I, dinitrogenase) reflecting their metal compositions. The Fe protein (64 kDa) is a γ_2 homodimeric protein containing four Fe atoms organized in a Fe_4S_4 cluster. The MoFe protein is an $\alpha_2\beta_2$ tetramer (»250 kDa) containing 30 Fe atoms and two Mo atoms organized into two pairs of metalloclusters (P clusters) and FeMo-cofactors. Each αβ dimer contains one FeMo-cofactor paired with one P cluster. Nitrogenase metalloclusters play critical roles in electron transfer

and substrate reduction.

During catalysis, electrons are delivered one at a time from the Fe protein to the MoFe protein in a gated process involving the association and dissociation of the component proteins and the hydrolysis of at least two MgATP molecules for each electron transfer (Dean et al., 1993; Peter et al., 1995; Mayer et al., 2001). During MgATP binding and hydrolysis, electrons are transferred from the Fe_4S_4 cluster to the P cluster, which then mediates the intramolecular transfer of electrons to the FeMo-cofactor, providing the substrate-binding and reduction site (**Figure 1.3**).

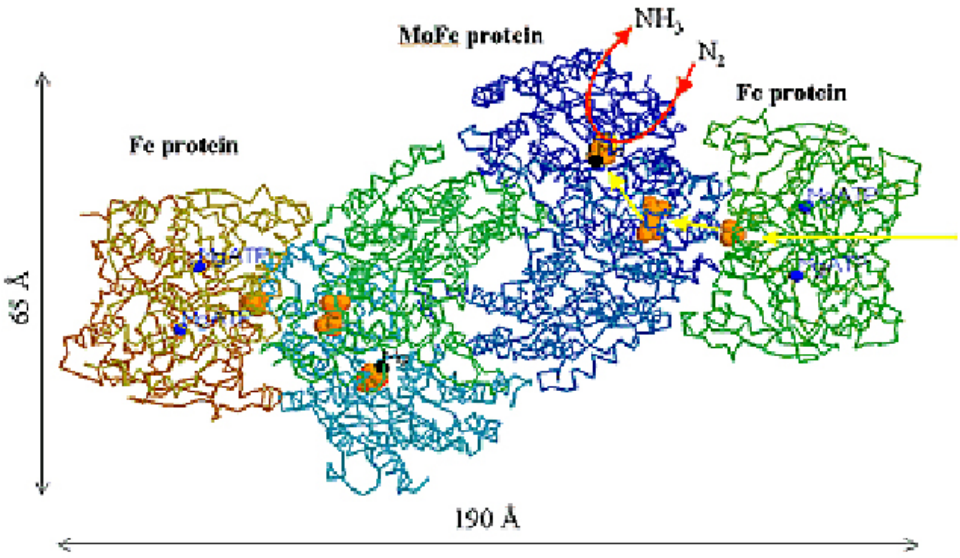

Figure 1.3. Structure of a molybdenum nitrogenase consisted of MoFe and Fe proteins (Seefeldt et al., 2009). A MoFe protein binds two Fe proteins, with each αβ-unit functioning as a catalytic unit. One Fe protein is shown associating with one αβ-unit of the MoFe protein. The relative positions of two bound MgATP molecules, the Fe protein [4Fe-4S] cluster, the MoFe protein P cluster (8Fe-7S), and the FeMo-cofactor (7Fe-Mo-9S-homocitrate-X) are shown. The electrons flow from the Fe protein [4Fe-4S] cluster to the MoFe protein P cluster and then to the FeMo-cofactor. The electron flow is shown by yellow arrows and the clusters - by orange spheres. Red arrows indicate conversion of N_2 to NH_3 in the reduction site of the FeMo cofactor.

1.2.3.2. Nitrogenase component genes from *S. thermoautotrophicus*

Streptomyces thermoautotrophicus is a thermophilic, aerobic, chemolithoautotrophic bacterium that is naturally enriched in soil covering burning charcoal piles (Gadkari et al., 1990). The bacterium uses gases as sources of energy, carbon and nitrogen, and can fix N_2 with CO or H_2 plus CO_2 to use as growth substrates (Gadkari et al., 1992). This free-living dinitrogen fixer has an unusual nitrogenase system because it is constitutively expressed and can fix N_2 with H_2 and CO even though these gases usually inhibit nitrogenase activity in other bacteria. Additional unusual features of the *S. thermoautotrophicus* nitrogenase include its insensitivity to oxygen, acetylene, ethylene and CO (Gadkari et al., 1992). The *S. thermoautotrophicus* nitrogenase is unable to reduce acetylene to ethylene or ethane, regardless of whether H_2 or CO is provided as the energy source. Instead, purified *S. thermoautotrophicus* nitrogenase can reduce azide, cyanide and nitrite to ammonium, in the presence of MgATP and dithionite (Gadkari et al., 2001).

The *S. thermoautotrophicus* nitrogenase comprises three enzymes: CO dedehydrogenase (St3), a manganeso-superoxide oxidoreductase (St2) and a molybdenum-dinitrogenase (St1) (**Figure 1.4**). All three components are insensitive to O_2 and H_2O_2, and can actually utilize O_2 in the reaction (Ribbe et al., 1997). The CO dehydrogenase generates electrons during CO oxidation ($CO + H_2O \rightarrow CO_2 + 2e^- + 2H^+$) and transfers them to O_2 thereby producing superoxide anion radicals (O_2^-) and hydrogen peroxide. The manganese component of the superoxide oxidoreductase reoxidizes the O_2^- anions to O_2 and transfers the electrons to the MoFeS dinitrogenase where they are used to reduce N_2 to ammonium:

$N_2 + 4\text{-}12\ MgATP + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2 + 4\text{-}12\ MgATP + 4\text{-}12\ Pi$

Ribbe et al. (1997) revealed the two-component nature of *S. thermoautotrophicus* nitrogenase in an experiment where St1 and St2 reduced N_2 to NH_4^+ whereas the separate enzymes did not. Denaturing PAGE revealed three non-covalently bound St1 subunits of 93, 33 and 18 kDa, designated L, M and S, respectively. This differs from other known dinitrogenases, which are $\alpha_2\beta_2$ tetramers. St2 was found to be a 48 kDa homodimer comprising two 24 kDa O subunits.

H_2 is a specific and competitive inhibitor of biological N_2 fixation even though it is an obligatory product of the fixation reaction. At least 25% of the energy used for N_2

fixation is used to produce H_2, resulting in substantial loss of efficiency.

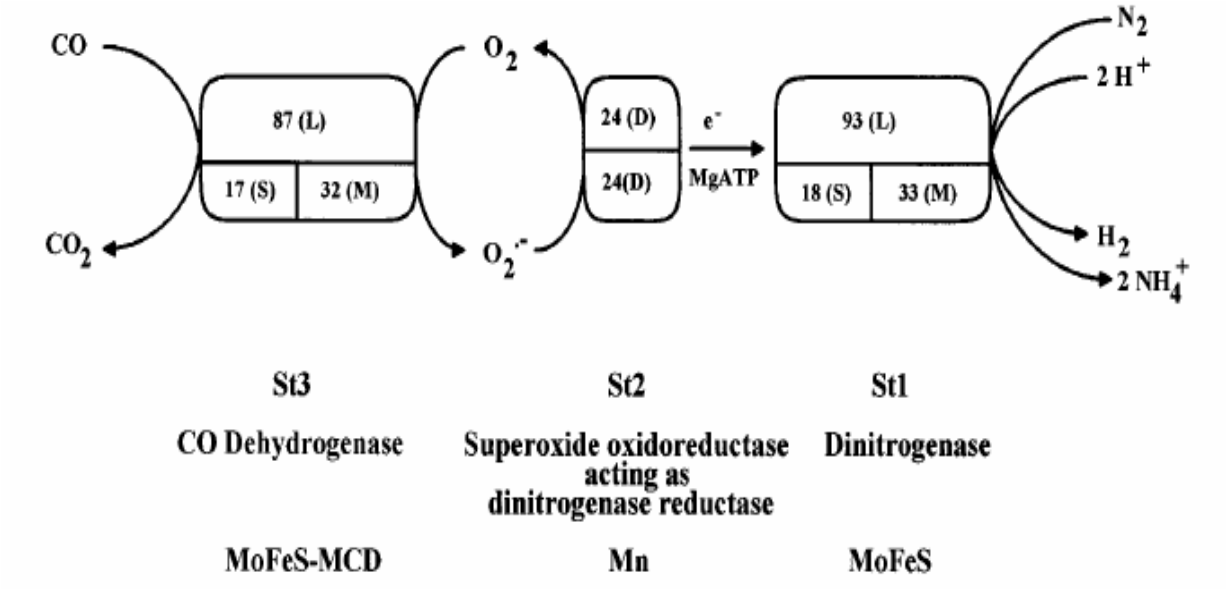

Figure 1.4. Schematic representation of N_2 fixation in *S. thermoautotrophicus* (Ribbe et al., 1997).

1.3. Nitrogen fixation in plants

1.3.1. Plants and symbiotic nitrogen-fixing microorganisms

In 1886, Hellriegel and Wilfarth discovered that the root nodules of leguminous plants (now reclassified as the family Fabaceae) contained microorganisms that were able to assimilate atmospheric nitrogen and convert it into ammonia (Hellriegel and Wilfarth, 1888). Two years later, Beijerinck isolated root-nodule bacteria from several Fabaceae and demonstrated that these bacteria induced nodule formation (Beijerinck, 1888). They were allocated the genus *Rhizobium* (*rhiza* = root; *bios* = life). These discoveries led to important agronomic practices such as inoculating Fabaceae seeds with *Rhizobium* spp. before planting to ensure the formation of nodules for effective nitrogen fixation (Brockwell, 1995). Even modern agricultural systems depend on nitrogen fixation by bacteria in alfalfa, clover and other Fabaceae to supplement chemical nitrogen fertilizers. This intimate relationship between plants and bacteria benefits both organisms, the bacteria receiving sugar in exchange for nitrogen.

Rhizobium is now divided into a number of distinct genera, including *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium* and *Mesorhizobium* (van Berkum and Eardly, 1998). The symbiotic interactions are highly specific, involving only the Fabaceae (Long 1996).

The symbiotic process begins when the plant secretes signaling molecules such as flavonoids, different compositions being produced by different species and by plants differing in age and physiological status (Long 2001). Flavonoids activate bacterial nodulation (*nod*) genes, which are expressed only in response to plants and not in free-living bacteria. The expression of bacterial *nod* genes induces the synthesis of lipochitooligosaccharides that elicit nodule formation on host plant roots and trigger the infection process. Isolated *nod* gene products can induce root hair deformation and/or curling, pre-infection thread formation and cortical cell division in host plants (Lhuissier et al., 2001), but nodules only form in the presence of the bacteria. The activation of Nod factors and proline-rich early nodulins (ENODs) located in the cell wall results in extensive morphological changes beginning 12–24 hours after bacterial infection, culminating in the formation of nodules on the plant and enlarged bacterial cells known as bacteroids.

The oxygen concentration inside the nodule must be closely regulated, because oxygen inhibits nitrogenase activity. This is achieved by the presence of leghemoglobin, an oxygen-binding protein similar to hemoglobin. The heme (oxygen-binding) portion is produced by the bacterium, whereas the globin (protein) portion is produced by the host plant, again illustrating the closeness of the symbiotic relationship. Oxygenated leghemoglobin turns the inside of an oxygen-free nodule red or pink, which indicates an active nitrogen-fixing nodule.

Although the majority of biological nitrogen fixation reflects the symbiotic relationship between bacteria and Fabaceae, two different organisms form similar relationship that results in nitrogen fixation under water: the water fern *Azolla* and the blue-green filamentous cyanobacterium *Anabaena azollae*. The bacteria enter the fern tissue through extracellular cavities at the tip of growing shoots (Hill 1977). Nitrogen fixation takes place in specialized *Anabaena* cells known as heterocysts that are photosynthetically inactive and represent up to 10% of the cells in a filament. Heterocysts develop thick cell walls that limit the influx of O_2 and other gases, while vegetative cells remain actively photosynthetic and pass the photosynthate to the heterocysts. The heterocysts in turn use this photosynthate to fix N_2, and they export

fixed nitrogen to the vegetative cells. Cyanobacteria photosynthesis is supplemented by that of the water fern.

This highly effective symbiotic system has been used in Southeast Asia for hundreds of years to add fixed nitrogen to rice fields. *Azolla* is grown in rice paddies early in the season. As the rice grows above the water surface, it shades out the fern, which dies, releasing the stored nitrogen. In this way, the paddy is fertilized without the application of chemical fertilizers. Over 100 kg of nitrogen is fixed per hectare per season, and as the water fern and cyanobacteria decompose, they release fixed nitrogen to the rice (Burris 1991).

1.3.2. Assimilation of nitrogen by plants

Plants take up nitrogen via the roots and it is transported to other parts of the plant through the xylem (**Figure 1.5**) (Masclaux-Daubresse et al., 2010). Within symbiotic nodules, nitrogen gas from the atmosphere is converted into ammonia and then transferred by the same mechanism to other parts of the plant. Fabaceae accumulate more nitrogen in seeds that are rich in proteins.

Plants assimilate nitrogen in the form of nitrate and (rarely) ammonium. The nitrate is first reduced to ammonium, and then combined into organic forms, generally via glutamate. Nitrates get into most plants by a proton-nitrate symport, powered by a V-type ATPase. Once inside, nitrate is converted to nitrite by cytoplasmic nitrate reductase.

$NO_3^- + 2e^- + 2H^+ \rightarrow NO_2- + H_2O$

Nitrite is rapidly translocated into the plastids because it is very toxic. Plastids contain nitrite reductase that converts nitrite to ammonia.

$NO_2^- + 6e^- + 8H^+ \rightarrow NH_4^+ + 2H_2O$

The ammonia is assimilated by glutamate synthase into the amino acid glutamine. The NH_2 group is then moved onto other carbon skeletons to form other amino acids by transaminases (aminotransferases). Glutamine synthetase produces the amino acid glutamine from glutamate and ammonia, and this is the main route by which ammonia enters plant metabolism.

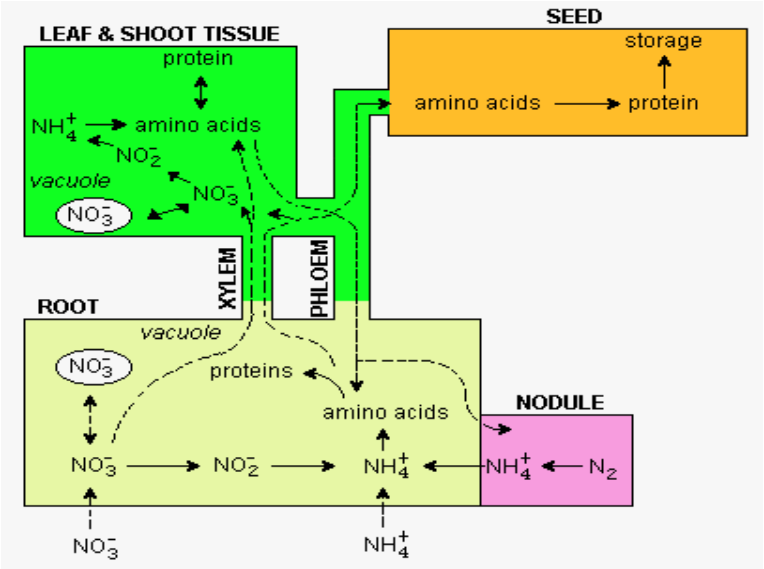

Figure 1.5. Assimilation of nitrogen by plants (Masclaux-Daubresse et al., 2010).

1.4. Genetic engineering of plants to enhance nitrogen fixation

The world population is expected to exceed nine billion by the year 2050 and much more food will be required. One of the main constrains affecting crop yields is nitrogen fixation, which is currently addressed by the use of chemical nitrogen fertilizers that cost $45 billion per year (Ladha and Reddy, 2000). In addition nitrogen-containing fertilizers are the major source of pollution.

Rice needs 1 kg of nitrogen to produce 15–20 kg of grain, and before chemical fertilizers were available, the productivity of rice depended almost entirely on biological nitrogen fixation (Reddy and Ladha, 2000). Since the Green Revolution, chemical nitrogen fertilizers have boosted rice yields by 100–200%, matching the demands of the growing human population (Ladha and Reddy, 2000). It is estimated that rice production must exceed 12 tons per hectare in 2020 (compared to the current levels of 4.37 tons per hectare) to sustain the population, and that fertilizer applications should be increased from 220 to 400 kg per hectare to achieve this. An alternative solution is therefore required to provide rice with inexpensive and environmentally-beneficial fixed nitrogen.

In the 1980s and 1990s, research was carried out to optimize biofertilizer technologies. Under favorable conditions, blue-green algae can fix 2040 kg of nitrogen per hectare (Roger and Watanabe, 1986) and cyanobacterial inoculation is a low-cost technology.

However, there are many constraints that limit its success such as phosphate deficiency, the presence of high nitrate levels in floodwater, low pH, and arthropod grazer populations that limit the growth of cyanobacteria in rice fields.

As an alternative, the water fern *Azolla* and the semiaquatic legume *Sesbania* can potentially produce 6–8 tons of nitrogen per hectare, roughly equivalent to the application of 100–200 kg urea (Watanabe and Liu, 1992). However, *Azolla* and *Sesbania* attract additional labor costs and therefore offer no economic advantages to farmers.

1.5. The need for *in planta* biological nitrogen fixation

It is clear that neither chemical fertilizers nor biofertilizers can address the increasing demand for rice production and that a second Green Revolution is now required, based on new technologies. The ability to fix nitrogen biologically in cereals would increase productivity significantly without damaging the environment. Potential solutions include the discovery of endophytic diazotrophs that can fix nitrogen in rice, and the genetic engineering of rice to endow them with the ability to fix their own nitrogen. We discuss these alternative strategies below.

1.5.1. Endophytic associations between rice and nitrogen-fixing bacteria

Endophytic diazotrophs have the potential to infect many non-Fabaceae plants. For example, *Acetobacter diazotrophicus* is an endophytic acid-tolerant nitrogen-fixing bacterium (Cavalcante and Dobereiner, 1988) associated with sugarcane, which provides abundant nitrogen to sugarcane crops (Gillis et al., 1989; Boddey et al., 1991). *A. diazotrophicus* can also form natural associations with wetland rice cultivated in the fields of South India (Muthukumarasamy et al., 2005). Unfortunately there is no evidence that this association results in nitrogen fixation for rice plants.

Herbaspirillum spp. are predominantly diazotrophic species, some of which colonize plants. The inoculation of wild rice (*Oryza officinalis* W0012) with *Herbaspirillum* spp. strain B501 in the presence of $^{15}N_2$ resulted in the assimilation of ^{15}N by the rice plants, but there was no evidence that the same bacterial strain can colonize cultivated rice (Elbeltagy et al., 2001).

Azoarcus spp. are endophytes of Kallar grass that can also colonize rice roots under laboratory conditions (Egener et al., 1999). The *nifH* gene was fused to *gusA* and *gfp* and introduced into *Azoarcus* spp. strain BH72. Quantitative GUS and nitrogenase assays based on acetylene reduction showed that cortex cells of rice roots were able to support the expression of *Azoarcus nif* genes, but the results were inconsistent. The next step would be to isolate *Azoarcus* strains that can colonize rice plants in the field.

Like *Acetobacter diazotrophicus* and *Azoarcus* spp. *Rhizobium* spp. can also grow endophytically in the roots of graminaceous plants. Since antiquity, rice in Egypt has been grown in rotation with clover, which replaces 25–33% of the recommended fertilizer input. Yanni et al. (1997) investigated whether this reflected the availability of fixed nitrogen through the mineralization of nitrogen-rich clover crop residues, or the endophytic colonization of rice by *Rhizobium leguminosarum* bv. *trifolii*. They found bacteria present naturally inside the roots of rice plants grown in rotation with clover in Egypt, but there were no nodule-like structures (Yanni et al., 1997). Pure cultures of *Rhizobium leguminosarum* bv. *trifolii* were able to colonize the upper portion of rice roots grown in sterile media or in the field, increasing the shoot and grain yields of rice plants in laboratory tests and in the field. However, the stimulation of growth is not likely to reflect enhanced nitrogen fixation within the rice plants because no acetylene reduction activity was detected.

Photosynthetic *Bradyrhizobium* strains are known specifically to induce nitrogen-fixing nodules on the stems and roots of aquatic legumes of the genus *Aeschynomene* (Molouba et al., 1999). These photosynthetic symbiotic bacteria also form a natural endophytic association with the wild rice species *O. breviligulata* (Chaintreuil et al., 2000). A low but significant level of nitrogen-fixing (acetylene reducing) activity was detected in 4-week-old rice plants inoculated with the photosynthetic strain ORS278, but not in controls. *Bradyrhizobium* strain ORS278 increased shoot and grain yields by 20% in the greenhouse, but the nitrogen-fixing activity detected by acetylene reduction was too low to support the observed increase, which instead probably reflected the secretion of indoleacetic acid, gibberellic acid and phytohormones that promote rice growth.

1.5.2. Engineering root nodule symbiosis in non-Fabaceae species

The Fabaceae are well known for their intimate relationship with diazotrophic microorganisms, which provide fixed nitrogen in exchange for carbohydrates. This relationship does not exist for most non-Fabaceae plants and the ability to engineer root nodule symbiosis (RNS) in non-Fabaceae plants, specifically in cereals, would be a massive breakthrough that would increase crop productivity significantly (Charpentier and Oldroyd, 2010).

Nodulation occurs in three major steps: mutual recognition, bacterial infection and root nodule development. Many specific molecular components are required for each step, so transferring RNS to agriculturally important non-nodulating plant species such as rice, maize and wheat is a significant challenge that will require engineering methods mimicking the evolutionary processes that originally occurred in the Fabaceae. Many components are needed to confer the ability to recognize rhizobial bacteria, to tolerate infection and to form root nodules, but some of the components are already present in cereals and the challenge is to understand the gaps and the role of plant hormones in the bacterial recognition and nodulation processes.

1.5.2.1. Engineering the symbiotic component pathway so that cereals recognize rhizobial bacteria

As stated above, the recognition process between Fabaceae and rhizobial bacteria begins when the plant secretes flavonoid compounds from the roots, which induce the production and secretion of lipochitooligosaccharides (Nod factors) by the bacteria (Spaink, 1996). These are recognized by Nod factor receptors, which bind the Nod factors produced by particular bacterial species. There are at least eight common symbiotic components (SYMs) acting downstream of the Nod factor receptors, including SYMRK, three components of the nucleopore (NUP85, NUP133 and NENA), two cation channels (CASTOR and POLLUX), a calcium/calmodulin-dependent kinase (CCaMK) and a nuclear-localized protein with coiled-coil motif (CYCLOPS) (Charpentier and Oldroyd, 2010).

RNS is thought to have evolved from the more ancient arbuscular mycorrhizal (AM) symbiosis, an interaction between land plants and fungi from the phylum Glomeromycota (Kistner et al., 2005). At least five of these eight SYM components

have AM functions in rice, and four of them can functionally restore AM symbiosis in the corresponding Fabaceae mutants. An understanding of how these SYM components evolved to function in RNS will help in the development of strategies to engineer the SYM pathway in cereals to recognize nitrogen-fixing bacteria (Charpentier and Oldroyd, 2010).

1.5.2.2. Engineering rhizobial entry in non-Fabaceae species

Rhizobial bacteria can invade host plants by root-hair-dependent and root-hair-independent routes. In the former case, bacteria enter via epidermal root-hair cells, whereas in the latter they enter at points of epidermal damage where lateral roots emerge, or between epidermal cells at the base of root-hairs. Root-hair-independent infection is the more primitive mechanism, and this may be the more realistic strategy for achieving rhizobial entry in non-Fabaceae plants.

Bacterial infection via root hairs is stringently regulated in the epidermis by the production of an infection thread (IT) by the plant host. The root-hairs deform and curl, trapping the bacteria in an infection pocket that elaborates the IT, an inward growing tubular structure. IT development and progression requires many components, including SYM components, Nod factors, Nod factor receptors, and proteins that reorganize the cytoskeleton. In *Medicago truncatula*, the VAPYRIN protein is essential for IT development, and is also necessary for mycorrhizal colonization (Pumplin et al., 2009). This suggests that some components of the infection machinery were recruited from the pre-existing mycorrhizal symbiosis, providing further encouragement for the development of nitrogen-fixing cereals.

1.5.2.3. Engineering of nodule organogenesis in non-Fabaceae species

Previously it was thought that nodules only form in the presence of the bacteria but Gleason et al. (2006) showed that bacterial infection and nodule organogenesis are genetically separable. When Nod factor is perceived by plant, Ca^{2+} *and* Ca^{2+} *calmodulin (CaM) levels are increased and trigger the activation of* Ca^{2+}/calmodulin-dependent protein kinase (CCaMK) that is known to play a major role in the development of diverse nodule structures. The activation of CCaMK is achieved by CaM suppression of the CCaMK autoinhibition domain. The specific removal of the autoinhibition domain

leads to constant activation of the nodulation signaling pathway and the spontaneous development of nodules in the absence of bacteria in *Medicago truncatula* (Gleason et al., 2006). Plant hormones are also known to be involved in nodule organogenesis because the addition of cytokinin or withdrawal of auxin promotes nodulation (Mathesius et al., 1998).

1.5.3. Engineering nitrogenase activity in non-Fabaceae species – how many genes are required?

The expression of nitrogenase genes in higher non-Fabaceae plants is a very complex task. The proteins must be targeted to the most appropriate cellular compartment, and the plastid has several advantages because its genes are organized and expressed like those of bacteria, it is the major site of ammonium uptake by glutamine synthetase and it is a major site of amino acid biosynthesis. Many electron donors are present in the chloroplast and may act together with nitrogenase so there is no need to introduce additional electron donors (Dixon et al., 1997).

One potential drawback of the plastid is that during the day it is the site of photosynthesis, producing oxygen that is harmful to nitrogenase. Temporal or spatial separation is needed for uninhibited nitrogenase activity. One solution is to fix nitrogen only in darkness, a method employed naturally by the aerobic nitrogen-fixing cyanobacterium *Gloeothece*, which provides ATP and a reductant generated during daylight to support the process. Alternatively, nitrogenase can be expressed in amyloplasts, which do not carry out photosynthesis.

The expression of 16 *nif* genes plus others is required to maintain nitrogenase activity. Most diazotrophs contain a common core of *nif* genes (*nif H, D, K, Y, T, E, N, X, U, S, V, Z, W, M, B* and *Q*) but several of these genes (*nifY, T, X, U, S, Q, W* and *Z*) can be mutated without eliminating nitrogenase activity completely (Dean and Jacobson, 1992; Merrick, 1993). Homologs of *nifS* and *nifU* are found in *E. coli* and chloroplasts.

Genes that contribute to the synthesis of the nitrogenase metallocluster (*nif*H, *nif*E, *nif*V and *nif*B) are also required for heterologous nitrogen fixing because the MoFe enzyme and its cofactor are not present in plastids. The primary translation products of *nif* H, D and K are inactive (Dean et al., 1993), and *nif*M is required for the activation and stability of the Fe protein. When *nif*H was expressed in *E. coli* or yeast in the absence of

*nif*M, only a small amount of the dimeric Fe protein was synthesized (Berman et al., 1985; Paul and Merrick, 1989).

The most straightforward approach is to express just one subunit Fe protein (which requires only *nif*H and *nif*M) The Fe_4S_4 cluster can be synthesized by endogenous genes and the activity of the Fe protein can then be measured in vitro by complementation with purified MoFe protein.

1.5.3.1. Introduction of *nif*H into the *Chlamydomonas reinhardtii* chloroplast genome

Gymnosperms, green alga, cyanobacteria and the photosynthetic bacteria *Rhodobacter capsulatus* possess a light-independent pathway for chlorophyll biosynthesis in which chlorophyll *a* and *b* are synthesized in complete darkness. Three chloroplast genes encoding protochlorophyllide reductase are required for this pathway (*chl*L, *chl*N and *chl*B) and these are related to the three nitrogenase subunits. ChlL and the dinitrogenase reductase Fe protein (NifH) show 32–35% identity; both exist as homodimers in solution and both bind ATP. In order to determine whether NifH can substitute for ChlL, the unicellular green alga *Chlamydomonas reinhardtii* was chosen as a model and chloroplast transformation was performed to introduce *Klebsiella pneumoniae nif*H into the plastid genome (Cheng et al., 2005). When *chl*L was replaed by *nif*H, the transgenic line was partially green in the dark, whereas the wild-type was green in the dark and *chlL*$^-$ mutants were yellow in the dark. Fluorescence emission studies showed that the transgenic algae possessed the characteristic chlorophyll *a* and *b* emission spectrum, confirming the conversion of protochlorophyllide to chlorophyll *a* and *b*. Structural and functional similarities between NifH and ChlL indicate that the chlorophyll iron proteins may have evolved from the nitrogenase Fe protein.

The Fe protein is the most oxygen-sensitive component of nitrogenase and its ability to partially replace ChlL suggests that oxygen-sensitive enzymes may function in chloroplasts when *C. reinhardtii* is grown in the dark. This is the first study indicating the potential activity of one of the major *nif* genes in a eukaryotic cell. Since *chl*N and *chl*B components of the protochlorophyllide reductase have sequence similarities with *nif*D and *nif*K from the MoFe protein of nitrogenase, it would be interesting to replace

the *chl*N and *chl*B with *nif*D and *nif*K to investigate if also the MoFe protein can be functional in chloroplast.

It may be possible to express *nif*H without *nif*U, *nif*M and *nif*S because NifH could substitute for ChlL, which contains a Fe_4S_4 cluster in a similar environment and is therefore likely to be processed by proteins functionally similar to those required for Fe protein assembly.

1.5.3.2. Import of the Fe protein into the tobacco chloroplast

The *nif*H and *nif*M genes have been fused to a transit peptide and individually placed under the control of the CaMV 35S promoter for transfer to the tobacco nuclear genome (Dowson-Day et al., 1991). Expression was confirmed by RT-PCR but western blots indicated only a low level of protein accumulation, reflecting either poor translation or protein degradation. This may reflect the short half-life of nitrogenase in air (10 minutes).

1.5.3.3. The introduction of *Klebsiella pneumoniae* oxygen-sensitive NifH and NifM into rice

The feasibility of nitrogen fixation in cereals can be determined by introducing the Fe protein, which is the most oxygen sensitive component of nitrogenase (it is inactivated by exposure to air with a half-life of 45 s). The *Klebsiella pneumoniae nif*H and *nif*M genes were therefore introduced into the rice nuclear genome under the control of the constitutive ubiquitin promoter, and with a transit peptide from the pea ribulose bisphosphate carbohylase small subunit to target the recombinant protein to the plastids (Fu et al., personal communication). The *nif*S and *nif*U genes were not cloned because there are housekeeping homologs in both prokaryotes (Nakamura et al., 1999) and eukaryotes (Kispal et al., 1999). Plants were regenerated under hygromycin selection and the analysis of 82 independent transgenic lines indicated that ~50% of lines were co-transformed with *nif*H and *nif*M. Some of the co-transformed lines expressed high levels of mRNA and protein in roots and leaves. Fe protein activity was measured in transgenic root extracts mixed with purified MoFe protein, revealing a slight increase in acetylene production compared to control, the first time a functional component of nitrogenase has been expressed in plants.

1.5.3.4. The introduction of *S. thermoautotrophicus* oxygen-insensitive nitrogenase genes into rice

The next step is to introduce both the Fe and MoFe proteins into rice plants. Recent studies focusing on the *S. thermoautotrophicus* nitrogenase revealed unique characteristics such as O_2 insensitivity, which is particularly useful for expression in plants. Therefore genes for St1 (S, M, L) and St2 (O) were introduced into the pAL76 vector containing a transit peptide for plastid targeting. The CO dexydrogenase (St3) was not included because endogenous plant genes are likely to provide the same activity. St3 generates superoxide anion radicals which are already present in the chloroplast so this enzyme is not necessary. In the subsequent chapters we describe rice transformation, molecular analysis of transgenic plants and biochemical assays.

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Objectives

1. Introduce four nitrogenase structural genes from *Streptomyces thermoautotrophicus* into rice plants

2. Determine nitrogenase enzymatic activity in transgenic rice lines as a prelude to developing an understanding of the key constraints in engineered nitrogen fixation in rice.

Chapter 2

Generation and molecular characterization of transgenic rice plants engineered with *S. thermoautotrophicus* nitrogenase structural genes

2.1. Introduction

The development of crops with the inherent ability to fix atmospheric nitrogen would create a second Green Revolution, helping to achieve the substantial yield increases that are needed to meet the demands of a fast-growing human population. This development would be particularly important in rice, which is the staple food for more than half of the world's population (IRRI, 2002; Coats, 2003). However the development of plants expressing nitrogenase structural genes is a complex task, with little progress over the last two decades. The *nif*H and *nif*M genes have been introduced into tobacco and expressed at high levels, but the corresponding proteins accumulated to only low levels in the chloroplast stromal fraction (Dowson-Day et al., 1991). In preliminary experiments, the *nif*H and *nif*M genes from *Klebsiella pneumoniae* were introduced into the rice nuclear genome (Fu et al., personal communication). The Fe protein was shown to be active in transgenic roots (resulting in the marginally higher production of hydrogen and acetylene) but the major constraint was oxygen sensitivity, resulting in the degradation of the protein within minutes.

The discovery of an oxygen-tolerant nitrogenase in *S. thermoautotrophicus* was an important breakthrough (Ribbe et al., 1997). This has three components: a CO dehydrogenase (St3), a manganese-superoxide oxidoreductase (St2) and a molybdenum-dinitrogenase (St1), all of which were found to be insensitive to O_2 and H_2O_2, with O_2 actually used in the reaction (Ribbe et al., 1997). St3 generates electrons through the oxidation of CO ($CO + H_2O \rightarrow CO_2 + 2e^- + 2H^+$) which are transferred to O_2 producing superoxide anion radicals (O_2^-) and hydrogen peroxide. The manganese atom in St2 reoxidizes the O_2^- anions to O_2 and transfers the electrons to St1 for the reduction of N_2 to ammonium. Ribbe et al. (1997) suggested the existence of a two-component nitrogenase in an experiment where St1 and St2 reduced N_2 to NH_4^+ whereas the separate enzymes did not. Denaturing PAGE revealed three non-covalently bound St1 subunits of 93, 33 and 18 kDa, designated L, M and S, respectively, contrasting with the

$\alpha_2\beta_2$ tetrameric structure of other dintrogenases. St2 is a 48-kDa homodimer comprising 24-kDa O subunits.

The oxygen tolerance of *S. thermoautotrophicus* nitrogenase makes it an ideal candidate for transfer to plant cells, and the chloroplast is the most suitable compartment because it provides an ample supply of ATP as well as the glutamine and glutamate synthetase pathways for ammonia assimilation. Glutamine synthetase produces glutamine from glutamate and ammonia, whereas the glutamate synthetase enzyme GOGAT (glutamine:2-oxyoglutarate aminotransferase) is responsible for the transamidation of glutamine-amidonitrogen to the amino position of 2-oxoglutarate, forming two glutamate molecules. The plastid is the ideal site for introduced *nif* genes because plastid genes are expressed in a prokaryotic fashion and homologs of the cofactors required to fold the nitrogenase subunits correctly are thought to be present in this organelle (Dixon et al., 1997).

Particle bombardment can be used to transform cereal crops such as rice with multiple transgenes (Christou, 1997). We therefore used particle bombardment to transform rice with the *S. thermoautotrophicus* nitrogenase structural genes, including transit peptide sequences that target the corresponding proteins to the plastids. We then confirmed transgene integration and expression in the transgenic plants over two generations.

Rice is a self-pollinated crop and the pollen remains viable for a very short time after anther dehiscence, probably less than 5 min under most conditions; there are also no known insect pollinators (Jodon, 1938). Natural cross-pollination is highly unlikely but can be achieved artificially in a growth chamber (Coffman and Herrera, 1980). We successfully crossed a non-fertile transgenic line (sterile pollen) expressing four *S. thermoautotrophicus* nitrogenase structural genes with wild type rice and examined the transgene content of the progeny. The ability to carry out crosses between rice lines can help to address the frequent problem of sterility among important transgenic lines and also to breed new transgenic plants with different agronomic characteristics.

2.2. Materials and methods

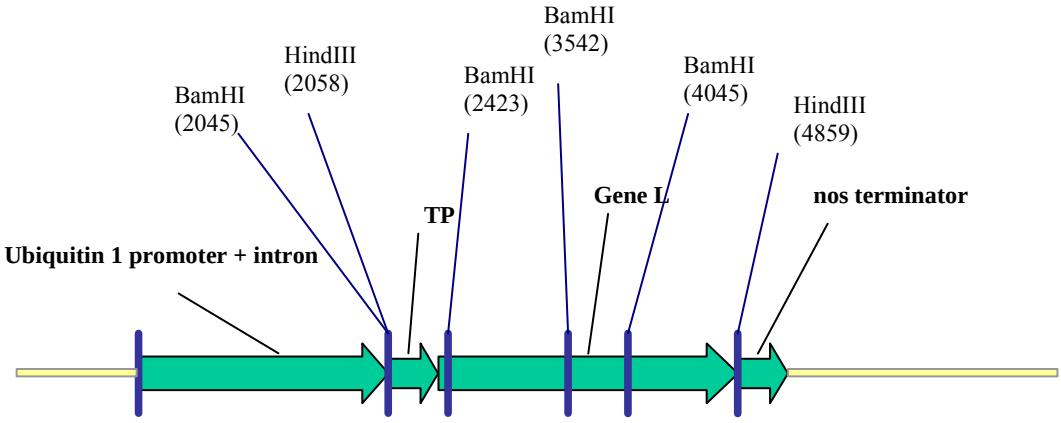
2.2.1. Construction of plant transformation vectors

Genes L, M, O and S were amplified from the plasmids SPAM-L, SPAM-M, SPAM-O

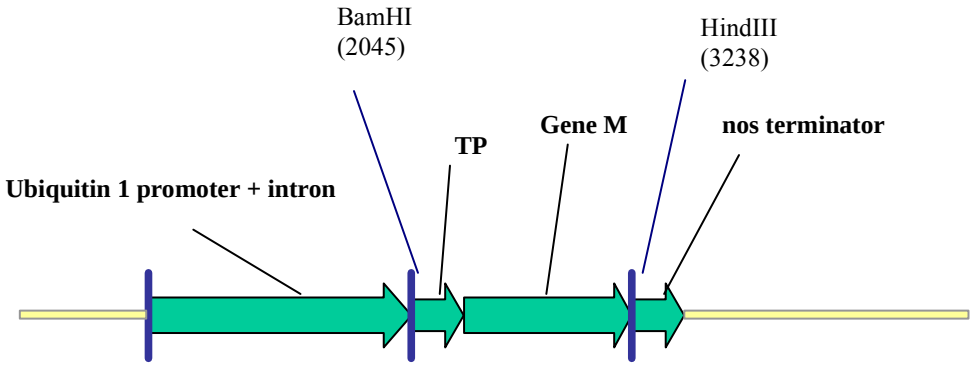
and SPAM-S provided by Prof. Fritz Kreuzaler (RWTH, Aachen, Germany) using the primers listed in **Table 2.1**. These were designed to amplify genes linked to the 3` end of the transit peptide (TP) (303 bp) forming fragments of 2796 bp for L, 1185 bp for M, 816 bp for O and 924 bp for S. The PCR was carried out in a 50-µl final volume containing 1 µl of plasmid template (100 ng/µl), 2.5 µl each of the forward and reverse primers (20 µM each), 1 µl dNTP mix (2.5 mM each dNTP), 10 µl Taq buffer x5, 1.25µl Taq polymerase (5 units/µl) and 31.75 µl of sterile water. After an initial denaturation step for 3 min at 95°C, 30 amplification cycles were carried out, each comprising a denaturation step at 94°C for 45 s, annealing at 60°C for 30 s, and extension at 72°C for 3 min for L, 1 min 30 s for M and 1 min for O and S. The PCR products were transferred to the pGEM-T-easy vector (Promega, Spain) for sequencing and analysis using the DANMA software package (UAB, Barcelona, Spain). The TP-M, TP-O and TP-S fragments were isolated from pGEM-T-easy by digestion with BamHI and HindIII and inserted into the same sites of the pAL76 vector. The TP-L fragment was isolated by digestion with HindIII and ligated to the pAL76 HindIII site. All the fragments were inserted between the ubiquitin-1 promoter/intron and the *nos* terminator (**Figure 2.1**). The orientation of the TP-L insert was verified by sequencing with primer 5`-ACC ATT GCT AGC AAT GGA AGT GAT GTC-3`. All the final constructs were verified by sequencing.

Gene	**Size of amplified fragment**	**Primer name**	**5´ to 3´ sequence**
L	2796 bp	L-For	CAAGCTTATGACGCACAATCCCACTATC
		L-Rev	TAAGCTTCACCCAGACGTAAGGCTC
M	1185 bp	TP-gene-For	AGGATCCATGACGCACAATCCCACTATC
		M-Rev	GTAAGCTTCATTGTATCCCCACCTTTCCTTGCAG
O	816 bp	O-Rev	TAAGCTTCACCGCCCGAACTTTTTCAGC
S	924 bp	S-Rev	TAAGCTTCAGTCTGCGGCAGTGCCACTG

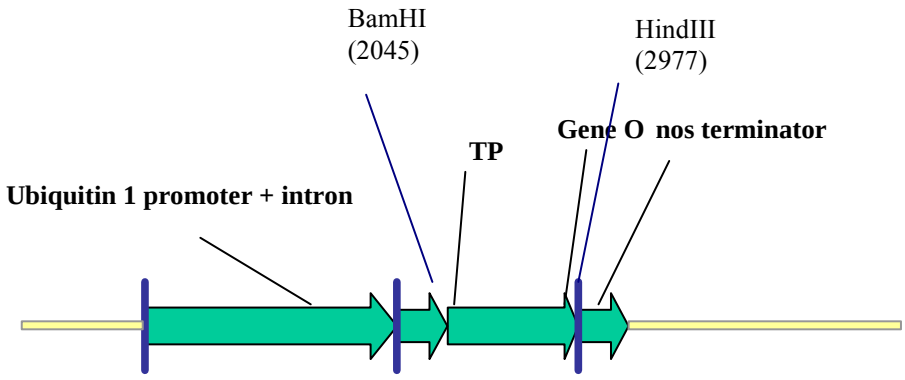
Table 2.1. Primers used to clone the *S. thermoautotrophicus* nitrogenase structural genes (the BamHI and HindIII restriction sites are shown in green and blue, respectively).



pAL76 + gene L
8199 bp



pAL76 + gene M
6578 bp



pAL76 + gene O
6317 bp

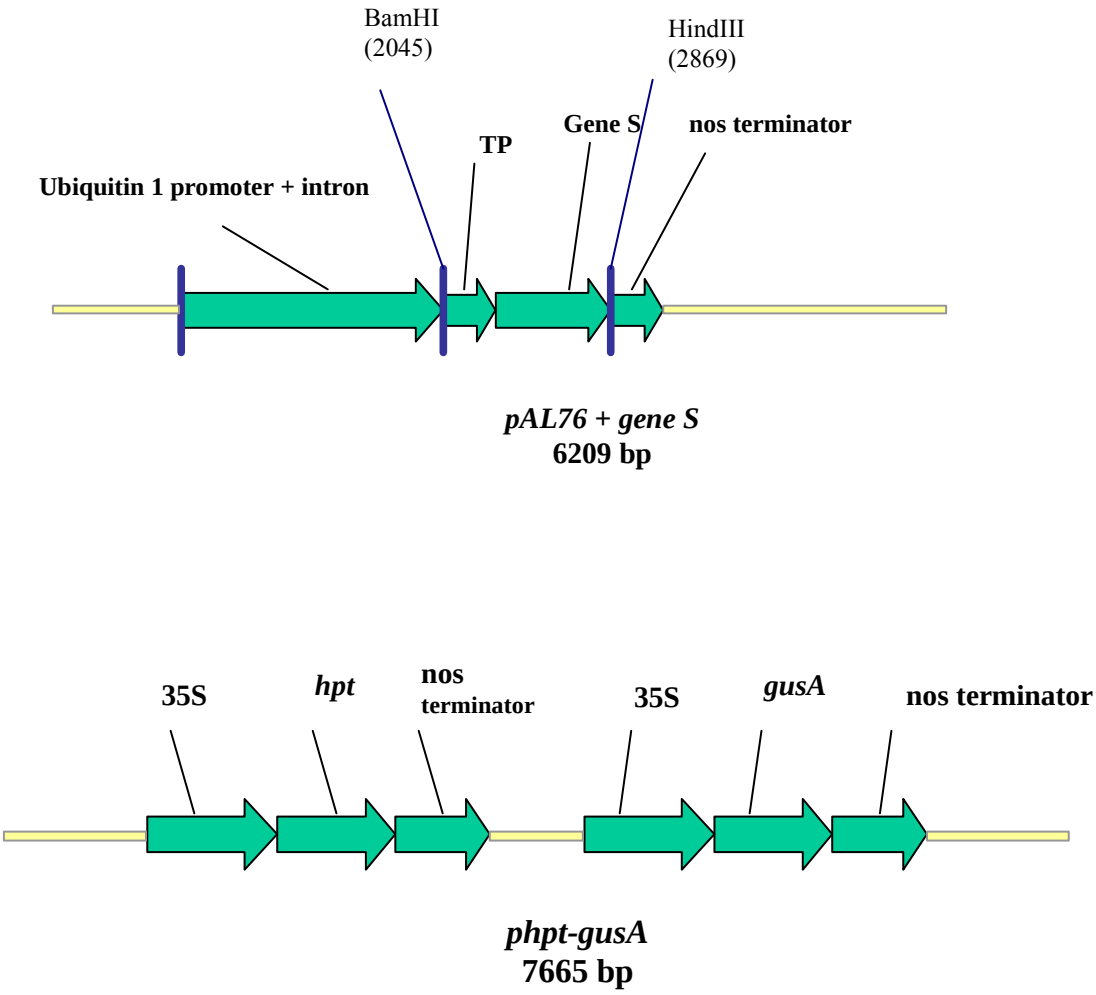


Figure 2.1. Schematic representation of the constructs used for rice transformation.

2.2.2. Creation and recovery of putative transgenic rice lines

The nitrogenase constructs were introduced into rice tissue along with the p*hpt-gusA* construct, carrying the *hpt* (hygromycin phosphotransferase) selectable marker gene conferring hygromycin resistance (**Figure 2.1**), at a 3:1 molar ratio. Therefore, the gold particles were loaded with 11 µg pAL76-L, 8.8 µg pAL76-M, 8.5 µg pAL76-O, 8.3 µg pAL76-S and 3.5 µg p*hpt-gusA*. Rice transformation, selection and regeneration were carried out as previously described (Sudhakar et al., 1998).

2.2.3. RNA blot analysis

Total RNA was extracted from the leaves of 2–3-month-old plants using Trizol® (Invitrogen, Carlsbad, CA, USA), and 30 μg of denatured RNA was separated by 1.2% agarose-formaldehyde gel electrophoresis in 1x MOPS buffer (Sambrook et al., 1989). The RNA was then transferred to a positively-charged nylon membrane (Roche, UK). The membrane was washed in 2 x SSC for 30 min and then prehybridized at 50°C for 2 h using the DIG-easy hybridization solution (Roche, UK). Specific probes were synthesized using the PCR DIG probe synthesis kit (Roche, UK) according to the manufacturer's instructions. After denaturation for 3 min at 95°C, 35 amplification cycles were carried out, each comprising a denaturation step at 94°C for 45 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. For gene L, we used primers Forw-L-third (position +1011) and Rev-L-third, amplifying a fragment 880 bp in length. For gene M, we used primers M-Forw-probe (position +765) and MRev-New, amplifying a fragment 423 bp in length. For gene O, we used primers O-Forw-probe (position +351) and New TP-O-Rev, amplifying a fragment 576 bp in length. For gene S, we used primers S-Forw-probe (position +372) and TP-S-Rev-New, amplifying a fragment 447 bp in length. The probes and primer sequences are shown in **Table 2.2** (the positions cited above defining position +1 as the ATG in the transit peptide. To avoid cross-hybridization, none of the probes contained the transit peptide sequence. Probes were denatured at 68°C for 10 min and hybridized to the membrane overnight at 50°C. After high-stringency washing, the membranes were incubated with CSPD® chemiluminescent substrate (Roche, UK) and exposed to BioMax light film (Kodak, Spain) at 37°C.

Gene	Size of amplified fragment	Primer name	5´ to 3´ sequence
L	880 bp	Forw-L-third	CGAACACATGATTCAGGTCATTTCG
		Rev-L-third	TTCTTCGACCACGACGTCGTCGAC
M	423 bp	M-Forw-probe	CGATACGTTTGCAACCGCTTTAAATGAAG
		MRev-New	GTAAGCTTCATTGTATCCCCACCTTTCC
O	576 bp	O-Forw-probe	GTATTTTGACGCCAAGACGATGGAAATTC
		NewTP-O-Rev	TAAGCTTCACCGCCCGAACTTTTTCAGC
S	447 bp	S-Forw-probe	GTACTTTCTGCGCGAGGAATTGAAGTTG
		TP-S-Rev-New	TAAGCTTCAGTCTGCGGCAGTGCCACTG

Table 2.2. Primers used for the synthesis of RNA blot probes.

2.2.4. Pollination of transgenic plants

For pollination, we isolated transgenic panicles 2-3 days after booting. Panicles were carefully released from the covered flag leaf. One third of each spikelet was cut from the upper part with scissors 17–18 hours before pollination. Panicles were emasculated by cleaning out all the anthers with a vacuum pump (UN842.3FTP, KNFLab, LABOPORT®, Germany) so that they did not present a physical barrier to the wild type pollen. The panicle was left overnight enclosed in a glassine bag. Pollination was carried out in the growth chamber the next morning after 11 am, when new anthers appeared bearing mature pollen. The panicles of wild type and transgenic plants were brought together carefully and the wild type pollen was released onto the transgenic stigmata by gentle shaking. The panicles were then tied together, covered with a glassine bag and left for several days.

2.2.5. DNA extraction and PCR

DNA was isolated from rice leaves following a modification of the procedure described by Edwards et al. (1991). Samples were ground to powder under liquid nitrogen and extracted in 4 ml extraction buffer containing 300 µl 20% SDS. After vortexing for 10 min, samples were heated to 65ºC for 10 min and extracted with phenol and phenol:chloroform:isoamylalcohol (25:24:1) to remove proteins. After centrifuging, the aqueous phase containing nucleic acids was transferred into a fresh tube and incubated with 15 µl 10mg/ml RNAse A at 37ºC for 1 h. The DNA was then precipitated with one volume of isopropanol, centrifuged at 5000 rpm for 15 min, washed with 70% ethanol, air dried and dissolved in 50 µl distilled water. The DNA concentration was measured by NanoDrop® ND-1000 (Thermo Scientific, Wilmington, DE, USA) and the quality assessed by agarose gel electrophoresis.

The purified DNA was diluted to 50 ng/µl and amplified with nitrogenase-specific primers (**Table 2.3**). The whole M, O and S genes (including the transit peptide sequence and 122 bp of the promoter) were amplified, whereas for the L gene we amplified a 1313-bp fragment starting at the position +1011. Amplification was carried out in a 20-µl final volume containing 2 µl of genomic DNA (50 ng/µl), 1 µl each of the

forward and reverse primers (20 µM each), 0.4 µl dNTP mix (2.5 mM each dNTP), 4 µl Taq buffer x5, 0.5 µl Taq polymerase (5 units/µl) and 11.1 µl sterile water. After initial denaturation for 3 min at 95°C, 35 amplification cycles were carried out, each comprising denaturation (94°C, 45 s), annealing (55°C, 30 s), and extension (72°C, 1 min 30 s).

Gene	**Size of amplified fragment**	**Primer name**	**5´ to 3´ sequence**
L	1313 bp	Forw-L-third	CGAACACATGATTCAGGTCATTTCG
		Rev-L-second	TGCACCTGACCTTCGACGATCATC
M	1307 bp	pAL76-Forw	ATGCAGCAGCTATATGTGGATTTT
		MRev-New	GTAAGCTTCATTGTATCCCCACCTTTCC
O	938 bp	pAL76-Forw	ATGCAGCAGCTATATGTGGATTTT
		New TP-O-Rev	TAAGCTTCACCGCCCGAACTTTTTCAGC
S	1046 bp	pAL76-Forw	ATGCAGCAGCTATATGTGGATTTT
		TP-S-Rev-New	TAAGCTTCAGTCTGCGGCAGTGCCACTG

Table 2.3. Primers used to detect nitrogenase transgenes in transgenic plants.

2.3. Results

2.3.1. Molecular analysis of putative transgenic lines

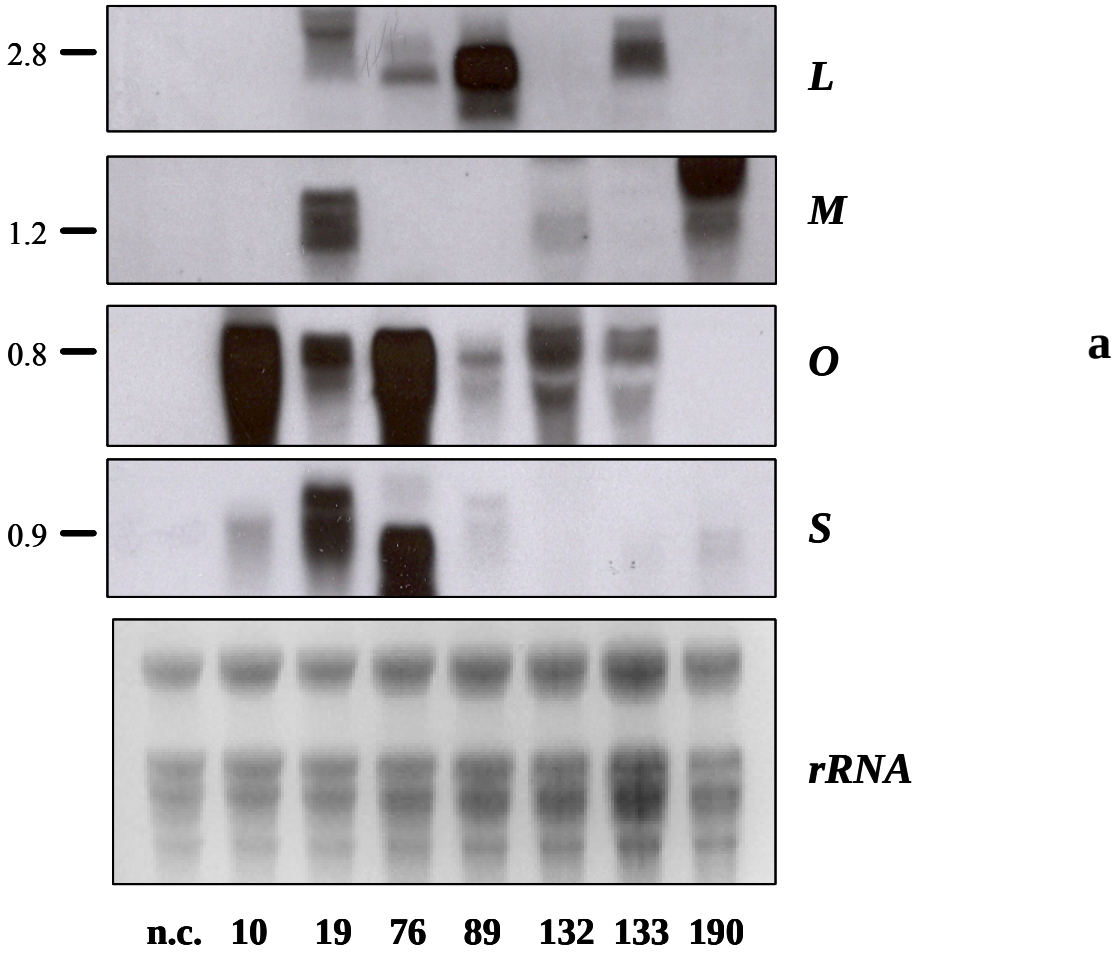
Following the first round of transformations, we recovered 22 independent rice lines, seven of which did not express any of the introduced transgenes except of the selectable marker. The remaining 15 lines expressed different combinations of transgenes, one expressing a single transgene, seven expressing two genes, six expressing three genes and one expressing all four. Nine additional transgenic lines were recovered in further transformations, two of which expressed none of the genes, one expressed a single gene, one expressed two genes, two expressed three genes and three expressed all four genes. The lines are described in **Table 2.4**.

Line 19 expressed of all four genes but was infertile, although vegetative propagation

was possible (**Figure 2.2**). Line 9 also expressed all four genes and was fertile (**Figure 2.3**). Among 18 T1 plants from line 9 analyzed by RNA blot, eight expressed all the transgenes whereas 10 did not express any, confirming segregation according to Mendel´s law and integration of all the transgenes at the same locus (**Figures 2.4a, b**).

Line #	O	M	S	L	Fertility
8	v	v		v	yes
10	v		v		no
18					
19	v	v	v	v	no
24	v			v	yes
25		v			no
26					
27	v		v		no
30					
42	v	v	v		yes
76	v		v	v	no
88	v			v	no
89	v		v	v	yes
94					
101					
103					
129	v	v	v		yes
130					
132	v	v			no
133	v			v	no
190		v	v		yes
1-5		v	v	v	yes
6	v	v		v	yes
9	v	v	v	v	yes
11	v				yes
60	v	v	v	v	yes
84	v	v	v	v	yes
86	v	v	v		yes
98					
116					
24-2	v		v		no

Table 2.4. Transgenic rice lines expressing at least one nitrogenase transgene, indicating fertility status.



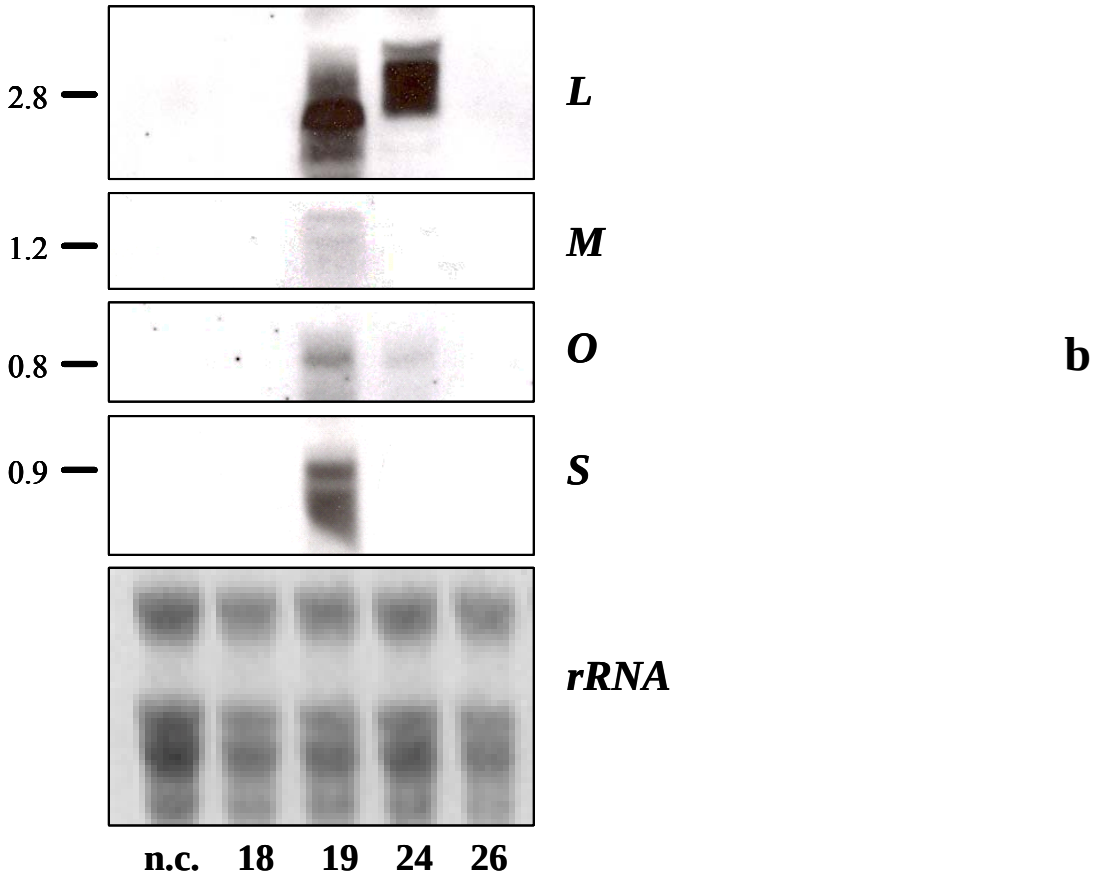


Figure 2.2a, b. RNA blot analysis of plants transformed with four structural nitrogenase genes, and probed with different sized fragments for each transgene (L, 880 bp; M, 423 bp; O, 576 bp; S, 447 bp). **a** – transgenic lines 10, 19, 76, 89, 132, 133 and 190. **b** – transgenic lines 18, 19, 24 and 26. n.c. – negative control (wild type rice plant). Exposure time was 1 h 30 min. Lower panel shows fluorescence of the ethidium bromide-stained gel to confirm equal loading of total RNA.

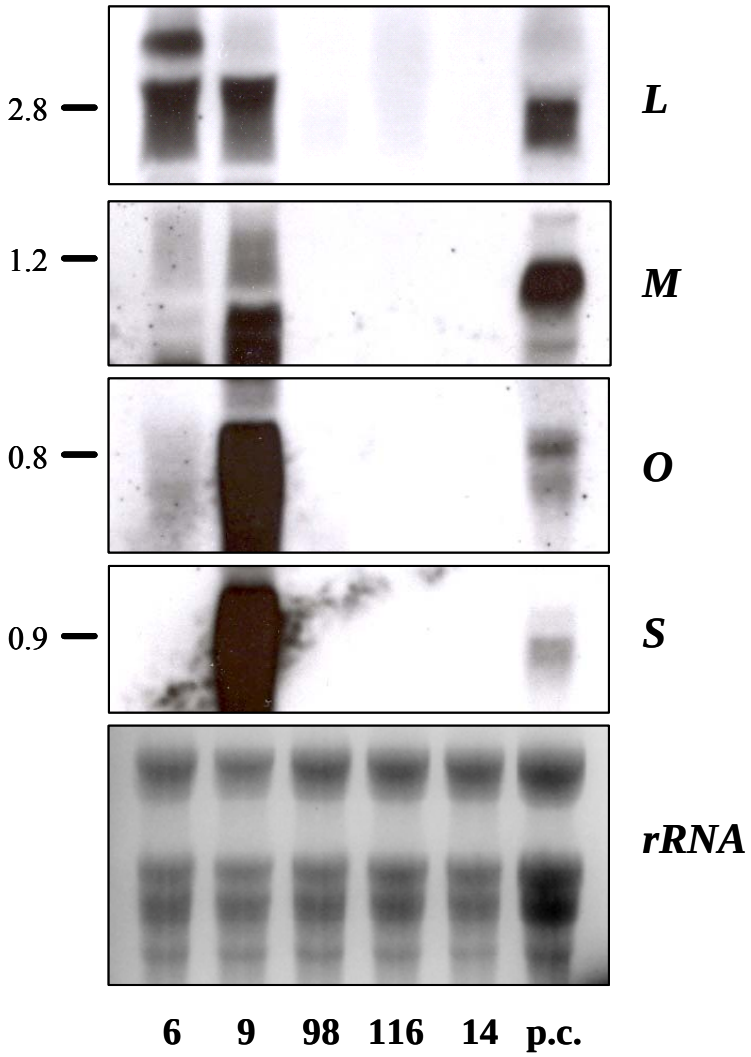
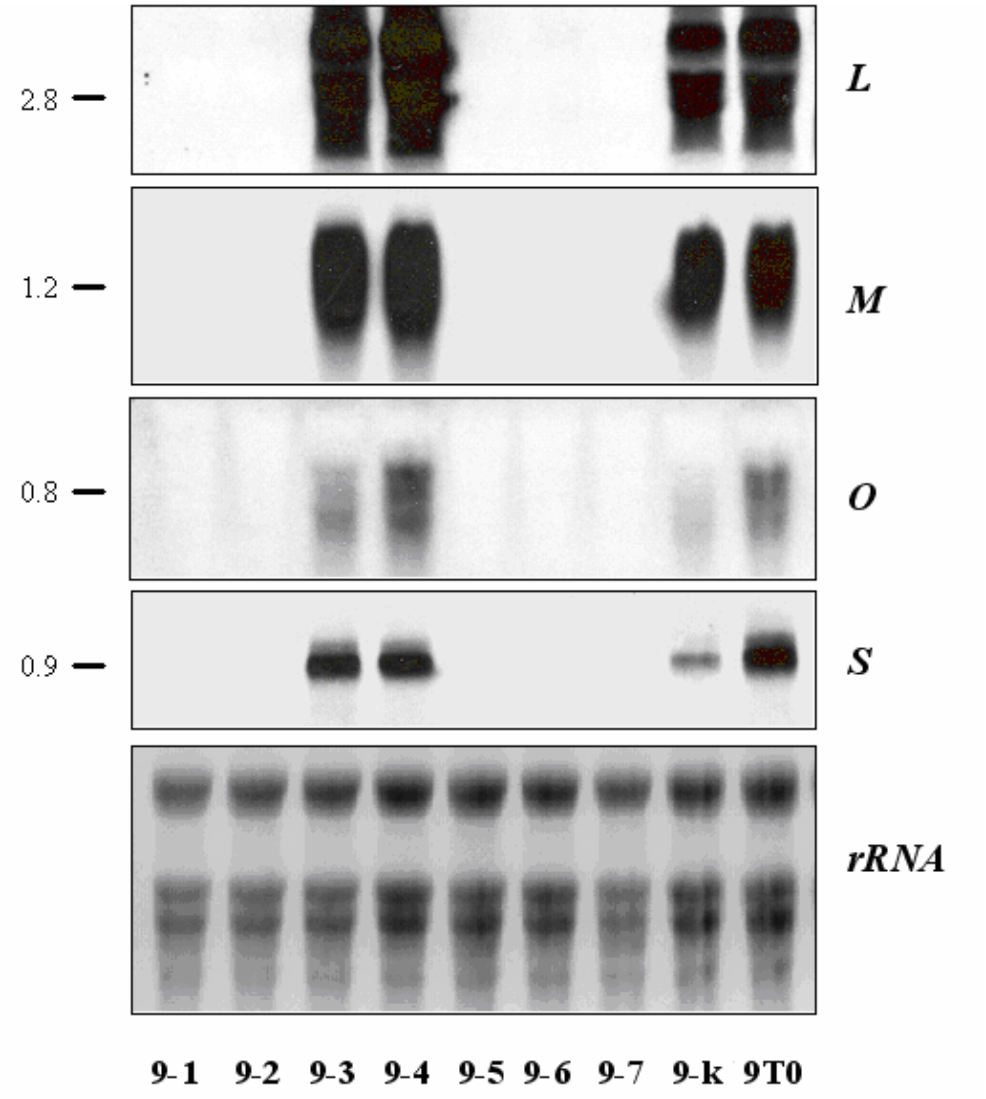


Figure 2.3. RNA blot analysis of plants transformed with four structural nitrogenase genes, and probed with different sized fragments for each transgene (L, 880 bp; M, 423 bp; O, 576 bp; S, 447 bp). Exposure time was 1 h and 30 min. p.c. – positive control for L and O genes (line 24) and for M and S genes (line 190). Lower panel shows UV fluorescence of the ethidium bromide-stained gel to confirm equal loading of total RNA.



a

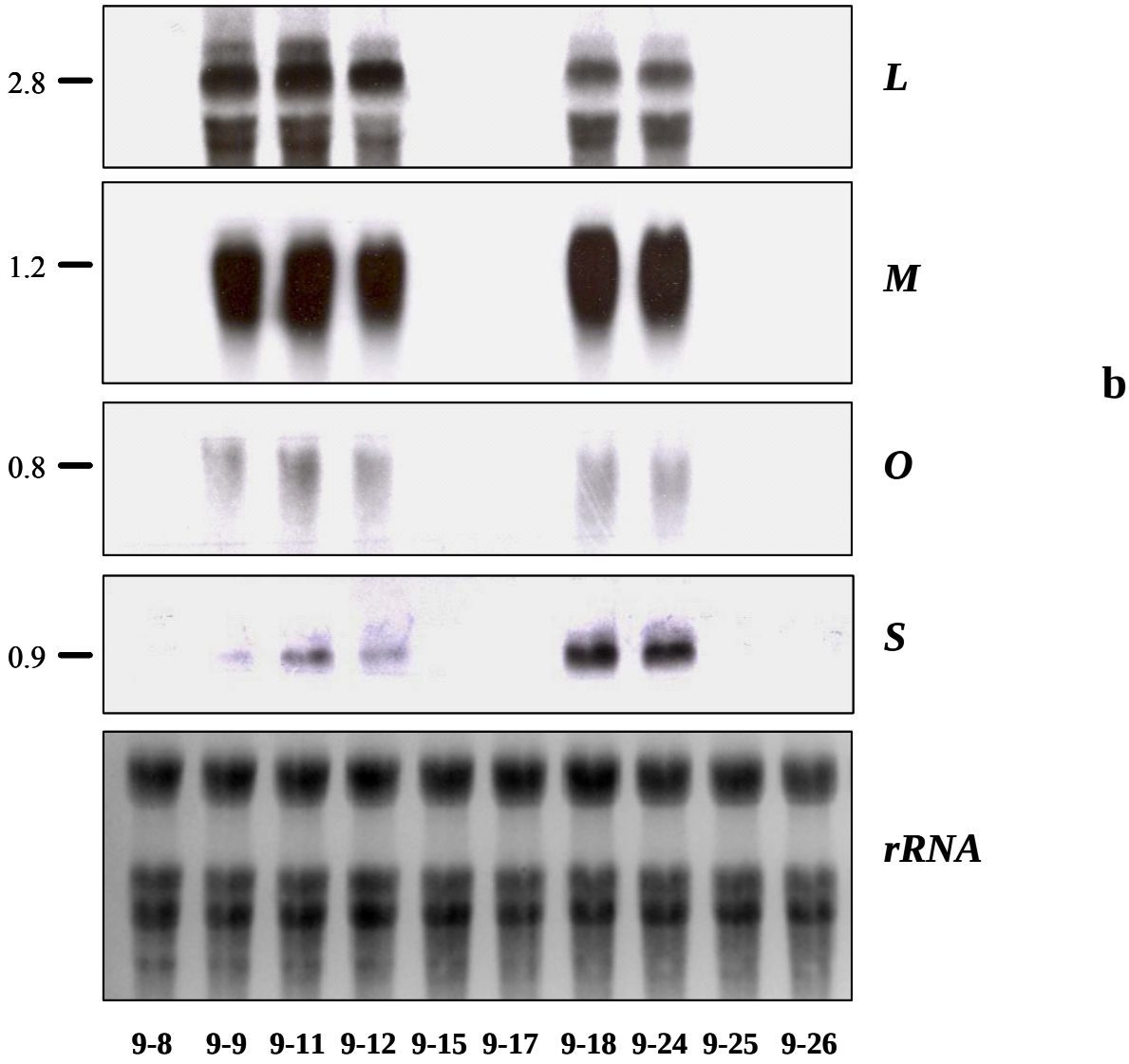


Figure 2.4a, b. RNA blot analysis of T1 progeny plants from transgenic line 9, probed with different sized fragments for each transgene (L, 880 bp; M, 423 bp; O, 576 bp; S, 447 bp). **a** – transgenic lines 9-1, 9-2, 9-3, 9-4 9-5, 9-6, 9-7, 9-k and 9T0. **b** – transgenic lines 9-8, 9-9, 9-11, 9-12, 9-15, 9-17, 9-18, 9-24, 9-25 and 9-26. Exposure time was 1 h and 30 min. Line 9 (T0) is used as the positive control. Lower panel shows UV fluorescence of the ethidium bromide-stained gel to confirm equal loading of total RNA.

2.3.2. Successful cross of the sterile line 19 and wild type rice

The vegetative propagation of sterile line 19 (containing all four nitrogenase structural genes) was slow and produced only a small amount of vegetative tissue for enzyme analysis. Fertile seeds would allow faster breeding and biomass accumulation, and would also allow the line to be preserved. We therefore made three attempts to cross line 19 with wild-type plants. The first two experiments produced a total of two seeds, which were found to be non-transgenic when DNA was extracted from the resulting plants and tested by PCR (data not shown). The third experiment yielded 11 seeds, four of which grew into plants. Plants 2 and 3 were found to have L, M, O and S genes and plants 1 and 4 contained just O and S genes (**Figure 2.5**).

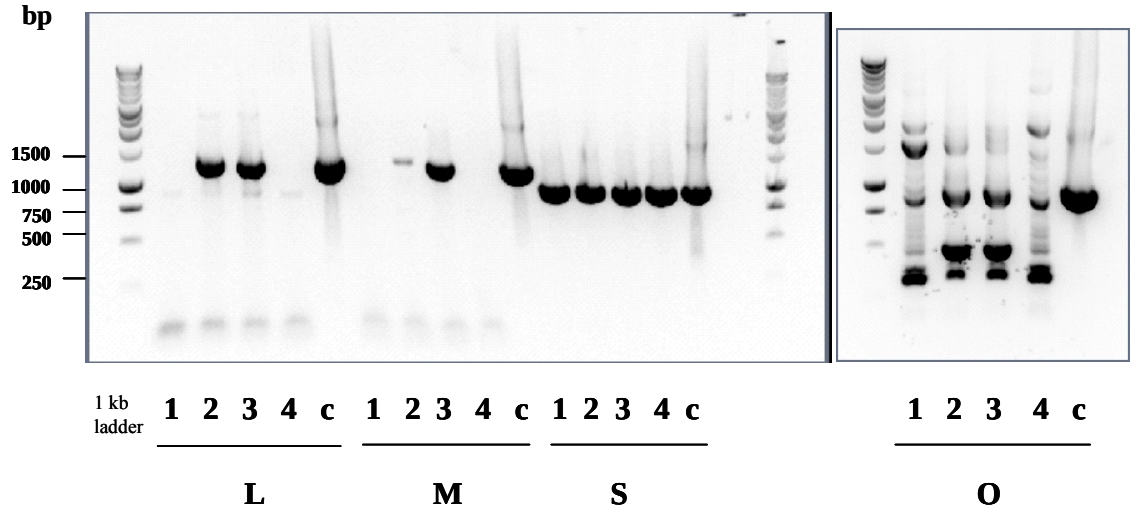


Figure 2.5. Identification of nitrogenase transgenes in T1 progeny of a line 19 x wild type hybrid. The positive controls were pAL76-L, -M, -S and -O.

2.4. Discussion

Nitrogenase structural genes from *S. thermoautotrophicus* were successfully introduced into rice and were expressed at the mRNA level. Twenty-two transgenic lines expressing at least one of the four transgenes were recovered. Nine percent of transgenic lines expressed just one transgene, 36% expressed two transgenes, another 36% expressed three transgenes, and 18% expressed all four transgenes. This is a typical distribution of transgenes given the number of transgenic lines examined, and matches well to the frequencies expected through random sampling. The results are similar to those from previous studies in which rice plants were transformed with five unrelated

genes including the *hpt* selectable marker (Agrawal et al., 2005).

Four lines contained all four genes (lines 9, 19, 60 and 84) two of which (lines 9 and 19) expressed the genes at a high level. Differences in expression levels among the four lines were probably due to position effects, which reflect the physical position of transgene integration and interactions with flanking sequences (Miki et al., 2009).

Lines 9 and 19 were the most promising candidates for further analysis and characterisation because of the high expression levels. DNA analysis was omitted as we were interested in the expression of the transgenes. DNA analysis would give information on transgene presence but would not permit measurement of expression levels.

Line 19 was sterile but could be regenerated by vegetative propagation, and it also was successfully crossed with wild type rice. This cross was carried out so that the seeds could be used to generate biomass more rapidly, and to ensure that the transgenic line could be preserved. Seeds from the cross were germinated and DNA analysis confirmed that the cross was successful. These plants and their progeny can be used to further investigate the accumulation and activity of the nitrogenase. This crossing method could be useful to eliminate sterility in other transgenic lines, or to cross transgenic lines with different traits. For example, transgenic rice with enhanced vitamin A levels could be crossed with transgenic rice resistant to drought to obtain a new line with both valuable traits.

Line 9 was fertile, and T1 seeds were germinated so that the expression of the nitrogenase structural genes could be investigated. Transgenic plants generated by particle bombardment tend to have all the transgenes at a single locus (Kohli et al., 1998) and this was clearly the case in line 9 progeny, where the transgenes cosegregated in the expected 3:1 Mendelian ratio.

Previous attempts to introduce *nif* genes into plants have focused on dinitrogenase reductase in tobacco (Dowson-Day et al., 1991) and rice (Fu et al., personal communication). In both cases, the *nif*H encoding dinitrogenase reductase was introduced along with *nif*M, which is required to process the immature NifH protein and generate the active enzyme. Both genes were fused to transit peptide sequence, individually placed under the control of a constitutive promoter (CaMV 35S in tobacco, ubiquitin-1 in rice) (Dowson-Day et al., 1991; Fu et al., personal communication). The

mRNA levels were determined by RT-PCR and were shown to be relatively high in both studies, and we have achieved the same outcome in our transgenic lines. We identified two lines expressing both dinitrogenase reductase and dinitrogenase mRNA at high levels, and these were selected for further evaluation of nitrogenase enzymatic activity.

2.5. Conclusions

The oxygen insensitivity of *S. thermoautotrophicus* nitrogenase is a unique and advantageous characteristic that should allow the enzyme to function in the oxygen-rich environment of the plant cell. Particle bombardment allows the efficient introduction of multiple genes into the rice genome, and we used this method to transfer four nitrogenase structural genes representing one subunit of dinitrogenase reductase (St2) and three subunits of dinitrogenase (St1). All four transgenes were expressed in two independent transgenic lines (lines 9 and 19). RNA analysis of line 9 T1 progeny confirmed that the expression of all four transgenes was maintained at the level seen in the primary transformant, indicating the transgenes have stably integrated into the same genetic locus. Sterile transgenic line 19 was regenerated by vegetative propagation and also crossed with wild-type rice to generate transgenic progeny containing all four transgenes.

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Chapter 3

Characterization of nitrogenase activity in bacteria and plants

3.1. Introduction

The successful transfer of the *nif* gene cluster from *Klebsiella pneumoniae* to *E. coli* created the first engineered diazotroph (Dixon and Postgate, 1972). In turn, this gave rise to a more challenging objective: the development of transgenic plants that can fix atmospheric nitrogen. The expression of *nif* genes in plants would be favored by maintaining the prokaryotic environment needed to regulate and coordinate their expression. Plastids are the ideal destination because they feature prokaryotic gene organization and expression, and plastids are also a major site for ammonia assimilation by glutamine synthetase and amino acid biosynthesis (Dixon et al., 1997). However, plastids also produce oxygen during photosynthesis and this inactivates the Fe protein of the nitrogenase complex within minutes (Parker and Scutt, 1960). The discovery of an oxygen-insensitive nitrogenase in *S. thermoautotrophicus* provided a candidate suitable for transfer to the plastid environment (Ribbe et al., 1997).

The introduction of nitrogenase biosynthesis genes into plants is an extremely complex task and few attempts have been made to achieve it. One example is the introduction of the *K. pneumoniae nif*H gene (encoding the Fe protein) and *nif*M gene (required for Fe protein stability) into tobacco plants (Dowson-Day et al., 1991) and another is the transfer of the same genes into rice (Fu et al., personal communication). However, in both cases the Fe protein was oxygen-sensitive and degraded rapidly. We have repeated the transformation experiments in rice but have chosen the oxygen-insensitive nitrogenase structural genes from *S. thermoautotrophicus*. We transferred the genes encoding the Fe protein and MoFe protein into rice and monitored their expression and activity at the protein level.

We used two different methods to determine nitrogenase activity, one using nitroprusside to test the activity of the entire nitrogenase complex (Ribbe et al., 1997) and other using nitroblue tetrazolium and superoxide oxidoreductase (as the dinitrogenase reductase component) to measure photoreduction activity (Beauchaml and Fridovich, 1971).

3.2. Materials and Methods

3.2.1. Plasmid constructs, protein purification from bacteria and SDS-PAGE

Plasmids pACYCDuet-L, pETDuet-M, RSFDuet-O and CDFDuet-S, containing the four nitrogenase structural genes L, M, O and S for expression in *E. coli*, were provided by Mehtap Oksaksin (RWTH, ***Aachen***, Germany). Each of the four nitrogenase structural genes was modified to include six additional codons at the 5′ end encoding a His_6 tag. Each plasmid also contained the T7 promoter and the *lac* operator and ribosome-binding site. The plasmids were introduced into *E. coli* strain BL21DE3^{+} and cultured overnight in LB medium containing antibiotics (**Table 3.1**). On the next day, 500 ml aliquots of fresh LB medium were seeded with 5 ml of the overnight culture and incubated without antibiotics until the OD_{600} reached 0.6. Protein expression was then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h at 28˚C. The cells were centrifuged at 8000 rpm, 15 min, 4˚C and the pellets were frozen at –80˚C overnight. On the next day, the pellets were thawed and resuspended in 20 ml ice-cold lysis buffer (25 mM HEPES pH 7.5, 300 mM NaCl, 10% glycerol, 5 mM imidazole, 25 μM phenylmethylsulfonyl fluoride (PMSF) and 0.4 mg/ml lysozyme). The lysate was incubated for 30 min with gentle shaking at 4˚C and then sonicated three times for 3 min 30 s with a 30 s gap. The lysate was centrifuged at 19000 rpm, 50 min, 4˚C and the supernatant was transferred to a new tube.

The proteins were purified by passing the supernatant through a column loaded with Ni-NTA superflow resin (Qiagen, Hilden, Germany) that had washed in 20 ml ultrapure water and equilibrated with 10 ml lysis buffer containing 12.5 μM PMSF. The column was then washed twice with washing buffer (25 mM HEPES pH 7.5, 300 mM NaCl, 10% glycerol, 20 mM imidazole) and the proteins were eluted with 8 ml elution buffer (25 mM HEPES pH 7.5, 300 mM NaCl, 10% glycerol, 300 mM imidazole). We collected 1-ml aliquots of the eluate and determined the proteins concentration using the Bradford assay (Bardford, 1976). Proteins were concentrated using a Vivaspin 4-ml Concentrator according to the manufacturer's instructions (Vivaspinproducts, Littleton, MA, USA).

Protein quality and quantity was determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 30 μg of protein per lane and 10% acrylamide according to Laemmli (1970). The gel was stained overnight with a 9:9:2 mix of

methanol, water and acetic acid containing 0.5% Coomassie brilliant blue, destained using the same solution lacking the dye and photographed.

Plasmids	Resistance to:	Protein size kDa
pACYCDuet-L	chloramphenicol	91
pETDuet-M	ampicilin	32
RSFDuet-O	streptomycin	24
CDFDuet-S	kanamycin	18

Table 3.1. Plasmids containing nitrogenase structural genes and antibiotic resistance genes. The sizes of the corresponding proteins are indicated.

3.2.2. Crude protein extracts from whole leaves

Proteins were extracted from 2.5 g of wild type or transgenic leaves (line 19) expressing the L,M, O and S gene transcripts. Leaves were ground to powder under liquid nitrogen and resuspended in 8 ml 50 mM phosphate buffer (pH 7.5) containing 200 µl 2-mercaptoethanol. The samples were centrifuged at 10,000 rpm for 10 min and the supernatant was transferred to a 50-ml Erlenmeyer flask containing 1 ml of Dowex^{TM} resin (Dow, Midland, Michigan, USA). The mixture was stirred on ice for 30 min, centrifuged at 15,000 rpm for 30 min and the supernatant was used as a crude extract for further experiments.

3.2.3. Chloroplast isolation

Two-month-old rice plants were grown in darkness overnight to reduce the starch content of the leaves and facilitate chloroplast isolation. The next day, 2 g of leaf tissue was cut into small pieces and homogenized at 4ºC for 3–4 s in a polytron (Kinematica PT20) with a small rotor (13-mm diameter, V40% max speed) containing 80 ml grinding buffer (50 mM HEPES/KOH pH 8.0, 10 mM EDTA, 0.33 M sorbitol, 1 mM $MgCl_2$, 0.5 g/l BSA, 1g/l sodium ascorbate). The homogenate was filtered through a double layer of miracloth and the debris was homogenized again as above. The combined homogenate was centrifuged at 1000 x g for 10 min and the pellet was gently

resuspended in 1 ml SH buffer (50 mM HEPES/NaOH pH 8.0, 0.33 M sorbitol) using a brush. The resuspended chloroplasts were loaded onto a two-step Percoll gradient that was prepared in 13.2-ml thin-wall polyallomer tubes (Beckman Coulter, Germany). This comprised a bottom layer (4 ml) of 80% Percoll and 20% SH buffer, and a top layer (4 ml) of 40% Percoll and 60% SH buffer. The two-step gradient was centrifuged in a SW 41 Ti rotor (Beckman Coulter, Germany) at 40,000 x g for 20 min (no breaking). The band that appeared between the phases contained intact chloroplasts and the upper band contained broken chloroplasts. Broken and intact chloroplasts were collected in separate tubes using a 1-ml Pasteur pipette. One volume of SH buffer was used to wash Percoll from the chloroplasts. The chloroplasts were centrifuged at 6000 x g for 5 min (brake on). The supernatant was decanted and discarded, and the pellet was resuspended in SH buffer.

3.2.4. Protein isolation from chloroplasts and SDS-PAGE

Proteins were extracted separately from intact and from broken chloroplasts by adding one volume of extraction buffer (0.05 M HEPES-NaOH pH 7.5, 2 mM EDTA, 5 mM $MgCl_2$ and 0.1% Triton X-100), incubating for 10 min on ice and centrifuging at 6000 x g, 10 min, 4˚C. After centrifugation, the supernatant and pellet were separated and the pellet was dissolved in the same volume of extraction buffer as the supernatant. The protein concentration of each sample was determined using the Bradford assay (Bradford, 1976) with BSA as the standard. SDS-PAGE was carried out as described above (section 3.2.1).

3.2.5. Nitroblue tetrazolium photoreduction to determine superoxide oxidoreductase subunit activity in rice plants

Crude extracts were prepared from the leaves of wild type and transgenic plants (lines 19 and 24) and were separated by 10% SDS-PAGE as described by Van Camp et al. (1994). To detect superoxide dismutase in the crude extract, the gel was stained according to the method described by Beauchaml and Fridovich (1971).

3.2.6. In vitro nitroprusside assay for nitrogenase activity in bacteria

We tested two formats of the *in vitro* nitroprusside assay to test nitrogenase activity in *E. coli*, one using the purified L, M, O and S proteins mixed together and the other using lysates from bacteria expressing all four proteins. The bacteria were lysed as described above for protein isolation although in this case the lysate was resuspended in 20 ml of 50 mM KH_2PO_4/NaOH buffer (pH 7.5). In both forms of the assay, 0.1 ml of the pure protein mix or bacterial lysate was used.

The nitrogenase assay was performed as described in Ribbe et al. (1997) with some modifications. The nitrogenase assay comprised two steps: 1) reaction between the pure proteins or lysate and atmospheric nitrogen to form ammonia; and 2) the detection of ammonia. The assay was carried out using serum-stoppered 30-ml vials (Chroma-Globe, Düren, Germany) containing 2 ml 50 mM KH_2PO_4/NaOH buffer (pH 7.5) plus 25 mM $Na_2O_4S_2$, 5.5 mM Na_2ATP, 5 mM $MgCl_2$ and 0.1 ml of pure proteins or lysate. N_2 was introduced into the sealed vials for 3 min and the vials were incubated at 37˚C for 4 h. We then transferred 0.5 ml of the reaction to a mixture comprising 4 ml 2% sodium phenolate, 0.005% nitroprusside and 0.2 M sodium hypochlorite in 0.125 M NaOH. This mixture changed from colorless to blue in the presence of ammonia. We added 0.5 ml 2.5 M $(NH_4)_2SO_4$ to the reaction mixture as a positive control, whereas water was used as a negative control.

3.2.7. In vitro nitroprusside assay for nitrogenase activity in rice plants

The nitrogenase assay was applied to rice plants essentially as described above for bacteria, although 2 µl 3.7 mM molybdenum co-factor was also added to the reaction.

3.3. Results

3.3.1. Protein analysis in *E. coli*

The extraction of His_6-tagged nitrogenase proteins from *E. coli* was carried out in Germany by our collaborators (Mehtap Oksaksin, PhD student, RWTH, Aachen, Germany) because we were unable to induce the bacterial cultures with IPTG, resulting in poor expression levels of the L and S proteins (**Figure 3.1**). Both the supernatant and pellet derived from the cell lysate were resuspended in phosphate buffer (pH 7.5) but

neither contained any trace of the L protein (91 kDa) and only the supernatant contained an 18-kDa protein likely to represent the S subunit, but the same amount was produced in control cultures suggesting that the *lac* operator was leaky (**Figure 3.1**).

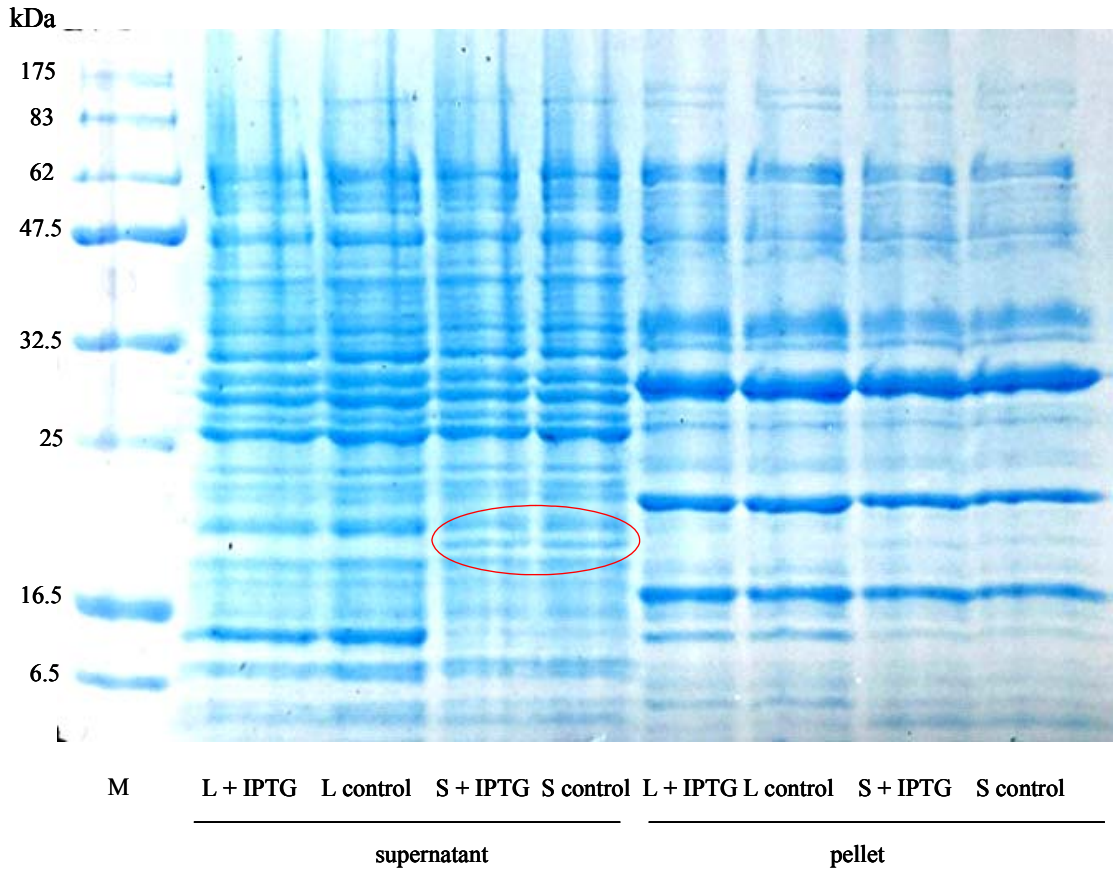

Figure 3.1. SDS-PAGE of the bacterial lysate for the L and S subunits of dinitrogenase. After centrifugation, both supernatant and pellet fractions were separated by SDS-PAGE. The red circle indicates the subunit S band that differs between the two lysates.

3.3.2. Protein analysis of putative transgenic plants

Chloroplasts were isolated from wild type plants and transgenic line 9 (**Figure 3.2**). Proteins from both wild type and line 9 chloroplasts were extracted and separated by SDS-PAGE to detect the nitrogenase subunits. We detected a single band in the extracts from intact line 9 chloroplasts that differed from wild type plants, and the size of this band (approximately 18 kDa) corresponds to that of the S subunit (**Figure 3.3**).

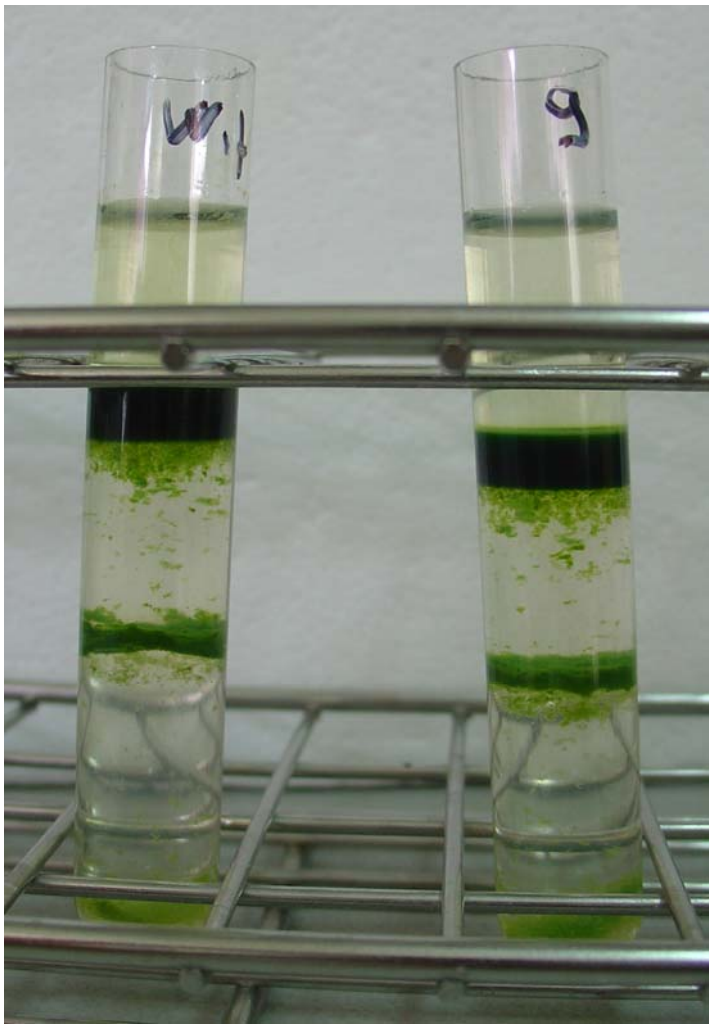

Figure 3.2. Chloroplast isolation from rice leaves using a Percoll gradient. Wild type extracts are shown on the left, and those from transgenic line 9 are shown on the right. The lower green phase is intact chloroplasts and the upper green phase is broken chloroplasts.

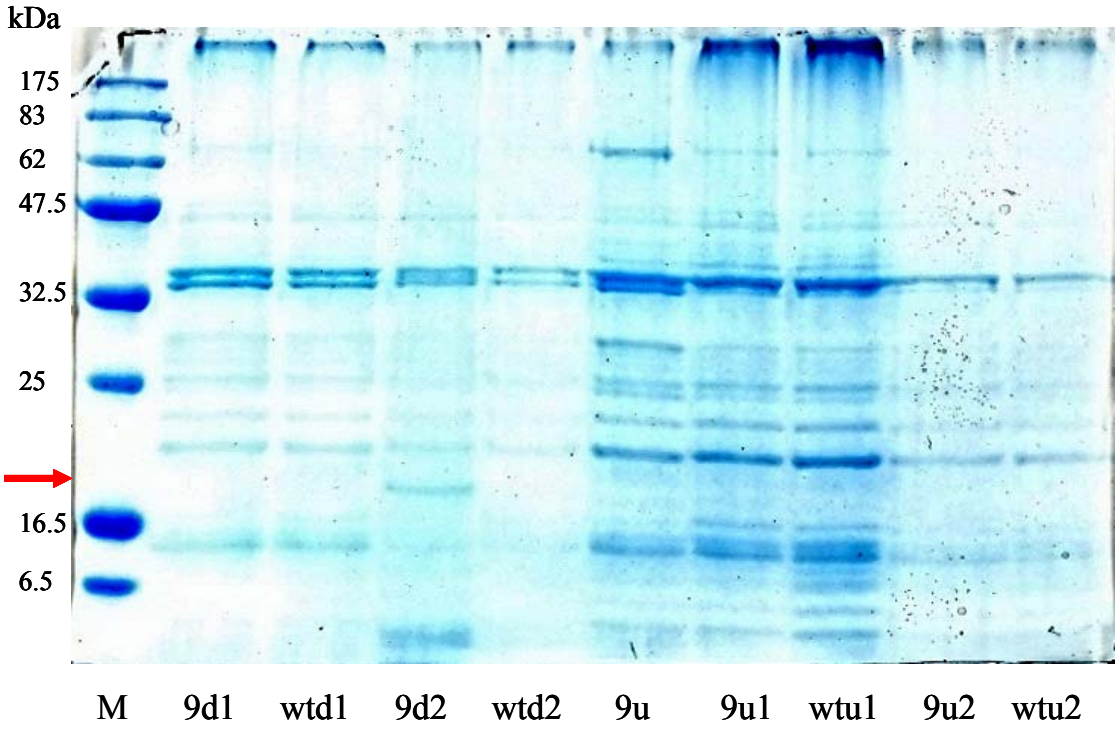

Figure 3.3. SDS-PAGE of crude protein extracts from wild type chloroplasts and those from transgenic line 9. M – protein marker, d – lower phase of intact chloroplasts, u – upper phase of broken chloroplasts, d1 – supernatant of lower phase after centrifugation, d2 – pellet of lower phase after centrifugation, u1 – supernatant of upper phase after centrifugation, u2 – pellet of upper phase after centrifugation. Arrow shows the band of approximately 18 kDa that corresponds to the size of S subunit in the 9d2 well.

3.3.3. Nitroblue tetrazolium photoreduction to evaluate superoxide oxidoreductase subunit activity in rice plants

One of the two components of the nitrogenase enzyme is a manganese-superoxide oxidoreductase (component O) that reoxidizes the O_2^- anions to O_2 and transfers the electrons to a MoFeS dinitrogenase allowing the reduction of N_2 to ammonium. Line 19 was previously shown to express the L, M, O and S genes at the mRNA level and line 24 was previously shown to express the O and S genes at the mRNA level (see Chapter 2). We did not use line 9, which also expressed all four genes, because not enough plant material was available at the time the experiments were carried out. To determine whether the high levels of O gene mRNA gave rise to similar amounts of active protein, we ran two non-denaturating gels in parallel, and one was stained with Coomassie brilliant blue (**Figure 3.4a**) while the other was used as the substrate for the nitroblue

tetrazolium photoreduction assay (**Figure 3.4b**). The first gel allowed us to detect the presence of the O protein, whereas the second allowed us to detect its activity. Purified protein O from bacteria was provided by our collaborators (Prof. Fritz Kreuzaler laboratory, RWTH, Aachen, Germany) and was used as the positive control, whereas a wild type rice extract was used as the negative control. Two samples from each plant line were prepared, an untreated crude extract and an extract that was boiled for 10 min to destroy endogenous plant enzymes but preserve the thermostable O protein (Zou et al., 2011). The O protein is a homodimer (48 kDa) comprising 24-kDa subunits. No bands of that size were visible in the extracts (**Figure 3.4a**) but the assay gel revealed two clear bands, one at 48 kDa (positive control) and the other at approximately 24 kDa, indicating the presence of undimerized subunits in transgenic line 19.

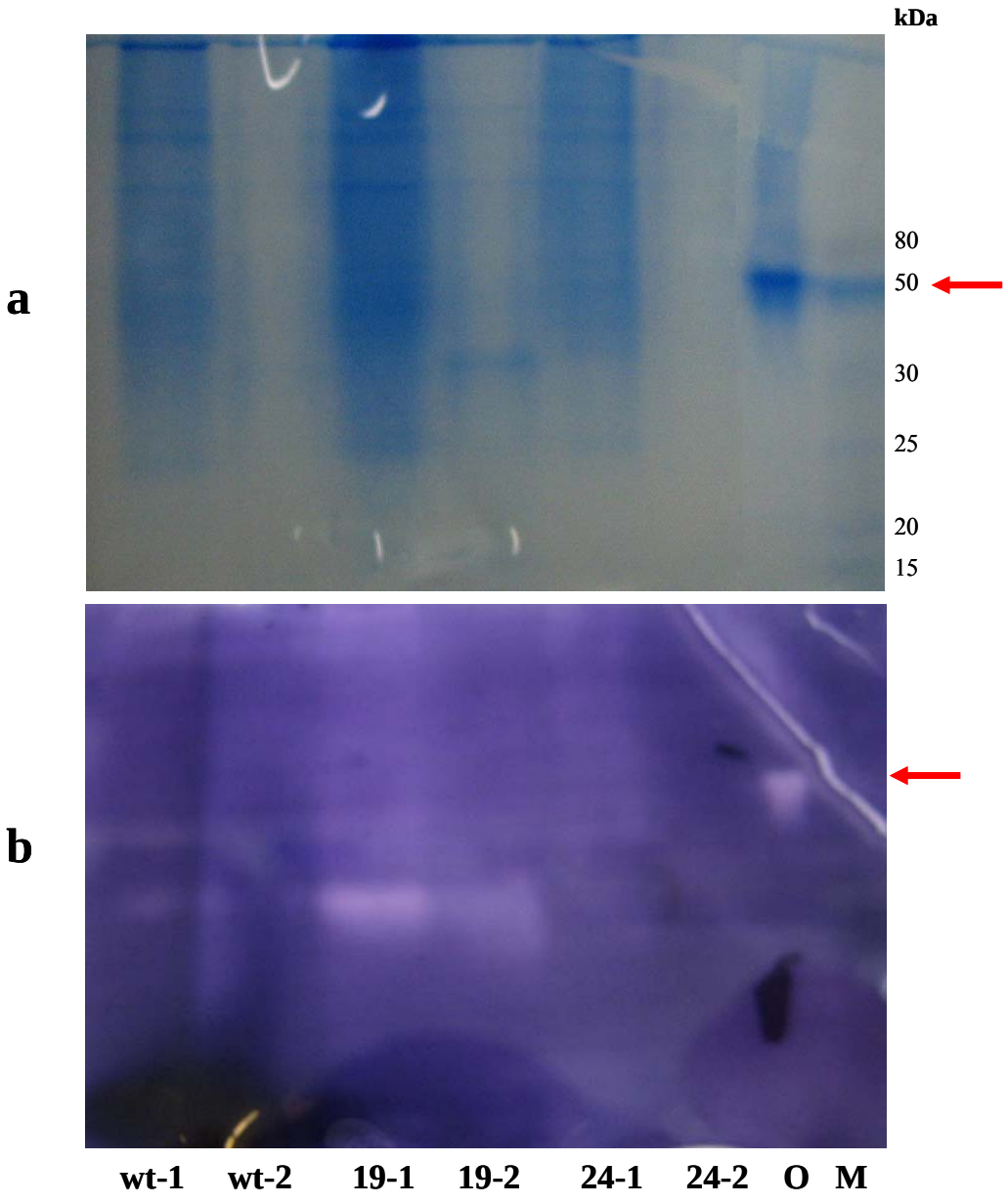

Figure 3.4a, b. Non-denaturing gels of crude extracts from lines 19 and 24 stained with Coomassie brilliant blue (**a**) or used for the nitroblue tetrazolium photoreduction (**b**). O is the purified protein from *E. coli* (positive control), wt-1, 19-1 and 24-1 are the untreated crude extracts and wt-2, 19-2 and 24-2 are the boiled extracts. M – protein marker. The position of the component O from bacteria is indicated by red arrows in the gel.

3.3.4. In vitro nitroprusside assay for nitrogenase activity in *E. coli* and rice plants

The in vitro nitroprusside test for nitrogenase activity was originally developed to characterize the *S. thermoautotrophicus* enzyme (Ribbe et al., 1997). The assay was adapted for *E. coli* as described in Materials and Methods, and was carried out by Mehtap Oksaksin (RWTH, Aachen, Germany) under the supervision of Prof. Dilip Gadkari. We adapted the assay for plants using two samples, one from wild type rice and the other from line 19. No ammonium was detected in either sample (**Figure 3.5**).

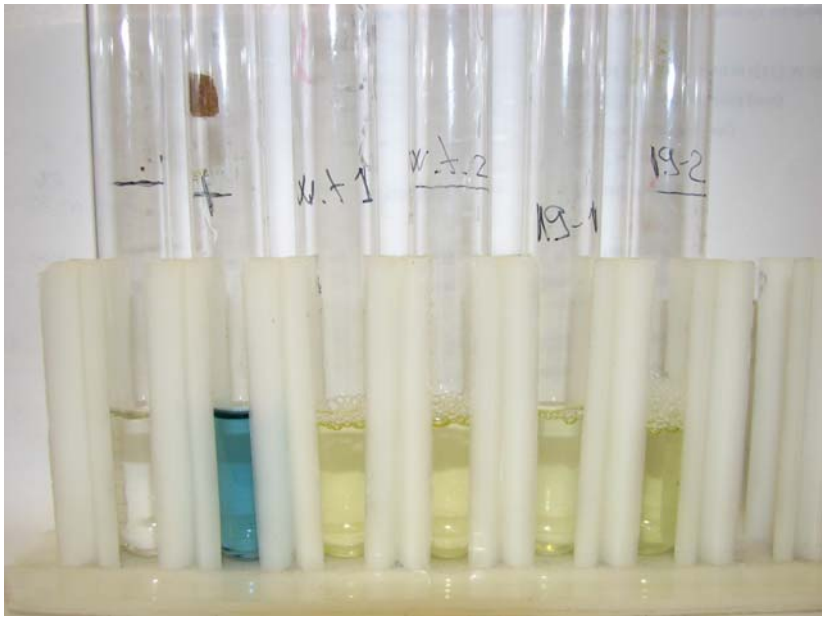

Figure 3.5. In vitro nitroprusside assay for nitrogenase activity in line 19. From left to right: negative control, positive control, crude extract from wild type sample 1, crude extract from wild type sample 2, crude extract from line 19 sample 1, crude extract from line 19 sample 2.

3.4. Discussion

3.4.1. Analysis of recombinant nitrogenase complex components by SDS-PAGE

We detected a single band in chloroplast protein extracts from transgenic line 9 that was not present in wild type chloroplasts, and its size (18 kDa) indicated it was probably the smallest dinitrogenase component, subunit. This means either that the other subunits were expressed at a level below the detection threshold of SDS-PAGE or that they were rapidly degraded after expression.

3.4.2. Nitroblue tetrazolium photoreduction assay

An assay for superoxide dismutase (SOD) activity in polyacrylamide gels was developed by Beauchaml and Fridovich (1971) and was based on observations that (1) photochemically-reduced flavins generate O_2^- following reoxidation in air, and (2) O_2^- reduces nitroblue tetrazolium to blue formazan. SOD inhibits the formation of blue formazan by converting O_2^- anions to O_2 and can be quantified on this basis. When the reactions are performed on acrylamide gels, the location of SOD is revealed by the appearance of achromatic zones on an otherwise uniformly blue gel. The in-gel assay is advantageous because it allows the presence of SOD to be confirmed by the size of the band and also shows whether the enzyme is active. Superoxide oxidoreductase also reduces O_2^- anions to O_2 and could be tested using the same assay.

In the nitroblue tetrazolium photoreduction assay, superoxide oxidoreducate purified from bacteria was used as a positive control, and a corresponding 48-kDa band was detected in both the nondenaturating gel and the photoreduction assay. The activity of the enzyme was preserved even after boiling for 10 min (personal communication, Prof. Fritz Kreuzaler laboratory, RWTH, Aachen, Germany) demonstrating a thermostability comparable to other SODs (Zou et al., 2011). However, there was no corresponding band in crude plant extracts of the line 19 and 24, and a 48-kDa band was not detected in any of the gels. A 24-kDa band was detected in extracts from line 19, but only in samples that were not boiled. Superoxide oxidoreductase is a homodimer of two 24 kDa subunits, so this band probably represents a superoxide oxidoreductase monomer. The presence of monomers instead of dimers could be an artifact of sample preparation, but may also reflect the absence of a *nif*M gene which is required to convert the immature form of the Fe protein into the active form and maintain its stability (Dean et al., 1993).

When *nif*H is expressed in *E. coli* or yeast in the absence of *nif*M, a very low level of dimeric Fe protein is produced (Berman et al., 1985; Paul and Merrick, 1989). However, in the plant extract, the monomer band retained a low level of enzyme activity, as shown by the appearance of weak bands on the in-gel assay. The levels of O gene mRNA were lower in line 24 than line 19 (see Chapter 2), and this probably explains why no protein was detected in this extract. Heating the plant crude extracts did not improve results and the assay was not repeated using chloroplast extracts.

3.4.3. In vitro nitroprusside assay for nitrogenase activity

No ammonium was formed in the crude extracts from line 19 indicating there was no nitrogenase activity. This assay was previously carried out using purified components of the superoxide oxidoreducate and dinitrogenase from *S. thermoautotrophicus* so the concentrations of the proteins were much higher (Ribbe et al., 1997). It is possible that the level of active nitrogenase is too low to make the reaction visible in line 19. Alternatively, the nitrogenase complex might not have formed properly, a proposition supported by the absence of the essential L and M subunits in SDS-polyacrylamide gels.

It is also possible that the immature L and M proteins are synthesized but not processed into their mature forms due to the lack of accessory proteins, and therefore are degraded. It is still not clear how many genes in addition to the structural genes encoding nitrogenase components are required for nitrogen fixation in plants. The CO dexydrogenase (St3) gene was not cloned because equivalent functions are thought to be provided by the plant (St3 generates superoxide anion radicals that are already present in the chloroplast). The *nif*S and *nif*U genes have housekeeping homologs in both prokaryotes (Nakamura et al., 1999) and eukaryotes (Kispal et al., 1999). The mutation of certain *nif* genes such as *nifY, T, X, U, S, Q, W* and *Z* does not completely eliminate nitrogenase activity (Dean et al., 1993). However, the genes required to synthesize the nitrogenase metallocluster may be required. Furthermore, the molybdoenzyme is not present in plastids and the *nif*H, *nif*E, *nif*V and *nif*B genes may therefore be required.

3.4.4. Advantages and disadvantages of *S. thermoautotrophicus* nitrogenase

The main advantage of *S. thermoautotrophicus* nitrogenase is its insensitivity to oxygen, which is abundant in the chloroplast. However, the optimal temperature for nitrogenase

activity in *S. thermoautotrophicus* is 65˚C (Gadkari et al., 1992). It is therefore possible that plants provide a suboptimal environment and that inadequate levels of the enzyme are expressed to support plant nitrogen demand.

The superoxide radical is the electron donor for superoxide oxidoreductase acting as dinitrogenase reductase and it is formed under stress conditions. This enzyme will therefore become active in the chloroplast only under photooxidative stress (Ladha and Reddy, 2000). Introducing this enzyme into chloroplasts might make plants more stress-tolerant, but nitrogenase activity would require stress conditions to be imposed.

3.5. Conclusions

It is important to find an appropriate location to support the synthesis and activity of nitrogenase in higher plants. The chloroplast is an ideal candidate because it has prokaryote-like gene organization and it is also the primary compartment for the assimilation of ammonia. However, the chloroplast also produces large amounts of oxygen during photosynthesis and this is incompatible with most nitrogenases. The *S. thermoautotrophicus* nitrogenase is not oxygen-sensitive, but the molybdoenzyme that is essential for nitrogenase metallocluster biosynthesis is not present in plastids, so it may be necessary to include this component in future engineering strategies.

Among the four structural genes of the *S. thermoautotrophicus* nitrogenase complex expressed in rice plants, only one S subunit was detected by SDS-PAGE and probably one O subunit of superoxide oxidoreductase was detected in the nitroblue tetrazolium photoreduction assay. However, further experiments need to be carried out to ensure that these subunits are actually needed for nitrogenase activity. The L and M subunits were not detected in these experiments, suggesting that the expression of nitrogenase structural genes alone is not sufficient to confer nitrogenase activity, and that other genes from the *nif* cluster might be required for the efficient formation and activity of the nitrogenase enzyme.

3.6. References

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Future prospects

The engineering of nitrogen-fixing plants is a long-term goal because it requires the assembly of a complex nitrogenase enzyme that is normally provided by bacteria, and which naturally functions in a prokaryotic intracellular environment. In addition, the presence of oxygen in plant cells places major constraints on the activity of most nitrogenases because they are oxygen sensitive. However, plastids could be the ideal site for recombinant nitrogenase expression *in planta* because they provide a prokaryotic-like environment and oxygen is only present in daylight, allowing nitrogenase to function in the dark when no photosynthesis takes place. The *Azotobacter vinelandii* Shethna (FeS II) protein (Moshiri et al., 1994) could also be expressed in plastids, providing conformational protection that would temporarily stabilize nitrogenase in the presence of oxygen and thus prevent its degradation. The FeS II protein binds to the MoFe and Fe proteins during oxidative stress to create an inactive tripartite complex that does not degrade in the presence of oxygen.

S. thermoautotrophicus nitrogenase is advantageous because it is not sensitive to oxygen, but the bacterium grows optimally at 65˚C so the nitrogenase is likely to be most active at this temperature. *S. thermoautotrophicus* nitrogenase also requires superoxide radicals for electron transfer from dinitrogenase reductase to dinitrogenase. Although plastids produce superoxide radicals, they are generally sequestered by antioxidants and therefore may not be abundant enough for the recombinant nitrogenase unless the plants are subjected to oxidative stress. Other *S. thermoautotrophicus nif* gene products are also likely to be required to ensure that nitrogenase functions correctly in the plant cell environment (e.g. NifM converts the immature form of dinitrogenase reductase into the catalytically-active form, whereas NifE, NifV and NifB are required for the biosynthesis of the MoFe protein).

Nitrogenase works slowly, so high levels must be expressed in plants to achieve efficient nitrogen fixing. This could be accomplished by tailoring each of the introduced genes using plastid 5' and 3' regulatory signals to optimize expression or by exploiting similarities between the bacterial and plastid transcriptional machinery, e.g. using bacterial sigma factors and transcription factors to achieve high-level plastid expression.

As an alternative to plastids, *nif* genes could be targeted to mitochondria because they provide an energy-rich, reducing environment (Beatty and Good, 2011). Mitochondria

have efficient oxygen-consuming respiratory enzymes, functioning oxygen-sensitive enzymes, and enzyme complexes with iron-sulfur clusters highly similar to nitrogenase. The *nif* genes could be introduced into the nuclear genome to allow spatiotemporal regulation, with the proteins targeted to mitochondria.

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General conclusions

1. We introduced *S. thermoautotrophicus* nitrogenase structural genes encoding the superoxide oxidoreductase component (gene O) and the dinitrogenase component (genes L, M and S) into rice and recovered transgenic plants.

2. Two transgenic lines (9 and 19) were identified that expressed all four nitrogenase structural genes at the mRNA level.

3. Line 19 was infertile, but biomass was produced by vegetative propagation and the line was also crossed successfully with wild type rice.

4. At the protein level, we detected the 18-kDa S subunit (the smallest component of the dinitrogenase) when protein extracts from line 9 were separated by SDS-PAGE, but the other subunits were not detected, suggesting either limited accumulation or degradation of the protein after synthesis.

5. A nitroblue tetrazolium assay for photoreduction revealed a single dinitrogenase reductase monomer with minimal enzyme activity in extracts from transgenic line 19.

6. An *in vitro* nitroprusside assay did not reveal any nitrogenase activity, indicating that the level of active nitrogenase was too low or that the nitrogenase complex was not formed properly.

Outcomes

Publication in preparation

1. **Dashevskaya S.**, Horn R., Chudobova I., Schillberg S., Capell T. and Christou P. Abscisic acid and cyprosulfamide cooperate to enhance abiotic stress tolerance in rice by maintaining the expression of *OsLEA3*, putative fumarylacetoacetate hydrolase and mitochondrial translocase genes.

Other publications

1. Ramessar K., Naqvi S., **Dashevskaya S.**, Peremartí A., Yuan D., Gomez-Galera S., Maiam S., Farré G., Sabalza M., Miralpeix B., Twyman R., Zhu C., Bassié L., Capell T. and Christou P. (2009). The Contribution of Plant Biotechnology to Food Security in the 21st Century. Nova Science Publishers. Hauppage, NY 11788 (www.novapublishers.com) pp 453-455 ISBN 978-1-60692-977-3

2. Peremarti A., Twyman R.M., Gomez-Galera S., Naqvi S., Farré G., Sabalza M., Miralpeix B., **Dashevskaya S**., Yuan D., Ramessar K., Christou P., Zhu C., Bassié L. Capell T. Christou P (2010) Promoter diversity in multigene transformation. Plant Molecular Biology 73: 363-378.

3. Yuan D., Bassie L., Sabalza M., Miralpeix M., **Dashevskaya S.,** Farre G., Rivera S., Banakar R., Bai C., Sanahuja G., Arjo G., Avilla E., Zorrilla-Lopez U., Ugidos – Damboriena N., Lopez A., Almacellas D., Zhu C., Capell T., Hahne G., Twyman R. and Christou P. (2011). The potential impact of plant biotechnology on the Millennium Development Goals. Plant Cell Rep. DOI: 10.1007/s00299-010-0987-5.

4. Yuan D., **Dashevskaya S.**, Twyman R., Capell T. and Christou P. (2011). Crop Plants: Transformation Methods. In: Meyers RA (ed) *Encyclopedia of Sustainability Science and Technology.* Springer NY (in press). DOI: 10.1007/978-1-4419-0853-3