Identification of Plant Genes for Abiotic Stress Resistance

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Dit onderzoek is uitgevoerd binnen de onderzoekschool ‘Experimental Plant Sciences’

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
Prof. Dr. M.J.Kropff
in het openbaar te verdedigen
op vrijdag 14 maart 2008
des namiddags te half twee uur in the Aula

Shital Dixit (2008)

Identification of Plant Genes for Abiotic Stress Resistance

PhD thesis Wageningen University, The Netherlands
With summaries in English and Dutch

ISBN 978-90-8504-905-0

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This thesis is dedicated to
my lovely Parents

Chapter 1

General introduction and scope of the thesis

Introduction

Plant growth and productivity is under constant threat from environmental challenges in the form of various abiotic and biotic stress factors. Plants are frequently exposed to a plethora of abiotic stress conditions such as low temperature, salt, drought, flooding, heat, oxidative stress and heavy metal toxicity. Plants also face challenges from pathogens including bacteria, fungi, and viruses as well as from herbivores. All these stress factors pose a challenge for plants, preventing them from reaching their full genetic potential and limit crop productivity worldwide. Abiotic stress in fact is the principal cause of crop failure world wide, among which drought and salinity affect more than 10 percent of arable land, with desertification and salinization rapidly increasing on a global scale declining average yields for most major crop plants by more than 50 percent (Bray et al., 2000). Understanding plant tolerance to drought and salinity is therefore of fundamental importance and forms one of the major research topics for stable plant production.

Plants can perceive abiotic stresses and elicit appropriate responses with altered metabolism, growth and development. The regulatory circuits include stress sensors, signaling pathways comprising a network of protein-protein interactions, transcription factors and promoters, and finally the output proteins or metabolites. Genetic studies revealed that stress tolerance traits are mainly quantitative trait loci (QTLs), which make genetic selection of traits difficult. Nevertheless, cultivars with respectable stress tolerance have been obtained by classical breeding,

Combating Abiotic stress

As water and salt stresses occur frequently and can affect many habitats, plants have developed several strategies to cope with these challenges: either adaptation mechanisms, which allow them to survive the adverse conditions, or specific growth habits to avoid stress conditions. Stress-tolerant plants have evolved certain adaptive mechanisms to display different degrees of tolerance, which are largely determined by genetic plasticity. Differential stress tolerance could be attributed to differences in plant reactivity in terms of stress perception, signal transduction and appropriate gene expression programs, or novel metabolic pathways that are restricted to tolerant plants. The hypothesis that the genetic program for tolerance is at least to some extent also present

in non tolerant plants is supported by the observation that gradual acclimation of sensitive plants leads to acquisition of tolerance to some degree. These plants may need gradual adaptation for proper expression of genes responsible for acquisition of tolerance (Zhu, 2001).

Exposure to drought or salt stress triggers many common reactions in plants (Verslues et al., 2006). Both stresses lead to cellular dehydration, which causes osmotic stress and removal of water from the cytoplasm into the extracellular space resulting in a reduction of the cytosolic and vacuolar volumes. Another consequence is the production of reactive oxygen species which then in turn affects cellular structures and metabolism negatively. Prevention of osmotic stress caused by drought depends upon minimizing stomatal and cuticular water loss and maximizing water uptake (through root growth and osmotic adjustment), and during salt stress osmotic adjustment appears to play a major role in maintaining osmotic homeostasis. Early responses to water and salt stress are largely identical except for the ionic component. These similarities include metabolic processes such as, for example, a decrease of photosynthesis or hormonal processes like rising levels of the plant hormone abscisic acid (ABA). High intracellular concentrations of sodium and chloride ions are an additional problem of salinity stress. As part of plant stress responses, regulation of gene expression also involves both universal and unique changes in transcript levels of certain plant genes (Shinozaki and Yamaguchi-Shinozaki, 2000). Based on the presence of these general and specific abiotic stress tolerance mechanisms, it is logical to expect plants to have multiple stress perception and signal transduction pathways, which may cross-talk at various steps in the pathways. These pathways act in cooperation to alleviate the effect of stress.

Salt and drought signaling pathways

Salt and drought stresses affect virtually every aspect of plant physiology and metabolism. In nature, for a plant to sacrifice a part of its structure constitutes an adaptive strategy to survive a stress period. For adaptive or presumed adaptive responses, it may be helpful to conceptually group them into three aspects: (*a*) homeostasis that includes ion homeostasis, which is mainly relevant to salt stress, and osmotic homeostasis or osmotic adjustment; (*b*) stress damage control and repair, or detoxification; and (*c*) growth control (Zhu, 2001). Accordingly, salt and drought stress signaling can be divided into three functional categories: ionic and osmotic stress signaling

for the reestablishment of cellular homeostasis under stress conditions, detoxification signaling to control and repair stress damages, and signaling to coordinate cell division and expansion to levels suitable for the particular stress conditions (Fig. 1). Homeostasis signaling negatively regulates detoxification responses because, once cellular homeostasis is reestablished, stress injury would be reduced, and failure to reestablish homeostasis would aggravate stress injury. Homeostasis and detoxification signaling lead to stress tolerance and are expected to negatively regulate the growth inhibition response, i.e., to relieve growth inhibition of the stress factors.

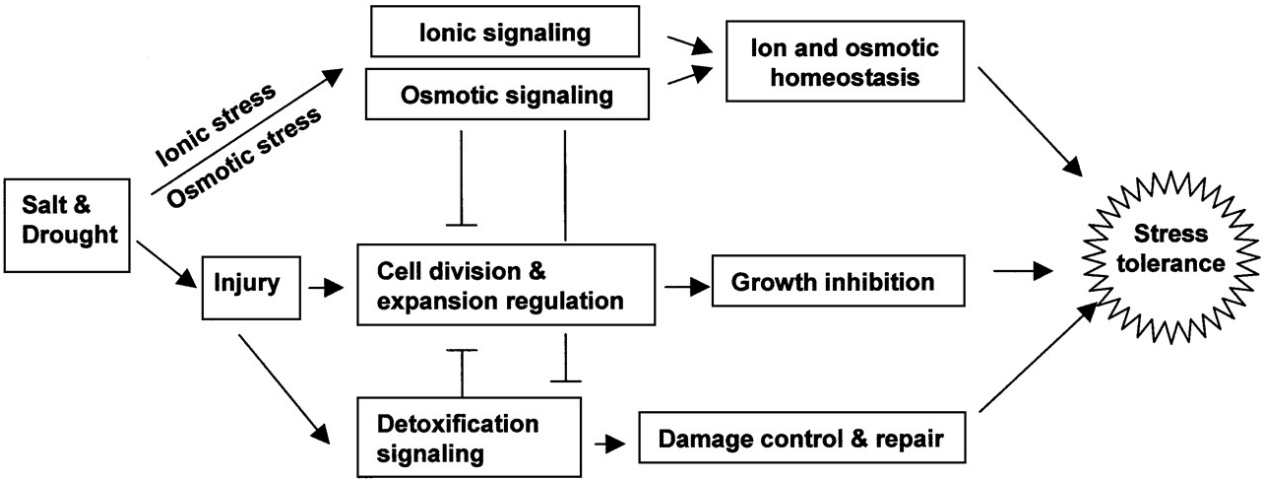

Fig. 1. Functional network of salt and drought stress signaling pathways. The inputs for the signaling pathways are ionic (excess Na^+) and osmotic (e.g., turgor) changes. The output of ionic and osmotic signaling is cellular and plant homeostasis. Direct input signals for detoxification signaling are derived stresses (i.e., injury), and the signaling output is damage control and repair (e.g., activation of dehydration tolerance genes). Interactions between the homeostasis, growth regulation, and detoxification pathways are indicated (Zhu, 2002).

Salt and drought exert their influence on a cell by disrupting the ionic and osmotic equilibrium resulting in a stress condition. Thus excess of Na^+ ions and osmotic changes in the form of the turgor pressure are the initial triggers of this pathway. This leads to a cascade of events, which can be grouped under ionic and osmotic signaling pathways, the outcome of which is ionic and osmotic homeostasis, leading to stress tolerance (Fig. 1). This stresses are marked by symptoms of stress injury including chlorosis and necrosis and may also exert their negative influence on cell division resulting in growth retardation of the plant. Reduction in shoot growth, especially leaves, is beneficial for the plant as it reduces the surface area exposed for

transpiration, hence minimizing water loss. Plants may also sacrifice or shed their older leaves, which is another adaptation in response to drought. Stress injury may occur through denaturation of cellular proteins/enzymes or through the production of reactive oxygen species (ROS), Na^+ toxicity and disruption of membrane integrity. In response to stress injury plants trigger a detoxification process, which may include changes in the expression of LEA/dehydrin type genes, synthesis of molecular chaperones, proteinases, enzymes for scavenging ROS and other detoxification proteins. This process functions in the control and repair of stress induced damage and results in stress tolerance.

A number of studies have shown that salt tolerance of plants can be greatly improved by regulating the expression of effectors or regulators functioning in a re-establishment of ion homeostasis under stress conditions. Among these cases, overexpression of a vacuolar Na^+/H^+ antiporter AtNHX1 (Apse et al., 1999) or a plasma membrane Na^+/ H^+ antiporter SOS1 (Shi et al., 2003) was shown to confer salt tolerance to transgenic plants. In addition, the expression of calcineurin (Pardo et al., 1998) or HAL1 (Gisbert et al., 2000), regulators of K^+ and Na^+ homeostasis in yeast, also resulted in improved salt tolerance of transgenic plants.

The regulation of salt and drought responsive genes is very complex and has been distinguished into “early responsive genes” and “delayed-responsive genes”. Early responsive genes are induced within minutes and often transiently and their induction does not require new protein synthesis because all signaling components are already in place. In contrast, delayed-response genes, which constitute the vast majority of the stress-responsive genes, are activated by stress more slowly (within hours), and their expression is often sustained. The early response genes typically encode transcription factors that activate downstream delayed-response genes (Zhu, 2002) (Fig. 2).

Several examples of early-response genes in salt, drought, cold, and ABA regulation have emerged. They include, e.g., the CBF/DREB gene family (Stockinger et al., 1997; Gilmour et al., 1998; Shinwari et al., 1998), RD22BP, AtMyb2 (Urao et al., 1996; Abe et al., 1997), and ABF/ABI5/AREB (Choi et al., 2000; Finkelstein and Lynch, 2000; Uno et al., 2000). These genes are all rapidly induced by either ABA or one or more of the stress signals. Defining the *cis*-regulatory promoter elements in these genes that confer stress inducibility could help to identify

transcription factors that bind these elements and activate the early-response genes. The upstream transcription factors are typically constitutively expressed and are regulated by stress at the posttranslational level, i.e., by phosphorylation changes (Fig. 2).

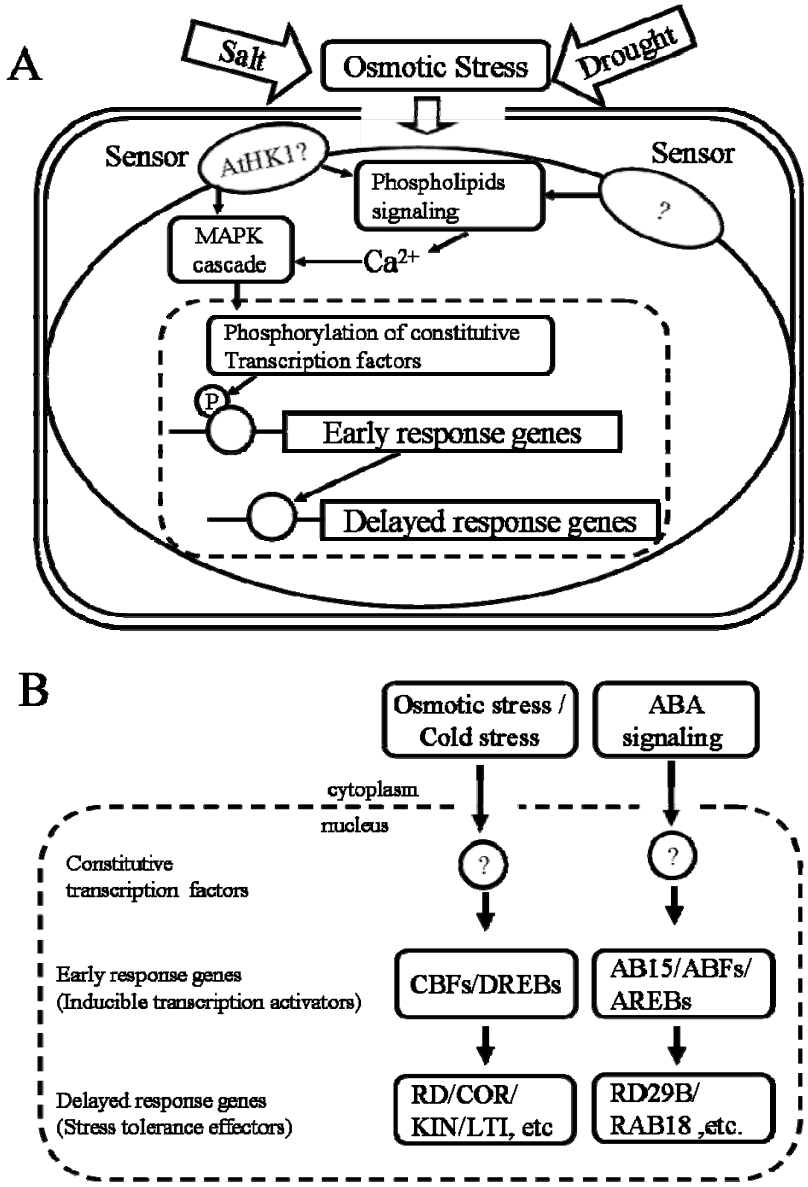

Fig. 2. Model showing osmotic stress regulation of early-response and delayed-response genes. (*A*) Model integrating stress sensing, activation of phospholipid signaling and MAP kinase cascade, and transcription cascade leading to the expression of delayed-response genes. (*B*) Examples of early-response genes encoding inducible transcription activators and their downstream delayed-response genes encoding stress tolerance effector proteins. Question marks denote unknown transcription factors that activate the early-response genes (Zhu, 2002).

ABA and abiotic stress signaling

ABA is an important phytohormone and plays a critical role in response to various stress signals. The application of ABA to plants mimics the effect of a stress condition. As many abiotic stresses ultimately result in desiccation of the cell and osmotic imbalance, there is an overlap in the expression pattern of stress genes after cold, drought, high salt or ABA application. This suggests that various stress signals and ABA share common elements in their signaling pathways and these common elements cross talk with each other, to maintain cellular homeostasis (Leung and Giraudat, 1998; Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Finkelstein and Gampala, 2002).

The functions of ABA include seed dormancy and delay in germination as well as the promotion of stomatal closure. ABA levels are induced in response to various stress signals. ABA actually helps the seeds to surpass the stress conditions and germinate only when the conditions are favourable for seed germination and growth. ABA also prevents the precocious germination of premature embryos. Stomatal closure under drought conditions prevents the intracellular water loss and thus ABA is aptly called a stress hormone.

The main function of ABA seems to be the regulation of plant water balance and osmotic stress tolerance. Several ABA deficient mutants namely *aba1*, *aba2* and *aba3* have been reported for Arabidopsis (Koornneef et al., 1998), as well as for tobacco, tomato and maize (Liotenberg et al., 1999). Without any stress treatment the growth of these mutants is comparable to wild type plants. Under drought stress, ABA deficient mutants readily wilt and die if the stress persists. However under salt stress ABA deficient mutants were able to germinate (Barrero et al., 2005) showing salt tolerance. In addition, ABA is required for freezing tolerance, which also involves the induction of genes in response to dehydration stress (Llorente et al., 2000; Xiong et al., 2001).

Processes that trigger activation of ABA synthesis and inhibition of its degradation result in ABA accumulation. Several ABA biosynthesis genes have been cloned which include zeaxanthin epoxidase (known as *ABA1* in Arabidopsis, (Marin et al., 1996), 9-*cis*-epoxycarotenoid dioxygenase (*NCED*) (Tan et al., 1997), ABA aldehyde oxidase (*AOO*) and *ABA3* also known as *LOS5*, encodes molybdenum cofactor sulfurase (Xiong et al., 2001).

Studies suggest that osmotic stress imposed by high salt or drought is transmitted through at least two pathways; one is ABA-dependent and the other ABA independent. Cold exerts its effects on gene expression largely through an ABA-independent pathway (Finkelstein and Gampala, 2002). ABA induced expression often relies on the presence of *cis* acting element called Abscisic acid responsive element (ABRE) (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Uno et al., 2000; Finkelstein and Gampala, 2002). Genetic analysis indicates that there is no clear line of demarcation between ABA-dependent and ABA-independent pathways and the components involved may often cross talk or even converge in the signaling pathway (Knight and Knight, 2001; Xiong and Zhu, 2001). Calcium, which serves as a second messenger for various stresses, represents a strong candidate, which can mediate such cross talk. Several studies have demonstrated that ABA, drought, cold and high salt result in rapid increase in calcium levels in plant cells (Sanders et al., 1999; Knight and Knight, 2000). The signaling pathway results in the activation of various genes, which play a significant role in the maintenance of cellular homeostasis.

Role of transcription factors in the activation of stress responsive genes

The promoters of stress responsive genes have typical *cis*-regulatory elements like DRE/CRT, ABRE, MYCRS/MYBRS and are regulated by various upstream transcriptional factors. These transcription factors fall in the category of early genes and are induced within minutes of stress induction. The transcriptional activation of some of the genes including *RD29A* has been well worked out. The promoter of this gene family contains both ABRE as well as DRE/CRT elements (Stockinger et al., 1997). Transcription factors, which can bind to these elements were isolated and were found to belong to the AP2/EREBP family and designated as CBF1/DREB1B, CBF2/DREB1C, and CBF3/DREB1A (Stockinger et al., 1997; Gilmour et al., 1998; Medina et al., 1999). These transcription factors (CBF1, 2 and 3) are cold responsive and in turn bind CRT/DRE elements and activate the transcription of various stress responsive genes. A novel transcription factor from soybean SCOF-1 (soybean zinc finger protein), responsive to cold as well as ABA, was found not responsive to drought or salinity stress (Kim et al., 2001). SCOF1 was shown to be a nuclear localized protein but did not bind directly to either CRT/DRE or ABA-

responsive elements (ABRE). Yeast 2-hybrid studies revealed that SCOF-1 interacted strongly with SGBF-1 (Soybean G-box binding bZip transcription factor) and in vitro DNA binding activity of SGBF-1 to ABRE elements was greatly improved by the presence of SCOF-1. This study supported that protein–protein interaction is essential for the activation of ABRE-mediated cold responsive genes (Kim et al., 2001). Transcription factors like DREB2A and DREB2B get activated in response to dehydration and confer tolerance by induction of genes involved in maintaining the osmotic equilibrium of the cell (Liu et al., 1998). Several basic leucine zipper (bZip) transcription factors (namely ABF/AREB) have been isolated which can specifically bind to the ABRE and activate the expression of stress genes (Choi et al., 2000; Uno et al., 2000). These *AREB* genes (*AREB1* and *AREB2*) are ABA responsive and need ABA for their full activation. These transcription factors exhibit reduced activity in the ABA-deficient mutant *aba2* as well as in the ABA insensitive mutant *abi1-1*. Some of the stress responsive genes, for example *RD22*, lack the typical CRT/DRE elements in their promoter indicating their regulation by other mechanisms. Transcription factors RD22BP1 (a MYC transcription factor) and AtMYB2 (a MYB transcription factor) could bind MYCRS (MYC recognition sequence) and MYBRS (MYB recognition sequence) elements, respectively, and could cooperatively activate the expression of the *RD22* gene (Abe et al., 1997). As cold, salinity and drought stress ultimately impair the osmotic equilibrium of the cell it is likely that these transcription factors as well as the major stress genes may cross talk with each other for their maximal response and help in reinstating the normal physiology of the plant.

Drought resistance mechanisms

In genetic sense drought resistance mechanisms can be grouped into three categories (Mitra, 2001), viz. drought escape, drought tolerance and drought avoidance. However, crop plants use more than one mechanism at a time to resist drought.

Drought escape is defined as the ability of a plant to complete its life cycle before serious soil and plant water deficits develop. This mechanism involves rapid phenological development (early flowering and early maturity), developmental plasticity (variation in duration of growth

period depending on the extent of water-deficit) and remobilization of pre-anthesis assimilates to grain.

Drought tolerance is the ability to withstand a water-deficit with low tissue water potential. The responses of plants to tissue water deficit determine their level of drought tolerance. The mechanisms of drought tolerance are maintenance of turgor through osmotic adjustment (a process which induces solute accumulation in the cell), increase in elasticity in the cell and decrease in cell size and desiccation tolerance by protoplasmic resistance. Osmotic adjustment increases drought resistance by maintaining plant turgor, but the increased solute concentration responsible for osmotic adjustment may have detrimental effect in addition to energy requirement for osmotic adjustment.

Drought avoidance is the ability of plants to maintain a relatively high tissue water potential (Ψ) despite a shortage of soil-moisture. Mechanisms for improving water uptake, storing in the plant cell and reducing water loss confer drought avoidance. Drought avoidance is achieved by the maintenance of turgor through increased rooting depth, efficient root system and increased hydraulic conductance and by reduction of water loss through reduced epidermal (stomatal and lenticular) conductance, reduced absorption of radiation by leaf rolling or folding and reduced evaporation surface (leaf area).

Plants under drought condition can survive by a balancing act between maintenance of turgor and reduction of water loss. However, most of these adaptations to drought have disadvantages. A genotype of short life duration usually yields less compared to that of normal duration. The mechanisms that confer drought resistance by reducing water loss (such as stomatal closure and reduced leaf area) usually result in reduced assimilation of carbon dioxide.

Water Use Efficiency

Water use efficiency (WUE) is the ratio of the dry matter produced to the amount of water used and is related to transpiration efficiency (TE) which is the dry matter produced per unit water transpired. Unlike other abiotic stresses, the water availability is directly related to productivity and one way to increase productivity under water scarcity is to increase crop WUE (Condon et al., 2004). Efficient water use under water limited conditions can maintain biomass and yield.

Attempts have been made in the past to identify quantitative trait loci (QTL) for transpiration efficiency (Virgona, 1990) in various crop species. Characterization of the QTLs identified for the WUE and localization of candidate genes involved in photosynthesis and transpiration would be useful information not only for traditional breeding programs but also in genetic manipulation of plants to obtain crops with high WUE. The analysis of the Arabidopsis *ERECTA* gene recently revealed its role in altering WUE by modifying leaf diffusive properties due to stomatal density, cell expansion and mesophyll capacity for photosynthesis (Masle et al., 2005).

Role of roots in drought resistance

Plant root systems perform many essential adaptive functions including water and nutrient uptake, anchorage to the soil and the establishment of biotic interactions at the rhizosphere. When plants are subjected to low Ψ, the growth of leaves and stems is rapidly inhibited (Acevedo et al., 1971; Nonami and Boyer, 1990). In contrast, roots may continue to elongate at low values of Ψ that completely inhibit shoot growth (Sharp et al., 1988; Spollen, 1993). This differential response of roots and shoots to low Ψ is considered to be an adaptation of plants to survive dry conditions since continued root elongation facilitates water uptake from the soil (Sharp et al., 1988; Spollen, 1993).

Changes in the architecture of the root system, therefore, can profoundly affect the capacity of plants to take up nutrients and water. Three major processes affect the overall architecture of the root system. First, cell division at the primary root meristem (i.e. of initial cells) enables indeterminate growth by adding new cells to the root. Second, lateral root formation increases the exploratory capacity of the root system; and third, root-hair formation increases the total surface of primary and lateral roots. Alterations to any of these three processes can have profound effects on root-system architecture and on the capacity of plants to grow in soils in which nutrient and water resources are limiting. Better understanding of root system structure and function is critical to crop improvement in water-limited environments.

Roots have the capacity to sense the osmotic changes in the environment and they can do so by the ‘osmo-sensors’ active in the root hair (Lew, 1996). It is known that plant responses are not triggered by changes in the turgor of the outer root cells, but by osmotic changes in the

environment. Evidence for this was provided by overexpression of the ATHK1-gene in Arabidopsis, which induced drought stress tolerance (Urao and Yamaguchi Shinozaki, 2002), indicating that improved ability to sense the environment leads to enhanced drought tolerance.

Several quantitative trait loci (QTLs) for root traits have been identified in rice and other crops which respond to environmental constraints like drought such as thickness, penetration ability and length (Ray et al., 1996; Ali et al., 2000; Zhang et al., 2001; Yadav et al., 1997). Hence root traits play a crucial role in determining stress tolerance in plants.

In addition to morphological and physiological changes, biochemical change involving induction of compatible solute biosynthesis is one way to impart drought tolerance. Under drought, plants try to maintain water content by accumulating various solutes that are nontoxic and do not interfere with plant processes and are, therefore, called compatible solutes (Yancey et al., 1982). Some of them are fructans, trehalose, polyols, glycine betaine, proline and polyamines. The different genes responsible for different enzymes involved in biosynthesis of these solutes have been identified and cloned from different organisms (bacteria, yeast, human and plant), and are, therefore, available for employment.

In synthesis, crop adaptation must reflect a balance among escape, avoidance and tolerance while maintaining adequate productivity. Drought resistance is, therefore, a complex trait for breeding, expression of which depends on action and interaction of different characters: morphological (earliness, reduced leaf area, leaf rolling, wax content, efficient rooting system, stability in yield and reduced tillering), physiological (reduced transpiration, high water-use efficiency, stomatal closure and osmotic adjustment) and biochemical (accumulation of proline, polyamine, trehalose, etc., increased nitrate reductase activity and increased storage of carbohydrate). Very little is known about the genetic mechanisms that condition these characters.

Rice and salt stress

Salinity is an ever-present threat to crop yields, especially in countries where irrigation is an essential aid to agriculture. Although the tolerance of saline conditions by plants is variable, crop species are generally intolerant to one-third of the concentration of salts found in seawater. Rice is the staple food for millions of people around the globe. It is also a crop whose requirement

for irrigation is huge compared to other major crops to achieve maximum yield. Also it is sensitive to salinity which causes major yield losses each year.

Investigations of the effects of salinity on rice have been underway for more than 50 years (Kapp, 1947; Pearson, 1959) and attempts to enhance the salt tolerance in rice through breeding date from the early 1970s (Akbar et al., 1972). In spite of considerable effort, through both international and national breeding programmes, progress in enhancing tolerance has been slow; with few new cultivars released (perhaps the most spectacular has been CSR10, developed at the Central Soil Salinity Research Institute in India).

Attempts to improve the salt tolerance of rice through conventional breeding programmes have met with very limited success, due to the complexity of the trait: salt tolerance is complex genetically and physiologically. Tolerance often shows the characteristics of a multigenic trait, with quantitative trait loci (QTLs) associated with tolerance identified in barley, citrus, rice, and tomato and with ion transport under saline conditions in barley, citrus and rice. Physiologically salt tolerance is also complex, with halophytes and less tolerant plants showing a wide range of adaptations. Attempts to enhance tolerance have involved conventional breeding programmes, the use of *in vitro* selection, pooling physiological traits, interspecific hybridization, using halophytes as alternative crops, the use of marker-aided selection, and the use of transgenic plants.

Recent research has shown that rice, transformed to overexpress genes that brought about the synthesis of trehalose, contained a reduced concentration of Na^+ in the shoot and grew better than non-transformed (control) plants when in the presence of 100 mM NaCl (Garg et al., 2002). In these experiments (Garg et al., 2002), the synthesis of trehalose was under the influence of a stress-inducible promoter, so that growth under control conditions was presumably no different from the wild type. The use of stress-inducible promoters may be an important tool to avoid growth inhibition under non-stressed conditions (Kasuga et al., 1999) as there are yield penalties from expressing genes under a constitutive promoter.

Arabidopsis and rice as model plants, for identification of stress resistance genes.

Arabidopsis and rice are the two model plants whose genome sequence has been completed. Amidst the complexities of environmental stress reactions in crop plants the use of the

simple model Arabidopsis, offers an opportunity for the precise genetic analysis of stress reaction pathways common to most plants. The relevance of the Arabidopsis model is evident in recent examples of improving drought, salt and freezing tolerance (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999) using genes identified in Arabidopsis. These genes are transcription factors of the EREBP/AP2 family that regulate the expression of a number of downstream genes conferring stress resistance in a number of heterologous plants.

Rice has a compact genome size (three times Arabidopsis) and is easy to transform, enabling the testing of gene constructs. Furthermore, extensive synteny exists between the rice genome and genomes of other important cereal species like maize, wheat and sorghum. In addition, large sets of ESTs are available, including from plants subjected to different growth conditions such as salt- or drought stress (www.stress-genomics.org), revealing many genes not expressed in normal grown plants. Furthermore, several international projects are underway to establish mutant collections that can be assessed on line (Hirochika et al., 2004). The rice genome sequence and comparative genomics tools have been used to find conserved functions to Arabidopsis as was demonstrated by Syngenta in an integrated approach to identify conserved stress mechanisms between monocots and dicots (Cooper et al., 2003).

Conservation of gene functions

The use of "model" species in biological research is based on the assumption that many of their features are shared among a wide range of related taxa. Thus, it is hoped that many of the genes associated with important traits in crop plants will be identified via homology with their counterparts in Arabidopsis. Monocot and dicot plants diverged ca. 200 million years ago (Wolfe et al., 1989). Although this 200 million years divergence has eroded conservation of gene order on the chromosomes, at the level of gene protein sequences we can still observe similarities between Arabidopsis and rice taken as models for dicotyledonous and monocotyledonous plants, respectively (Gale and Devos, 1998). Not only gene protein sequences but also the functions of many important genes have been documented to be conserved between dicots and monocots.

The “green revolution” dwarf gene used in the rice variety IR8 was identified as a deletion mutation of the *GA20ox-2* gene (Sasaki et al., 2002). This gene was identified on basis of

similarity to the conserved domain of the Arabidopsis *GA5* gene encoding GA 20-oxidase and also displays a dwarf mutant phenotype. The other "green revolution" genes in wheat (*Rht-B1*/*Rht-D1*) are orthologs of the Arabidopsis *Gibberellin Insensitive* (*GAI*) gene (Peng et al., 1999). Other agronomically important genes controlling flowering time in rice, *Hd1, Hd3a* and *Hd6* have been discovered to be orthologs of Arabidopsis *CONSTANTS*, *FT* and *CK2*, respectively (Yano et al., 2000; Takahashi et al., 2001; Kojima et al., 2002).

These examples exemplify the conserved function of genes of great importance to domestication and agriculture. Many others are incompletely described due to the lack of detailed analysis of genes and mutant phenotypes in a number of crop plants.

Identification of stress resistance genes by activation tagging.

The sequence of the Arabidopsis genome reveals that two-thirds of the genome is duplicated with about 15% of known genes involved in plant defense. Most signal transduction pathways in plant stress responses involve hundreds of interacting genes with redundant functions that are intractable through classical genetic analysis. The primary tool for dissecting a genetic pathway is the screen for loss-of-function mutations that disrupt such a pathway. However, a limitation of loss-of-function screens is that they rarely identify genes that act redundantly. The problem of functional redundancy has become particularly apparent during the past few years, as sequencing of eukaryotic genomes has revealed the existence of many duplicated genes that are very similar both in their coding regions and their non-coding, regulatory regions. To overcome these problems to genetically identify genes with functions useful for agriculture, strategies to overexpress genes and, subsequently, analyze their phenotypes would be an effective way of gene function identification. One such method is a candidate gene by gene approach that has been utilized for the transcription factors in Arabidopsis. This is supported by the identification of two AP2/ERF transcription factor genes from Arabidopsis that when overexpressed in Arabidopsis and other plants, render the transformants tolerant to drought, cold and salinity stress (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999). A strategy to overexpress plant genes in a high-throughput way and selection for stress resistant lines would, therefore, provide the most direct way of identifying genes that can confer stress resistance. Another way is activation tagging with

inserts that can activate adjacent genes to cause a gain in function phenotype. Activation tag populations have been obtained with inserted T-DNA or transposons. These 'Activation Tag' inserts contain a multiple enhancer of the CaMV35S promoter that can act on plant promoters located close by (~10kb) and enhance or activate their expression. Published results show that about 1% of the insertions cause dominant gain in function phenotype. When screened for resistance to stress about 1% display resistance to one of the stress parameters, suggesting that it is an effective method for gene function discovery. The most relevant criterion in this system is, that these selected overexpression phenotypes are directly applicable to other plant systems by constitutive or regulated expression. These genes are applicable for biotechnological use or for marker-assisted breeding towards a durable agroproduction system, providing stable production by helping plants fight environmental challenges.

Conclusion and prospects

Salt and drought stress signaling is an important area with respect to increase in plant productivity. Our knowledge of the molecular mechanisms underlying the responses of plants to such environmental stresses is still rather limited, but an increasing number of genes have been identified in recent years that mediate these responses. Some of these genes are induced by stress stimuli and encode products that confer tolerance to adverse conditions, whereas others encode upstream regulators that function within signaling pathways controlling the stress response. Therefore, the basic understanding of the mechanism underlying the functioning of stress genes is important for the development of stress tolerant crops. Each stress is a multigenic trait and therefore their manipulation may result in alteration of a large number of genes as well as their products. The ability of the transcription factors (TFs) to control both multiple pathway steps and cellular processes that are necessary for metabolite accumulation, offers much promise for the manipulation of metabolic pathways. This is particularly true for complex pathways whose components enzymes are poorly characterized, such as those of secondary metabolism. Similarly, overexpression of validated downstream stress responsive genes under control of tissue specific inducible promoters can also impart stress resistance (Zhu et al., 1998). A deeper understanding of

the transcription factors regulating these genes, the products of the major stress responsive genes and cross talk between different signaling remains an area of intense activity in the future.

Scope of the thesis

In this thesis, we have identified transcription factors from Arabidopsis conferring drought and salt tolerance in Arabidopsis. These TFs were used for improving salt tolerance in the heterologous monocot rice (*Oryza sativa*). Also we have demonstrated the use of high throughput screening for abiotic stress resistance.

In chapter 2, the Arabidopsis *HARDY* (*HRD*) gene, an AP2/ERF-like transcription factor (TF), that when overexpressed, confers drought resistance and increases water use efficiency (WUE) in Arabidopsis is described. It also confers high salt tolerance in Arabidopsis. Additionally, *HRD* overexpression in Arabidopsis exhibits an increased number of leaf mesophyll layers, an enhanced root cortex and a strong root network that increases the root strength. As the gene displayed various features useful for abitoic resistance, the gene was transformed into rice to study the salt tolerance phenotype and other physiological parameters.

In chapter 3, we describe another Arabidopsis gene *SHINE* (*SHN*), an AP2/ERF TF known to increase epicuticular wax and alter epidermal properties, in conferring drought resistance, salt tolerance and increasing WUE in Arabidopsis. We also tested the role of the Arabidopsis *SHN* gene in imparting salt tolerance in the heterologous system, rice.

The Arabidopsis gene called *BOUNTIFUL* (*BFL*), an AT-hook DNA binding protein is studied in relation to agronomic trait of increase in biomass in chapter 4. BFL overexpression in Arabidopsis exhibits a robust plant phenotype with increased rosette leaves of larger size. It also confers increased salt tolerance in Arabidopsis.

In chapter 5, we utilized a high throughput screening of an activation tagged (ATag) mutant population for abiotic stress resistance. We pre-selected candidate stress resistant mutants based on their phenotype of reduced fitness (e.g. low seed set, poor growth) and tested them for drought tolerance. We identified 8 drought resistant mutant lines, characterized them for their specific phenotypic parameters and identified the putative tagged genes by isolation of the DNA

flanking the activation tag insertions. In addition, we describe two of these mutants, *atal* and *vajra*, conferring increased water use efficiency and salt tolerance in Arabidopsis.

In chapter 6, the results from the experimental chapters are discussed together with our present understanding of the mechanism and function of these genes and how they could be best exploited for future use in engineering abiotic stress tolerant crops.

Chapter 2

HARDY, an Arabidopsis AP2/ERF-like transcription factor confers drought and salt tolerance and improves water use efficiency both in Arabidopsis and Rice

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Part of this work is published in (Karaba A and Dixit S, et al.) PNAS, 2007

Improvement of water use efficiency in rice by expression of HARDY, an Arabidopsis drought and salt tolerance gene.

Abstract

Drought and salinity are the major abiotic stresses that affect crop production worldwide. Freshwater is a limited and dwindling global resource, therefore efficient water use is required for food crops that have higher water demands such as rice. We show here that the expression of the Arabidopsis *HARDY (HRD)* gene, an AP2/ERF-like transcription factor, identified via a gain-of-function Arabidopsis mutant *hrd-D*, improves water use efficiency (WUE), drought resistance and salt tolerance. This mutant has roots with enhanced strength, an increased branching pattern and more cortical cells, which is accompanied by an enhancement in the expression of abiotic stress associated genes. In addition, overexpression of *HRD* significantly improves salt tolerance at the vegetative stage in rice and leads to higher biomass production and seed set both under non-stress and stress conditions compared to the WT. The results exemplify the application of a gene identified from the model plant Arabidopsis for the improvement of drought and salt tolerance in the crop plant rice.

Introduction

Plants are frequently exposed to a plethora of stress conditions such as low temperature, salt, drought, flooding, heat, oxidative stress and heavy metal toxicity. Plants also face challenges from pathogens including bacteria, fungi, and viruses as well as herbivores. All these stress factors are a challenge for plants that prevent them from reaching their full genetic potential and limit crop productivity worldwide. Abiotic stress in fact is the principal cause of crop failure worldwide, reducing average yields for most of the crops by more than 50% (Bray, 2000). Water deficit stress, which could be a result of drought or salt stress, is the critical factor responsible for these unpredictable losses in crop production. The physiological mechanisms governing plant responses to salinity and drought show high similarity, suggesting that both stresses must be perceived by the plant cell as deprivation of water (Verslues et al., 2006). High salt concentrations (most commonly NaCl) in the soil lead to a decrease in water potential, which affects water availability (Hasegawa et al., 2000). Therefore salt stress essentially results in a water deficit condition in the plant and takes the form of a physiological drought. In addition to the hyperosmotic shock and the generated subsequent oxidative stress (Borsani et al., 2001), the deleterious consequences of high NaCl concentration in the external solution of plant cells also include ion toxicity and nutrient imbalance (Hasegawa et al., 2000). As sessile organisms, plants had to develop various biochemical and physiological mechanisms to respond and adapt to these stresses and thus acquire stress tolerance. Adaptation to stress has been suggested to be mediated by both preexisting and induced defenses (Bray, 2000; Hasegawa et al., 2000; Pastori and Foyer, 2002).

Rice, uses two to three times more water than other food crops such as wheat or maize, using 30% of the freshwater used for crops worldwide (Barker, 1999). Rice is the primary source of food for more than half of the world's population, especially in developing countries in Asia where water scarcity and drought are an imminent threat to food security (Barker, 1999; Rosegrant and Cline, 2003). Furthermore, rice is also a salt sensitive crop (glycophyte). Therefore, a more sustainable use of global water resources in crop production is required by improvement of the rice crop for abiotic stress tolerance and water use.

Water use efficiency (WUE), measured as the biomass produced per unit of transpiration, describes the relationship between water use and crop production. In water-limiting conditions, it would be important to produce a high amount of biomass, which contributes to crop yield, using a low or limited amount of water. Plant resistance to drought stress can be improved through drought avoidance or drought tolerance (Price et al., 2002), among which drought avoidance mechanisms tend to conserve water by promoting WUE. Salt tolerance in plants could be achieved by regulating the expression of effectors or regulators functioning in a re-establishment of ion homeostasis under stress conditions (Zhu, 2001). The concomitant improvement in WUE, drought and salt stress resistance without yield penalties can offer long-term sustainable solutions to agricultural land use. In this study, we have identified an AP2/ERF-transcription factor gene called *HARDY*, which provides improved WUE, drought and salt tolerance in Arabidopsis and rice.

Materials and Methods

Arabidopsis plant growth and phenotype analysis

The Arabidopsis plants used are in ecotype Wassilewskija (Ws-3) and were grown in the greenhouse at around 22°C. For growth measurements, fresh weight (FW) of plants was determined immediately after harvest, and samples were oven-dried at 65°C for one week to obtain dry weight (DW). To measure the strength of the *hrd-D* and WT (wild-type) roots, we used 5-week old plants grown in well-watered soil in Aracon containers (BetaTech, Belgium). For each plant, a nylon cord was tied to the base of the stem and using an attached pulley with a container, sand was gradually added until the plant roots were pulled out or broke from the anchored pot below. The weight of sand required to pull the plants out was recorded and averages calculated for the different genotypes.

For leaf structure analysis, cryo-fracture Scanning Electron Microscopy (SEM) was done as described earlier (Aharoni et al., 2004). Cross sections of the Arabidopsis roots from 6-week-old plants were made by free-hand sectioning with a razor blade, stained with phloroglucinol-HCl (1% [w/v] phloroglucinol in 6 N HCl) and then observed under dark field microscope (Zhong et al., 2000).

DNA analysis and plant transformation

Plant DNA was isolated and 10 ng used for TAIL PCR (Marsch-Martinez et al., 2002), followed by sequencing of the flanking DNA to reveal the insert position in the Arabidopsis genome using BlastN (Altschul et al., 1990). Fragments encoding the *HRD* gene (At2g36450) were amplified from Arabidopsis ecotype Columbia genomic DNA using *pfu* DNA polymerase with primers HRDf (5'-CGGATCCATGCAAGGAACCTCCAAAGAC-3') and HRDr (5'-CGTCGACGGTTTGTTTAACTATCATGG-3'), cloned into the pGEM-T Easy vector (Promega) and sequenced before digestion and ligation to the binary vectors.

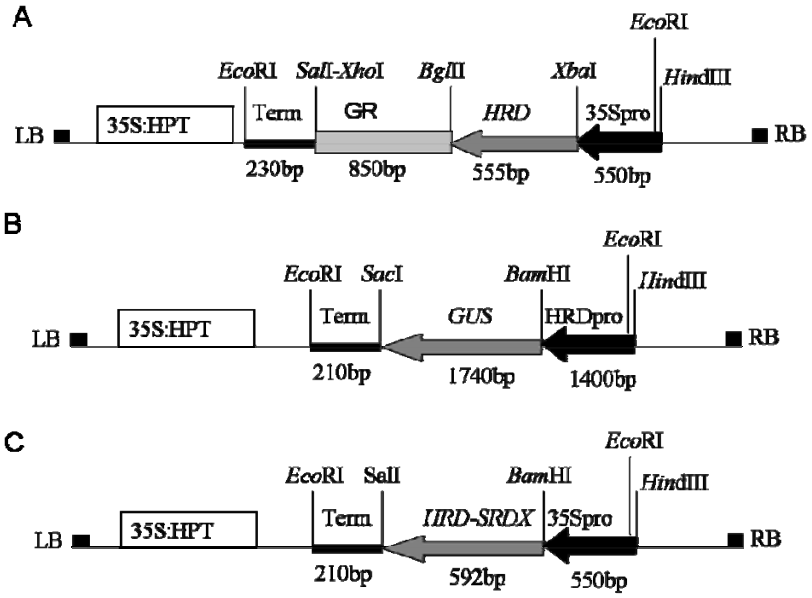

Fig. 1. *HRD* gene constructs.

(**A**) The 35S::HRD-GR construct contains the *HRD* gene fused in frame to the hormone binding domain of the rat glucocorticoid receptor (GR) fragment obtained from pMTL23 (Johnson et al., 2001) and cloned in binary vector pMOG22 using 35S:HPT for hygromycin resistance. The fragments ligated together are shown with respective sizes (in bp) and restriction enzymes used. **(B)** The HRDpro:GUS_HPT construct contains the *GUS* gene in the binary vector pMOG22 (Zeneca-Mogen, NL), driven by HRD-promotor and contains a chimeric CaMV35S-hygromycin phosphotransferase-tNos (35S:HPT) for hygromycin selection during transformation. The fragments used for cloning with their sizes in bp and restriction sites used for cloning are labeled. **(C)** The 35S:HRD-SRDX_HPT construct contains the *HRD* gene fused with ERF-associated amphiphilic repression (EAR) domain in the binary vector pMOG22 (Zeneca-Mogen, NL), which contains a chimeric CaMV35S-hygromycin phosphotransferase-tNos (35S:HPT) for hygromycin selection during transformation. The fragments used for cloning with their sizes in bp and restriction sites used for cloning are labeled.

For Arabidopsis transformation the 35S:HRD gene construct was made. The oligonucleotides introduced *Bam*HI and *Sal*I restriction sites to the *HRD* fragment at the 5' and 3', respectively, which were used to ligate the 555-bp coding region fragment to compatible sites in the pNEW1 binary vector (Marsch Martinez and Pereira, unpublished), in between a CaMV35S promoter (Pietrzak et al., 1986) and the nopaline synthase terminator (tNOS). To make an overexpression construct for rice transformation, the binary vector pMOG22 (Zeneca-Mogen, NL) was used, containing a chimeric CaMV35S-hygromycin phosphotransferase-tNOS for hygromycin selection during plant transformation. To generate the steroid inducible 35S::HRD-GR constructs (Fig. 1A), a *HRD* gene fragment was amplified (using *pfu* DNA polymerase) from Arabidopsis ecotype Columbia DNA using the oligonucleotides RP6 (5'-TTATTTCTAGAATGCAAGGAACCTCCAAAGAC -3') and RP7 (5'-TTATTAGATCTTGGAAAATTCCACAAGTAATCG -3') that introduced *Xba*I and *Bgl*II restriction sites at the 5' and 3' respectively. The *HRD* gene and other fragments, including a 0.85 kb *Bgl*II-*Xho*I GR fragment obtained from pMTL23 (Chang et al., 1987), were used in multi-point ligations with compatible ends to assemble the construct. For generating the *HRD*-promoter::GUS constructs (Fig. 1B), a fragment (1.4 kb) upstream to the ATG codon of *HRD* was amplified from genomic DNA (ecotype Columbia) using Taq DNA polymerase and oligonucleotides HRD-F (5'-GCTACCAACCATGCTTACAC – 3') and HRD-R (5'-AATAACCATGGGGAGGTTCCTTGCATATTG -3') which introduced *Hind*III & *Bam*HI restriction sites at the 5' and 3', respectively. The β-glucuronidase (GUS) reporter gene and the nopaline synthase terminator (tNOS) were digested out from pBI121 binary vector with *Bam*HI and *Eco*RI restriction sites at the 5' and 3', respectively. These fragments were used in multi-point ligations with compatible ends to assemble the construct in a binary vector pMOG22 (Zeneca-Mogen, NL). For making the *HRD* repression/knockout construct the 12 amino acid EAR sequence was introduced at the 3' end of the *HRD* gene sequence (Fig. 1C) and the fragment was amplified using oligonucleotides HRD-SRDX-F (5'-TTATTGGATCCATGCAAGGAACCTCCAAAGAC – 3') and HRD-SRDX-R (5'-CGTCGACTCAAGCGAAACCCAAACGGAGTTCTAGATCCAGATCCAGTGGAAAATTCC ACAAGTAATCGTCTCCTC -3') introducing *Bam*HI and *Sal*I restriction sites to the *HRD*-

SRDX fragment at the 5' and 3', respectively, which were used to ligate the 592-bp coding region fragment to compatible sites in a binary vector pMOG22 (Zeneca-Mogen, NL). In all cases PCR fragments were first A- tailed and introduced to the pGEM-T Easy vector as described by the manufacturer (Promega) and subsequently sequenced from both sides before digestion and ligation to the binary vector. PCR, restriction digests, plasmid DNA isolation and gel electrophoresis were performed using standard protocols. The constructs were introduced into Arabidopsis using the floral dip transformation method (Clough and Bent, 1998), the seeds selected for transformants on medium with 50 mg/L kanamycin or 20 mg/L hygromycin and subsequently transferred to the greenhouse. Agrobacterium-mediated transformation of *Oryza sativa* ssp. *japonica* cv. Nipponbare, plant regeneration and growth were performed (Greco et al., 2001), using the AGL-1 Agrobacterium strain. Regenerated transgenic plantlets were transferred to the greenhouse and grown in hydroponics culture with a regime of 12 hours light, 28°C, 85% relative humidity and 12 hours dark, 25°C, 60% humidity.

Arabidopsis dry-down drought resistance test

For the drought tolerance experiments, the soil mixture comprised one part of sand and perlite and two parts of compost (a mixture made up of 25% clay and 75% turf with EC = 1 [nitrogen, phosphorous, and potassium]; Hortimea, Elst, The Netherlands). Seeds were sown (after three days at 4°C) at a density of six plants per 4-cm pot in a tray with 51 pots (Aracon containers; BetaTech, Gent, Belgium). Nutrients (Hydroagri, Rotterdam, The Netherlands; 2.6 EC) were supplied 10 days after germination (DAG), and at 14 DAG the plants were subjected to drought (for 9, 10, 11, or 12 d) by transferring the pots to dry trays (after drying each pot from outside). Every 2 d in drought, the plants were moved within the tray to nullify pot position effects. Subsequently, plants were rehydrated and observed for recovery after 1 week. Experiments comparing drought tolerance between the WT and *hrd-D* were repeated three times.

Arabidopsis *g*ravimetric drought resistance test

Plants were sown in 7x7 cm plastic pots (4 plants per pot) in the greenhouse conditions as described above. Soil mixture used to grow the plants was the same as used in the dry-down

drought testing method. The soil was weighed accurately in each pot. The pots were well watered and maintained at 100% field capacity. (FC- the amount of water left in the soil after it has been saturated and allowed to drain by gravity for 24 hours). Two weeks after the germination, the bottom holes of the pots were sealed to avoid water drainage. A control set of pots (9 pots each line) both representing the WT and *hrd-D* mutant, were maintained at 100% field capacity for the rest of the experimental period. A similar set of WT and *hrd-D* mutant plants were exposed to drought by withholding water until the WT died (approx. 10-11 days of withholding water). Control pots ($n \geq 4$) with no plants were maintained at 100% FC throughout the experimental period and another set of pots without plants were maintained at 100% field capacity until the beginning of the drought treatment, when water was withheld until the end of the experiment. This allowed measuring water loss through evaporation in the control as well as the drought set of pots. These data were used as a correction factor for transpiration estimates. During the experimental period, the pots were weighed daily using a portable balance (0.001g accuracy). For the control set of pots ($n \geq 9$) the difference in the weight on subsequent days was corrected by adding water to maintain the 100% FC. For the drought set of pots the daily difference in the weight of pots was noted down. At the 15^{th} DAG or the 1^{st} day of dehydration (DOD), representative plants from both the WT and *hrd-D* were sampled to measure the initial biomass (B_{15}). The WT plants were carefully monitored daily for their appearance and when assessed as having wilted enough to survive for one more day in drought, a set of pots ($n \geq 4$) was rehydrated to be checked for recovery. In addition, biomass from each set of pots both representing WT and *hrd-D* mutants were collected, and this process of sampling was repeated daily until the WT had passed irreversible wilting point and could not recover the next day. This day of permanent wilting point was noted as day after WT's death (DAWD). In these experiments the WT died on 11^{th} DOD or 25^{th} DAG. The water added during the experimental period in the control set of pots maintained at 100% FC was summed up to arrive at the cumulative water lost (CWT) by the plants in process of transpiration from the control plants. The difference in the weight of pots of the drought set was calculated as the total water lost by transpiration in drought treated plants. The percentage of water present in each pot was measured at the beginning of the drought test and

when the WT died to determine the minimum threshold level of water beyond which the WT cannot survive. This was calculated using following formula,

Water pot^{-1}(g) = Total wt. of pot – Σ (dry wt. of soil pot^{-1} + wt. of empty pot + Fresh biomass of plant)

% water g^{-1} of soil = ($_{final}$ Water pot^{-1} / dry wt of soil pot^{-1}) x 100

Assuming that the growth was linear during the experimental period, WUE was computed as WUE= (B_{25}-B_{15})/CWT, where B_{25} and B_{15} are the total biomass (g pot^{-1}) measured on 25 and 15 DAG, respectively. CWT is the cumulative water transpired by the plants (g) during the experimental period and is computed as follows: For control pots,

CWT (g) = Σ (water added to the plants each day) - Σ (water lost by evaporation from the soil).

For pots exposed to drought CWT was calculated as follows,

CWT (g) = ($_{initial}$ Water pot^{-1} – $_{final}$ Water pot^{-1}) - Σ(water lost by evaporation).

Mean Transpiration Rate per plant per day were calculated as **MTR**= CWT/11(Days of observation).

All the fresh biomass collected was later oven dried at 65 °C for 5 days and the dry weights were measured separately. All experiments were tested for significance of parameters by analysis of variance (ANOVA), comparing the *HRD* lines vs. WT and control vs. drought treatments. Statistical analysis was done using Microsoft Excel 7.0 (Microsoft, 2000) and Graph Pad Prism version 4.00 for Windows, Graph Pad Software, San Diego California USA, www.graphpad.com.

Arabidopsis salt stress tolerance test

To screen for salinity tolerance (Cheong et al., 2003) we used seedlings grown at a density of 1-2 plants per 2-cm pot in a tray with potting soil (Hortimea, Elst, The Netherlands). Nutrients (Hydroagri, Rotterdam, Netherlands; 2.6 EC) were supplied at 14 DAG. At 21 DAG the plants were treated three times (at intervals of 3 days) with NaCl (200, 250 and 300 mM) and monitored for the development of chlorosis symptoms in the next 2 weeks. Survival rates were counted on the 10th day after the third application of NaCl. The experiment was repeated three times.

Rice salt stress tolerance test

Progeny (T2) of four independent lines (T1) of transgenic (HRD-A, HRD-B, HRD-C and HRD-D) plants and untransformed WT (Nipponbare) plants were used for the salt stress tolerance test. Seeds were germinated on wet filter paper in the growth chamber at 28 ºC using a day cycle (12h light, 12h dark) for 12 days. The germinated seeds were then transferred to the greenhouse with long day conditions (16h light, 8h dark) in larger pots (12cm X 12cm) with sand and were maintained on Hoagland's solution (Hoagland, 1950) throughout the experimental period. Three weeks after the plants were transferred to the greenhouse they were exposed to different NaCl concentrations (150 mM and 200 mM NaCl) for 14 days, assessed and photographed. Root of replicate plants (n=3) of each line were separately collected for fresh and dry weight determination. For the dry weight determination, the roots were dried at 70 ºC for 5 days and weighed.

The determination of Na^+ $Ca^{2+}$and K^+ Ion content

In Arabidopsis the seedlings and in rice the roots of the treated and control plants were rinsed with deionized water and dried at 65°C for one week. About 15 to 50 mg of dry material was digested with 1 ml of the digestion mixture (sulphuric acid - salicylic acid and selenium) and 2 carborundum beads and swirled carefully until all the plant material was moistened and treated overnight. Temperature was increased gradually in small steps to about 330 °C and later after cooling 0.1 ml of hydrogen peroxide was added and heated again. This step was repeated 3 times until the digest had turned colorless. On cooling down to room temperature 5 ml of demi-water was added to make up to the mark and left overnight. The Na^+ Ca^{2+} and K^+ ion content were determined by using an Atomic Emission Spectrophotometer (Elex, Eppendorf, Hamburg, Germany). Three individual plants were taken as replicate samples for each treatment in Arabidopsis and three dried roots of each line in rice were taken as replicates for this analysis.

Histological analysis and GUS staining

Impressions of leaf epidermis were done using a domestic nail polish (HEMA, The Netherlands) for Arabidopsis leaves. The nail polish was applied on the adaxial surface of leaves,

dried for about 10-15 minutes and the layer of imprint was removed and observed under a light microscope at 40x magnification. Four week old leaves of *hrd-D* and WT were used. For GUS staining, tissues from various organs of soil grown plants were analyzed for their GUS expression patterns. The GUS staining solution contained 100 mM sodium phosphate buffer, pH 7.0, 0.5 mg/ml 5-bromo-4-chloro-3-indolyl β-D glucoronic axid (X-Gluc, Duchefa, The Netherlands), 0.1% Triton, and 0.5mM each of potassium ferri/ferrocyanide. Samples were vacuum infiltrated and incubated at 37°C for 16 to 24 h and chlorophyll depleted out in 70% ethanol. Observations were conducted either under the binocular (WILD M3Z of Heerbrugg Switzerland, type-S), or with a light microscope (Zeiss) and an RS Photometrics CoolSNAP camera (MediaCybernetics®) was used to take the digital images, with the corresponding CoolSNAP software.

Microarray analysis

In the experiment we compared gene expression of the *hrd-D* mutant overexpressing the *HRD* gene and WT Ws, both under well watered conditions. Leaf was harvested at 21 DAG, with two biological replicates for each hybridization. Total RNA was isolated using TRIZOL reagent (Life Technologies, Inc.) and purified using RNeasy Minelute Kit (Qiagen, Carlsbad, CA, USA). First strand cDNA synthesis was carried out by reverse transcription of 3 μg RNA with the T7 Oligo (dT) primer, dsDNA synthesized in second strand buffer with DNA polymerase and RNase H. The purified cDNA was concentrated and used for RNA amplification using the Amino Allyl MessageAmp Kit (Ambion, Austin, TX, USA). For hybridization we used the long oligonucleotide Operon array comprising ~29,000 genes of Arabidopsis (http://www.operon.com/arrays/omad.php), available from the Galbraith laboratory. The aRNA samples were coupled to mono-reactive NHS esters of Cy3 or Cy5 (Amersham Biosciences), and after purification incubated with slides in a hybridization chamber for 9 h at 56 °C, washed, dried and used for scanning. The images were scanned and quantitated using ScanArray Express (Perkin Elmer Life and Analytical Sciences, Inc., Boston, MA USA), which, by default, performs a global lowess normalization of median intensities per microarray. Low intensity and saturated spots marked by the quantitation program were filtered out from the data before doing any further normalization or statistical analysis. A normalization pipeline in MIDAS (TM4 analysis suite)

(Condon et al., 2004; McLaren, 2005), with default parameters, was used (total intensity, per-block-lowess, standard-deviation regularization, low-intensity filter). Spots inconsistent across the replicates were removed. Analysis of differential expression was carried out using a regularized *t*-test (Masle et al., 2005) implemented in the software Cyber-T (http://visitor.ics.uci.edu/genex/cybert/). The Bayesian confidence estimate was set at 6 (thrice the number of replicates) and the sliding window at 101 (default). Many parameters around these values were tried with no major effects. The *p* values obtained were converted to *q* values (Story and Tibshirani, 2003) using the Q-Value software (http://faculty.washington.edu/jstorey/qvalue/) and a *q* value cut-off of 0.001 was used, identifying 211 genes as differentially expressed (SI dataset). Data from the 6h drought experiment (part of the drought stress time course in shoots (*ME00338)*, downloaded from TAIR) was normalized using RMA, and Mass5-Calls were used to filter low-intensity spots. 1548 genes (SI Dataset) were identified as significantly differentially expressed by a similar analysis using the regularized *t*-test, FDR control and the same *q* value cut-off. CLENCH (Shah and Fedoroff, 2004) was used for the enrichment analysis of GO terms in the set of differentially expressed genes in the *hrd-D* and the drought experiment.

Results

Identification of the *HARDY (HRD)* gene in Arabidopsis

In a phenotypic screen of an activation tagged mutant collection in Arabidopsis (Marsch-Martinez et al., 2002), a gain-of-function mutant, named *hardy* (*hrd-D* denoting the dominant effect), was identified with the remarkable feature of having roots that were difficult to pull out from the soil, and dark green leaves that were smaller and thicker than normal wild-type (WT) plants (Fig. 2A,B). Molecular characterization of the mutant revealed that the activation tag insert was adjacent to the At2g36450 gene, with the enhancer sequence of the tag located 3.1 kb upstream of the tagged gene. At2g36450 is an AP2/ERF-like transcription factor that was shown to be overexpressed in the *hrd-D* mutant (data not shown). A construct was made with the predicted coding region of the At2g36450 gene under control of the CaMV35S promoter and transformed into Arabidopsis. The transformants displayed the typical *hrd-D* phenotype (Fig. 2A) to various degrees of severity (data not shown).

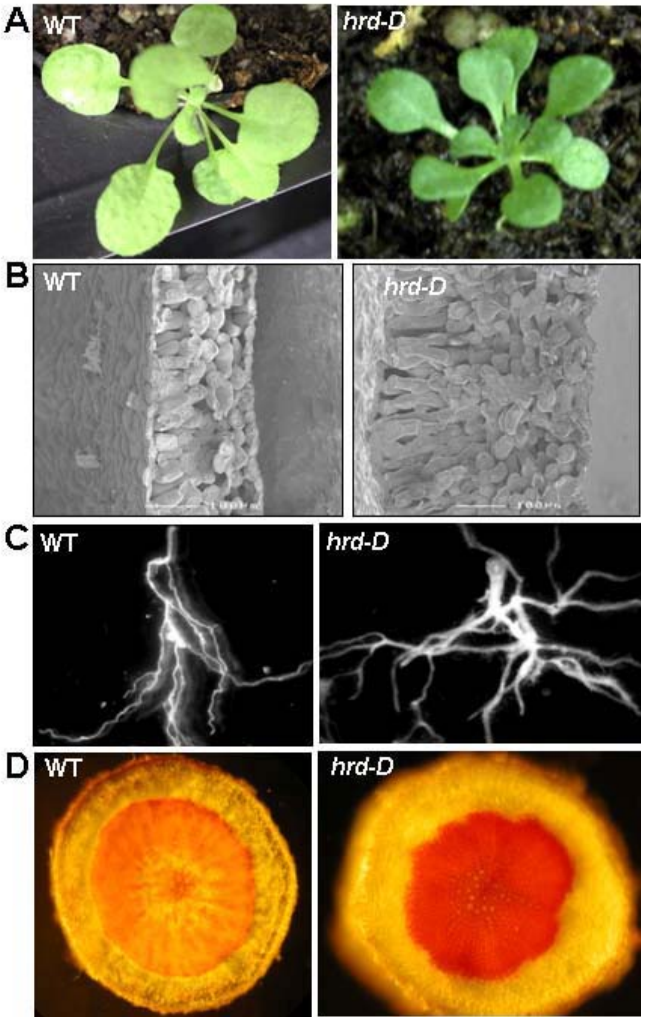

Fig. 2. The *hrd-D* mutant phenotype in Arabidopsis. **(A)** Rosette leaf phenotype of wild-type (WT) and *hrd-D* mutant with smaller, slightly curled, thicker, deep green leaves. **(B)** Cryo-fracture SEM section of leaf of wild-type (WT) and *hrd-D* mutant, showing more mesophyll cell layers. **(C)** Root structure of WT and *hrd-D* mutant, showing more profuse secondary and tertiary roots at the root base. **(D)** Cross section of WT and *hrd-D* roots showing increased cortical cell layers (lighter stained) and compact stele in the mutant.

Inducible *HRD* overexpression

To test if the *HRD* gene could be induced to exhibit the different phenotypes, we generated transgenic lines that express a fusion protein of HRD and the hormone binding domain of the rat glucocorticoid receptor (GR) (Chang et al., 1987) under the control of the CaMV35S promoter Fig. 1A). Arabidopsis plants containing this 35S::HRD-GR transgene (termed HRD-GR plants) displayed a wild-type phenotype and no phenotype like the *hrd-D* mutants (Fig. 2A). Seedlings grown in the greenhouse or growth chamber were treated with DEX at different times after germination (7 and 10 DAG) and either irrigated from below, sprayed from top, or both from top and below.

The HRD-GR plants, treated with 10 μM DEX from below by irrigation, gave a mild *hrd-D* like phenotype. These plants showed slight downward curled, deep green rosette leaves of almost normal size with an elongated stem, having normal looking cauline leaves and normal flowering time. The HRD-GR plants treated from the top by spraying DEX gave a similar phenotype as *hrd-D* with a small rosette, slightly curled leaves and with deep green color (Fig. 3). However, the main stem elongated normally up to 7-8 cm tall, and the flowering time ranged from normal to late in some plants. The HRD-GR plants treated from below (irrigation) and from top by spraying DEX, showed a very strong '*hrd-D* like' mutant phenotype with very small severely curled deep green rosette leaves and were late flowering. These experiments showed that DEX treatment of HRD-GR plant leaves could confer the leaf phenotype, while treatment through roots did not give a strong leaf phenotype. In all the above treatments WT showed no effect of DEX treatment. On basis of these phenotype recapitulation experiments, we termed the predicted At2g36450 gene *HARDY* (*HRD*), responsible for the *hrd-D* mutant phenotype.

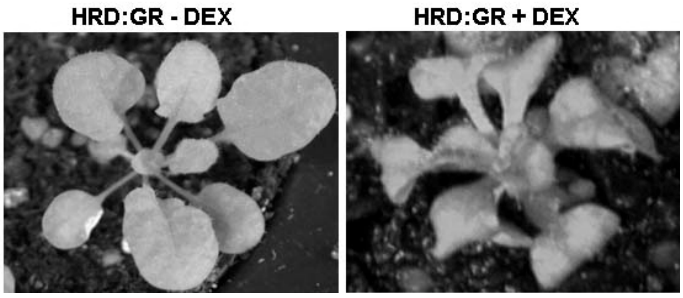

Fig. 3. Steroid DEX induction of HRD overexpression phenotype.
The HRD-GR (-DEX with WT phenotype) Arabidopsis plants sprayed from the top on the leaves with DEX (+DEX) gave a similar phenotype as the *hrd-D* mutant (Fig. 2A) with small rosette, slightly curled thicker leaves with deep green color.

Phenotype of *HRD* overexpression in Arabidopsis

Analysis of the *hrd-D* mutant leaf sections revealed extra palisade and spongy mesophyll layers compared to WT, contributing to the thicker leaf structure (Fig. 2B). The extended palisade layer bears abundant chloroplasts and contributes to the deeper green leaf color. The root phenotype was examined by growing *hrd-D* and WT plants (n=4) in sand with nutrients for 4 weeks and then analyzing the number and length of the primary, secondary and tertiary roots (Table 1). The distinguishing feature of the *hrd-D* mutant was the increased secondary and tertiary roots along 1 cm of the root base compared to the WT, giving rise to a denser root network (Fig. 2C).

The strength of the *hrd-D* mutant roots were quantified by an assay to measure the root pulling force for mature plants grown under well-watered conditions, showing that the *hrd-D* mutant required between 20-50 % more force to pull it out from the ground compared to WT (Fig. 4). Cross sections of the *hrd-D* root showed extra cortical cell layers and a more compact stele bearing vascular tissue (Fig. 2D).

Table 1: Root Structure of *hrd-D* plants

Line	Primary roots (number ± SE)	Secondary roots (number ± SE)	Tertiary roots (number ± SE)	Length of root base (cm ± SE)
WT	1 ± 0.00	1.50 ± 0.29	0.00 ± 0.00	0.55 ± 0.03
hrd-D	1 ± 0.00	4.25 ± 0.25	9.25 ± 0.63	0.28 ± 0.02

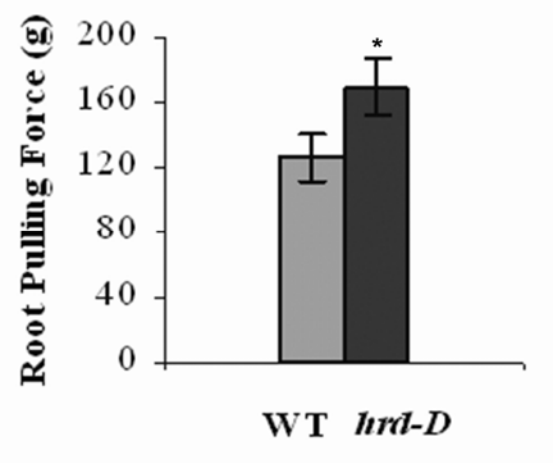

Fig. 4. Root pull assay of *hrd-D* compared to WT.
Greenhouse grown 5-week old Arabidopsis plants were tested in an assay for the weight (g) required to pull the plant from the soil. Bars indicate SE ($n \geq 4$), * indicates values significant at ($\alpha < 0.01$) compared to the WT.

Overexpression of *HRD* gave a small and thick leaf phenotype, hence to determine whether there was an alteration in the epidermal properties due to overexpression of HRD, we measured the stomatal and pavement cell density on the adaxial side of the *hrd-D* and WT using 4 weeks old leaves. The *hrd-D* leaf was around 3 times smaller in size compared to its WT and there was no significant difference observed in number of stomata per leaf between *hrd-D* and WT, which means that due to its relative size to the WT, *hrd-D* has 1.6 times more stomata compared to the WT (Table 2). The pavement cell number per leaf was less in *hrd-D* compared to

the WT; however this was compensated by the relative smaller size of its leaf compared to the WT (Table 2).

Table 2: Cell and stomatal density in mature leaves of Arabidopsis

Plant line*	Stomatal density/leaf (±SE)	Pavement cell density/leaf (±SE)	*Leaf Area (cm^2 ± SE)
Wild type	8223 ± 521	32497 ± 1235	3.82 ± 0.36
hrd-D	5011 ± 180**	11365 ± 330**	1.05 ± 0.12**
Ratio WS: *hrd-D*	1.64	2.86	3.64

* Indicates ($n \geq 4$); ** indicates, values significant at ($\alpha < 0.01$) compared to the WT.

HRD confers increased drought tolerance and water use efficiency in Arabidopsis

A quick high-throughput screen for drought resistance showed that the *hrd-D* mutant survived longer periods (3 days longer) of drought stress than WT plants (Fig. 5A) and recovered well to attain maturity and set seeds after subsequent rehydration. Since the size of *hrd-D* plants was smaller compared to its WT, it was necessary to know the exact water levels at which the plant can survive the drought stress. Hence, a more critical and intensive drought stress test was carried out, in which sets of replicate pots with 4 plants each were subjected to drought stress at 14 DAG by withholding water to the pots. The pots were weighed daily and the water transpired was replenished in the control set of pots maintained at 100% FC. The exact amount of water was calculated in the soil at the beginning of drought stress (14 DAG) and later in drought period when the WT died (11DOD) and this minimum level of water threshold was tested on the *hrd-D* mutant lines to see if they could survive at those water levels. In this test the WT could not survive beyond 12.7 % water levels on the 11DOD (Fig. 6A). On this day the *hrd-D* plants contained 14.2% water levels in the soil and survived the drought stress. On 12^{th} and 13^{th} DOD the *hrd-D* plants contained 7.8% and 5.8 % water respectively in the soil and survived on rehydration (Fig. 6A). The amount of water transpired per day, both under stress and non-stress condition, was measured for WT and *hrd-D*. The mean rate of transpiration (MTR) of *hrd-D* per day was much less compared to its WT both under stress and non-stress condition (Fig 6B). Also,

the relative average transpiration water loss by the plants to evaporation through soil throughout the experimental period of *hrd-D* was less compared to the WT, both under control and drought treated conditions (Table 3). Further we measured the WUE of *hrd-D* compared to the WT through a gravimetric method (see materials and methods) where WUE represents the ratio between the total gain in the biomass during the experimental period and the cumulative water transpired (CWT). The *hrd-D* line showed increased WUE, almost 2.7 times more under drought stress compared to the WT which showed 20% decrease in WUE under drought stress (Fig. 6C). The total dry biomass (shoots + roots) was calculated and *hrd-D* accumulated more total dry biomass (25-30%) under drought stress compared to the WT which showed decrease (42%) in dry matter accumulation under drought stress (Fig. 6D). This result suggests that *hrd-D* not only is able to survive below the minimum threshold of water levels required for WT but also is able to prolong survival under the drought stress situation by losing less water through transpiration. Also it is able to maintain its growth under drought stress conditions, by being able to use the water much more efficiently compared to the WT.

Table 3: Relative transpiration (T) water loss (mg/g fresh weight) to evaporation (E) water loss (mg/g fresh weight), (T/E).

	Number of plants (n=3)			Average
Plant line	**1**	**2**	**3**	**(± SD)**
WT (Control)	0.1789	0.1815	0.1821	0.1809 ± 0.0017
***hrd-D* (Control)**	0.1005	0.0995	0.1045	0.1015 ± 0.0026*
WT (11DOD)	0.0719	0.0832	0.0722	0.0758 ± 0.0064
***hrd-D* (11DOD)**	0.0248	0.0297	0.0247	0.0264 ± 0.0028*

* indicates, values significant at ($\alpha<0.01$) compared to the WT.

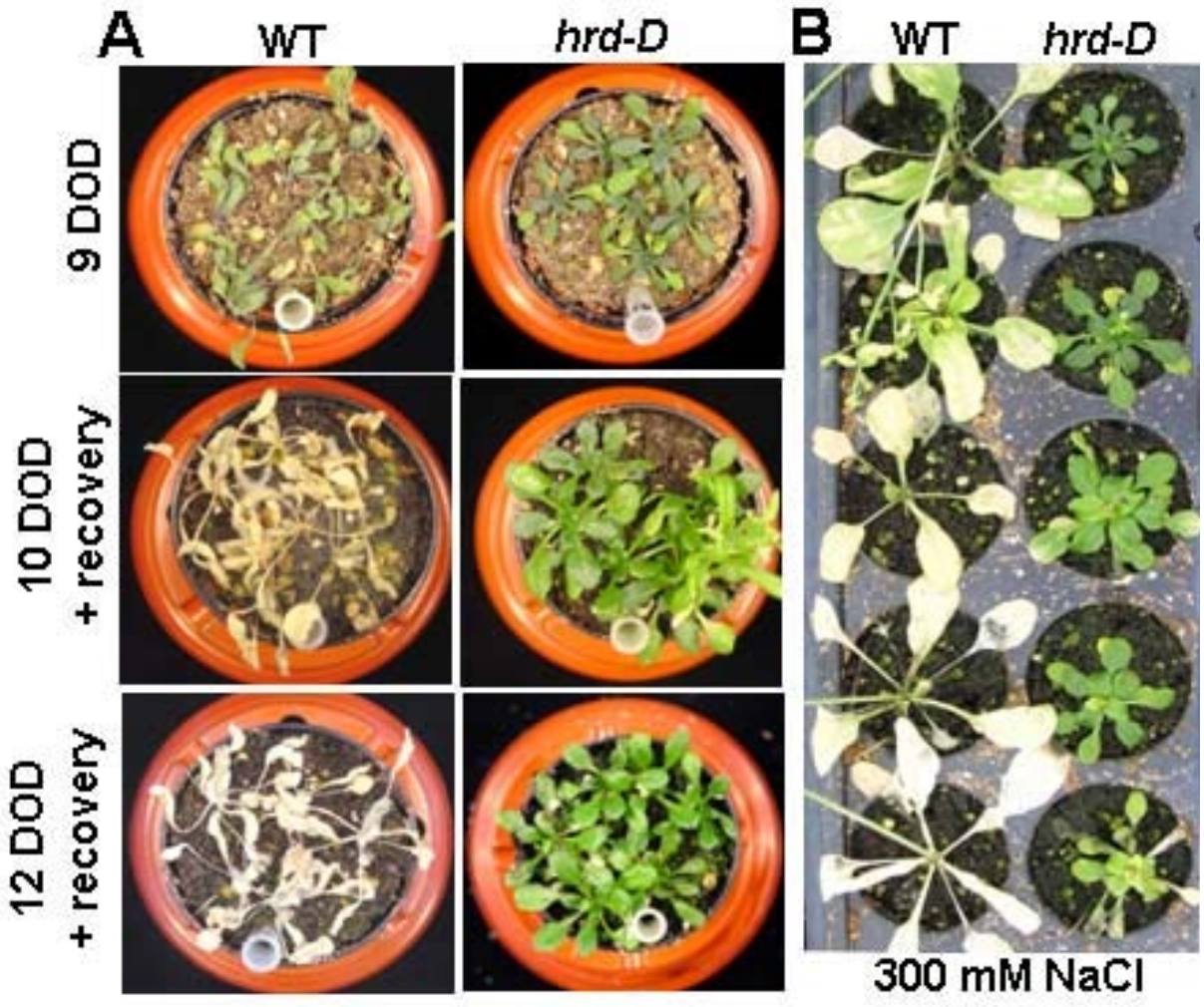

Fig. 5. Stress tolerance/resistance by overexpression of *HRD* in Arabidopsis. **(A)** Quick high-throughput drought resistance tests of Arabidopsis WT and the *hrd-D* mutant line, treated for a period of 9 to 12 days without water. The first row is at 9 DOD (days of dehydration); followed by plants treated for 11 and 12 DOD that were subsequently watered to reveal surviving plants. **(B)** Salt tolerance screen of mutant *hrd-D* and WT Arabidopsis treated at 300 mM NaCl concentrations showing bleached/dead plants and surviving *hrd-D* plants.

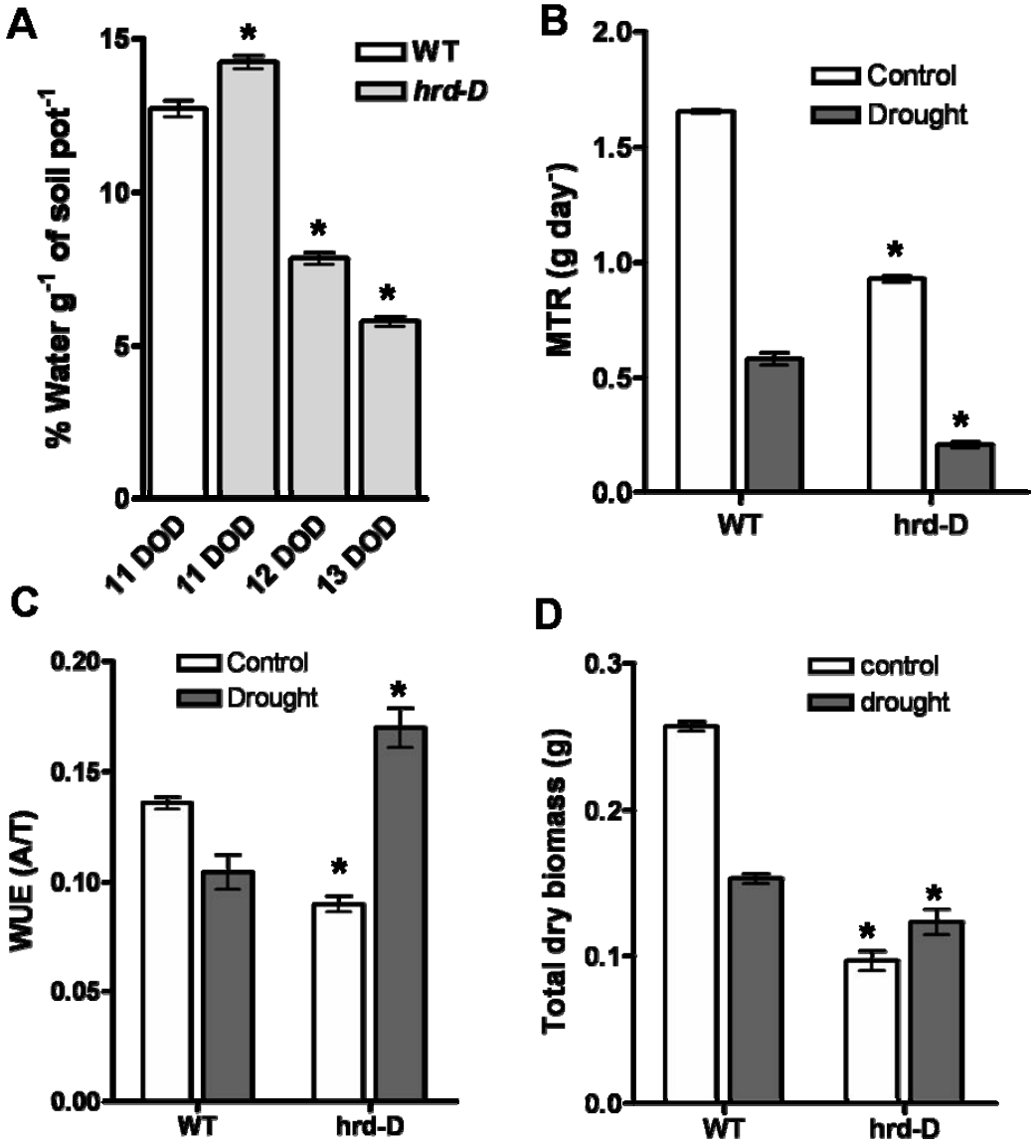

Fig. 6. *HRD* improves WUE in Arabidopsis.

hrd-D and WT plants tested under well-watered and drought stress conditions, bars indicate SE (n≥4); p-values shown for *hrd-D* vs. WT **(A)** % water per gram of soil, **(B)** Mean transpiration rate (MTR) (p-value 1e-04), **(C)** Water use efficiency (WUE) (p-value 8e-04), **(D)** Total dry biomass (p-value 1e-04). * indicates values significant at ($\alpha < 0.01$) compared to the WT in the same treatment.

Inducible drought tolerance assay

The HRD-GR transformants were tested for inducible drought tolerance to characterize the mechanism involved. The genotypes WT (wild-type), mutant *hrd-D*, and HRD-GR were germinated and either treated or untreated with DEX (provided either from below by irrigation, from top by spraying, or both from below and top). As DEX treatment of the WT also induced a level of tolerance, the day of WT death was noted for each treatment, and the survival (in %) of other genotypes and treatments were referred to in terms of the days after WT death (DAWD) (Table 4 and Fig. 7). The HRD-GR induced with DEX displays a drought tolerant phenotype equivalent to that of the *hrd-D* mutant. Comparison of experiments, of DEX treatments from below (irrigation to root), spraying from above (leaf) and treatment from both below and above, showed that all treatments showed drought tolerance of HRD-GR, though treatment by irrigation (from below) gave HRD-GR plants with almost wild-type leaf phenotypes and drought tolerance (Fig. 7). These results clearly demonstrated that the drought tolerance was not a secondary result of the smaller structured *hrd-D*-like mutant plants but by a resistance mechanism induced by the *HRD* gene.

Table 4: Induced Drought Tolerance (in % survival)

Plant line	Treatment	1 DAWD*	2 DAWD	3 DAWD
WT	+ DEX	0.00	0.00	0.00
WT	- DEX	0.00	0.00	0.00
hrd-D	+ DEX	93.33	92.31	96.55
hrd-D	- DEX	93.10	96.43	83.33
HRD-GR	+ DEX	83.33	75.00	71.43
HRD-GR	- DEX	0.00	0.00	0.00

*DAWD (days after WT's death) was used for the time periods, after WT death, to compare the survival (in %) of the experimental *HRD* expressing genotypes

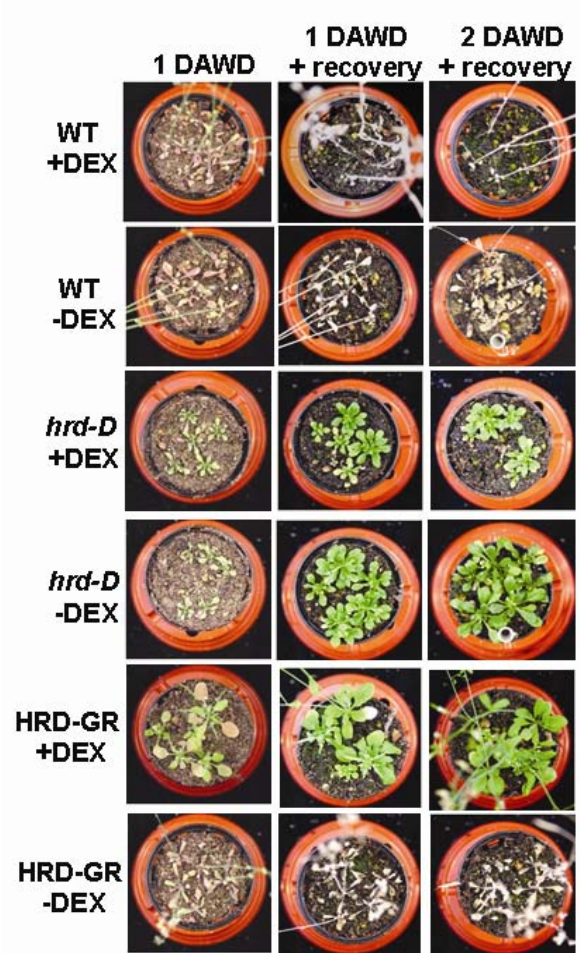

Fig. 7. Steroid inducible drought tolerance conferred by HRD-GR.
Seedlings of a DEX inducible HRD-GR line, the *hrd-D* mutant and wild-type (WT) Ws ecotype, were treated with DEX 7 days after germination (DAG), by irrigation from below, and compared to untreated genotypes in a drought tolerance assay. The day the WT death occurred was counted as 1 DAWD (day after WT death) and the survival of other genotypes counted in relation to this day. The first two rows show death of WT plants, and survival of the other genotypes are shown as 1-2 DAWD. The HRD-GR plants after DEX induction shows clear resistance compared to –DEX treatment, and similar to that as shown by the *hrd-D* mutant either + or – DEX treatment.

The *hrd-D* mutant shows increased salt tolerance in Arabidopsis

A salt tolerance assay was employed (Cheong et al., 2003), treating the WT and the *hrd-D* mutant with 200, 250 and 300 mM NaCl and then monitoring the plants for chlorosis and survival (Fig. 5B). The phenotype of the salt stressed plants was examined and the tolerant percentage was calculated on the basis of the undamaged non-bleached plants, which was higher at all NaCl concentrations for the *hrd-D* plants, which could reach full maturity in contrast to the WT (Fig. 5B and Table 5). The percentage reduction in the shoot/leaf dry matter accumulation of *hrd-D* under salt stress was 40% compared to WT that showed 76% reduction in growth. Chemical compositional analysis revealed that *hrd-D* accumulates lower amounts of Na^+ compared to the WT (Fig. 8B) under salt stress and maintains a higher K^+ / Na^+ ratio (Fig. 8D), which is a well known plant tolerance mechanism by maintaining osmotic balance of the cells.

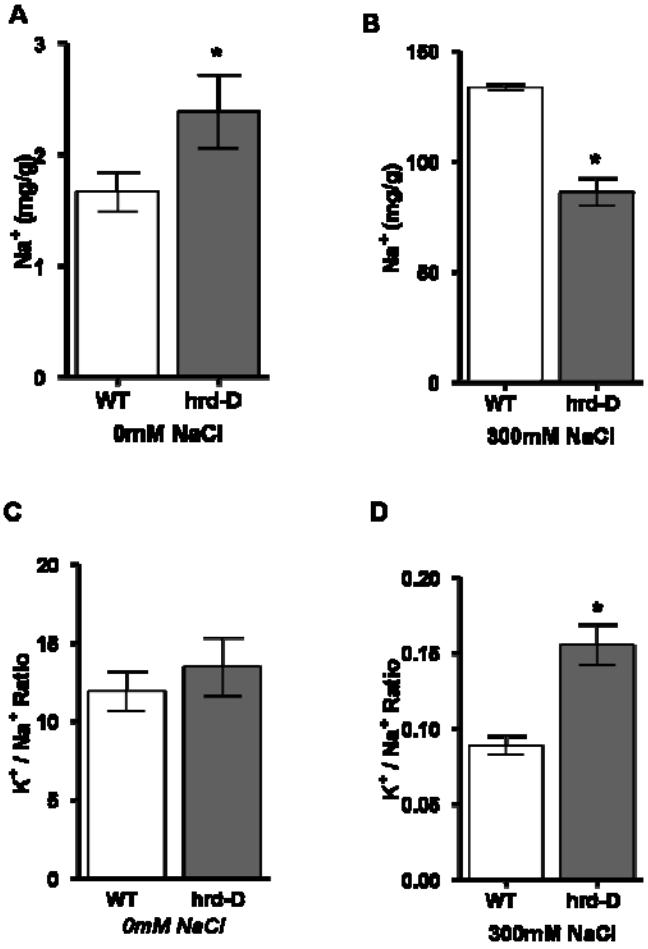

Fig. 8: Sodium accumulation in *hrd-D* vs. WT Arabidopsis leaves under no stress and 300 mM NaCl.

(A) Levels of Na^+ under normal soil with 0 mM additional NaCl in *HRD-D* compared to the WT plants; **(B)** *hrd-D* accumulates less amounts of Na^+ under 300 mM NaCl compared to the WT plants; **(C)** K^+/Na^+ ratio under normal soil with 0 mM NaCl are the same in *hrd-D* and WT; **(D)** K^+/Na^+ ratio under 300 mM NaCl is maintained much higher in *hrd-D* compared to its WT. Bars indicate SE (n≥3)

* indicates values significant at (α< 0.01) compared to the WT.

Table 5: Tolerance of *hrd-D* to NaCl stress

Genotype	NaCl treatment	Total plants	Non chlorotic	% Tolerant
WT	300 mM	36	6	16.67
hrd-D	300 mM	36	24 *	66.67
WT	250 mM	36	6	16.67
hrd-D	250 mM	36	22 *	61.11
WT	200 mM	36	9	25.00
hrd-D	200 mM	36	27 *	75.00

*All the values comparing WT and *hrd-D* were significant at (α<0.01) using chi-square test.

hrd-D displays enhanced pathogen resistance

In a screen of a number of activation tagged mutants for resistance to biotic and abiotic stresses, we also tested the *hrd-D* mutant for resistance to the non race-specific pathogen *Verticillium dahliae*. In replicated infection experiments under normal conditions with Verticillium, *hrd-D* mutants displayed high level of resistance displaying none of the wilt symptoms visible in WT (Jelle Hiemstra, unpublished data). No wilt symptoms or evidence of pathogen was visible at maturity suggesting an escape phenotype. The strong root network of *hrd-D* might be responsible for withstanding the fungal growth, providing continual water and nutrients to the plant, compared to WT where the roots get infected and are unable to support further plant growth. Also the resistance to drought and salt stress exhibited by *HRD* suggests that a general resistance mechanism is activated in the plant.

Expression analysis of *HRD*

To get further insight in the mechanism of *HRD* action, the expression of *HRD* was studied using promoter expression of the GUS marker in multiple transgenic lines. Significant consistent expression was observed in inflorescence tissue including petals, young inflorescence stem, mature pollen and seed (Fig. 9).

Microarray analysis of the leaf tissue of *hrd-D* mutant compared to WT (Fig. 10) using oligonucleotide microarrays revealed differentially expressed genes that were compared to publicly available microarray data. To identify significant biological pathways regulated by the HRD gene we examined the differentially expressed genes in terms of the gene ontology (GO) functional categories and compared these GO terms to data from drought experiments publicly available. The significant differentially regulated genes due to *HRD* overexpression are given with corresponding significant GO terms, along with the differentially regulated genes in a 6h drought induction experiment (Killian et al., 2007). Among the *HRD* and the drought up-regulated genes the abiotic stress regulated genes are evident by their annotation. This is further exemplified by the corresponding significant GO ID categories regulated by *HRD* and drought (Fig. 10A,B). This suggests that *HRD* promotes the expression of categories of genes that are also differentially expressed under drought, thereby promoting a drought resistance mechanism.

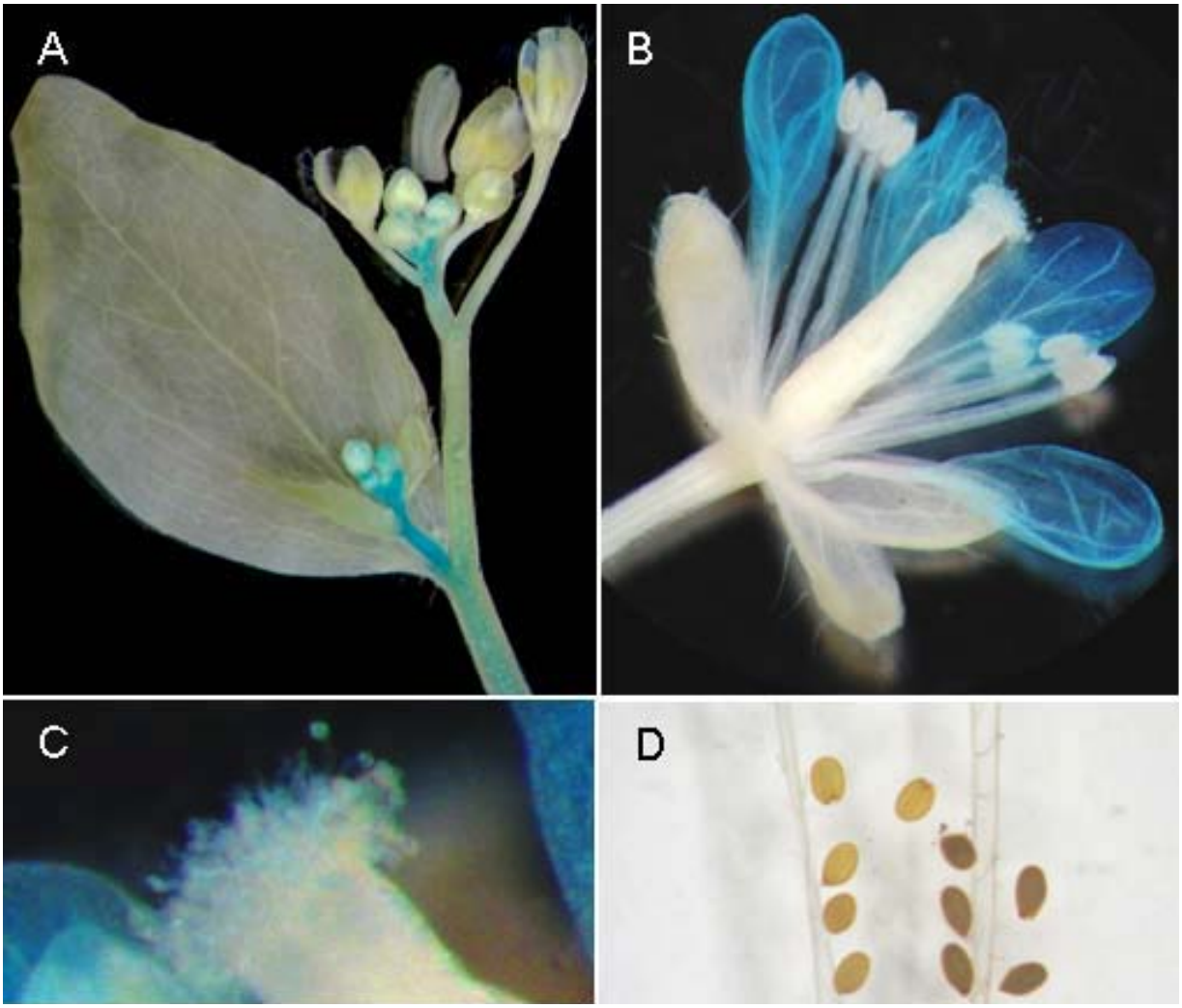

Fig. 9. Expression patterns of *HRD* detected in *HRD* promoter:GUS lines.
(A) Inflorescence expression in young inflorescence axis. **(B)** Expression in petals, and **(C)** mature pollen on stigma. **(D)** Seed expression conferred by *HRD* promoter (right) compared to WT (left) stained overnight for GUS activity.

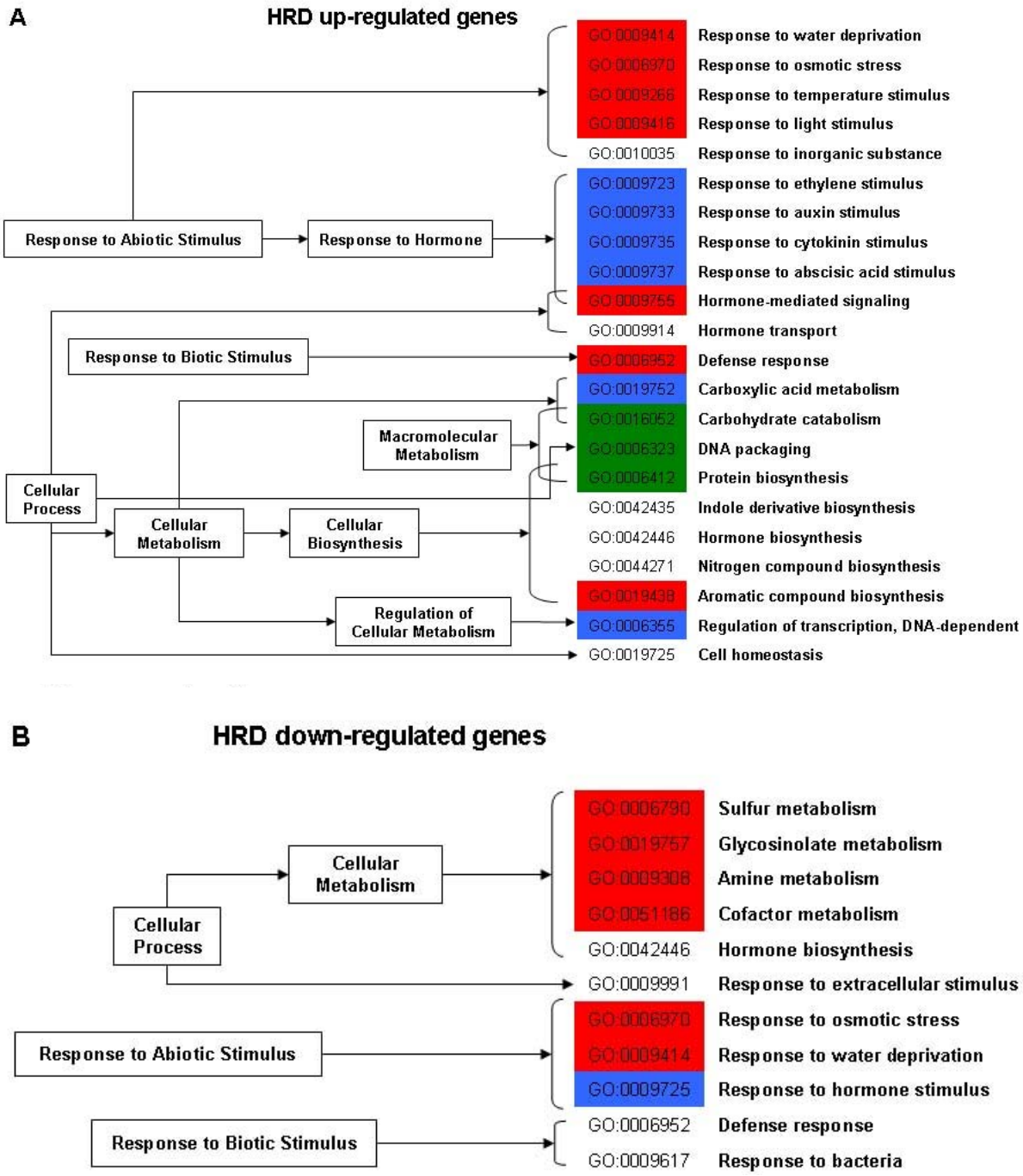

Fig. 10. The significant GO terms found by enrichment analysis among the *HRD* up-regulated **(A)** and down-regulated **(B)** genes. The corresponding GO terms enriched among the drought regulated genes are shown with the GO IDs boxed in colors representing the drought expression where Red: up-regulated; Green: down-regulated; Blue: up- & down-regulated (all w.r.t. drought regulated genes); and, White:

unique to *HRD* regulation. The hierarchy of the GO terms is also partially represented on the left. The GO-enrichment analysis was done using CLENCH (Shah and Fedoroff1), the 6h drought data and the *HRD* overexpression data were obtained from AtGenExpress (Killian et al., 2007).

HRD loss of function phenotype

Two knockout lines (from the SALK collection) with a T-DNA insert in the promoter region of the *HRD* gene were available and were analysed for phenotypic changes. However the segregating insertion lines did not show any difference in phenotype and appeared like the WT plants, probably due to the redundant function of the gene. To overcome this problem we designed a construct to create a dominant loss-of-function of the *HRD* gene (Fig. 1C) where the HRD protein was fused with the EAR-repressor domain (Hiratsu et al., 2003) under control of the constitutive 35S promoter (HRD-SRDX) and transformed to Arabidopsis. From five primary transformants with the HRD-SRDX obtained, T2 progeny were grown and analyzed for phenotypic changes (Fig. 11). The HRD-repressor transformed lines didn't show reduced plant height or thickening of leaves like in the *hrd-D* mutant, nor did they show any delay in plant development. They also produced normal fertile flowers. Some siliques were slightly shorter and wider than the WT and the rest appeared normal like the WT, but all siliques were fertile and full of seeds. The HRD-repressor plants developed main inflorescences with a reduction of main apical meristem activity and emergence of many lateral meristems lower down, showing complete loss of apical dominance (Fig. 11A). The cauline leaves were curled and twisted towards one side downwards, sometimes two cauline leaves appeared at the same node on the stem (Fig. 11B). The stems also appeared to be wavy and non-erect at the tips. The flowers and flowering time of the plants appeared to be normal like the WT. Some lateral meristems also showed emergence of several thin stems, some even emerging from the same node (Fig. 11C). Some transformants showed irregularities in senescence. Even when the siliques on older branches dried out, the plant continued to produce new meristem and leaves (Fig. 12D). This indicates that *HRD* might be involved in plant maturity signaling.

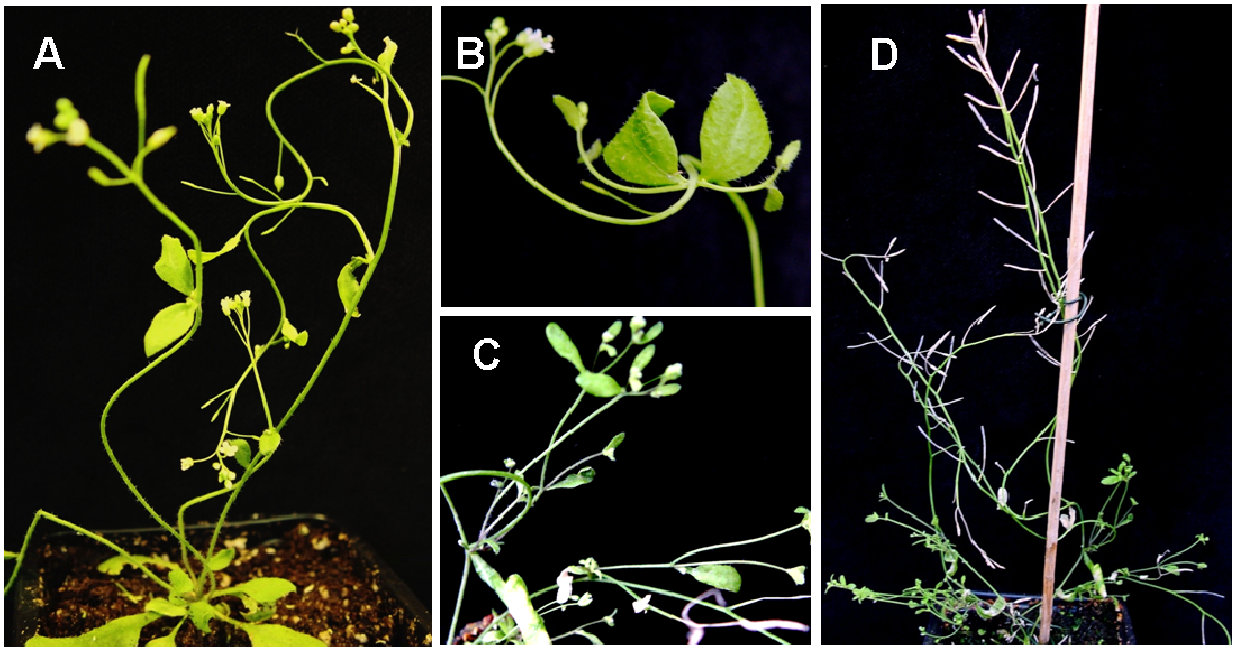

Fig. 11. 35S:HRD-SRDX phenotype in Arabidopsis. **(A)** HRD knockout transformant showing several non-erect lateral meristems, twisted and curled at the tips. **(B)** Two cauline leaves emerging at the same location on the HRD knockout mutant meristem. **(C)** Lateral meristems further developing many thin secondary stems, sometimes from the same node. **(D)** HRD knockout plant showing differential maturity pattern. Older stems (top part) already matured and dried up, whereas new stems still emerging (bottom) and continuing growth.

Transformation of an Arabidopsis *HRD* gene overexpression construct in rice

To analyze the effect of overexpression of the Arabidopsis *HRD* gene in rice, it was introduced into rice cultivar Nipponbare, controlled by the CaMV35S promoter, by means of Agrobacterium mediated transformation. Fifteen independent lines were generated, and analysis by semi-quantitative RT-PCR confirmed high levels of expression of *HRD* (data not shown). The overexpression of *HRD* gene in rice (referred to as rice HRD lines) exhibits a distinguishable phenotype, with darker green leaves compared to the leaves of Nipponbare WT plants. The transformants did not show any reduction in the growth, seed yield or germination when grown under normal greenhouse conditions, but surprisingly revealed a significant visual increase in leaf canopy with more tillers (Karaba et al., 2007). HRD lines in rice revealed extra bundle sheath cells (9-12%) compared to the WT. Also no change in the stomatal density was observed between

35S:HRD overexpressors and its WT (Karaba, 2007). Two independent HRD lines in rice tested for drought tolerance revealed a significant 50-100% increase in water use efficiency (WUE) under well-watered control conditions and about 50% increase under drought stress condition. These lines also showed a reduction in mean transpiration rate (MTR) that was more pronounced under well-watered conditions (Karaba et al., 2007).

***HRD* overexpression in rice improves salt tolerance**

In a replicated pot experiment for salt tolerance in rice, T3 generation lines of 4 independent transgenic plants (T2) tested for the presence of insert using oligonucleotides specific for the marker gene *HPT,* were treated with different NaCl concentrations in the greenhouse. The plants were grown on sand and maintained throughout the experimental period on Hoagland solution (Hoagland, 1950), and for salt stress treatment the NaCl was given to plants by adding it in the Hoagland's solution. Three-week old plants were given different concentrations of NaCl (0 mM, 150 mM and 200 mM) for 14 days and were later tested for their tolerance level based on their recovery, growth and survival. At the 10^{th} day under NaCl stress various growth data were collected such as the height of the plant, number of leaves, number of tillers (Table 6), fresh and dry weights of shoots & roots (Fig. 13) and documented by photographs (Fig. 12). At the end of the experiment when the plants had completed theire lifecycle, the seed yield per plant was obtained (Table 6).

HRD lines showed no consistent difference in height of plants, number of leaves and tillers compared to the WT under no-stress condition (Fig. 12A), except for HRD lines HRD-A & HRD-B which were slightly smaller in height compared to the WT (Table 6). Under 150 mM NaCl stress, HRD-C & HRD-D performed much better compared to the WT by producing more number of leaves and tillers and showed no difference in the height of the plants (Table 6 & Fig. 12B). Under 200 mM NaCl stress all the HRD lines performed better compared to the WT by enhancing their growth and growing taller, producing more number of leaves and tillers (Table 6 and Fig.12C). Lines HRD-C & HRD-D showed increase in the shoot (5-12%; 18-33%; 68-85%) and root (5-52%; 26-32%, 43-45%) fresh biomass and overall increase in the total fresh biomass (shoot & root) (5-29%; 20-26%; 38-44%) under no stress, 150 mM NaCl and 200 mM NaCl

conditions respectively (Fig. 13D,E,F and Fig. 12A-F) compared to the WT. HRD-A showed lower total fresh biomass accumulation under no-stress and 150 mM NaCl stress situation and showed slightly higher accumulation (24%) under 200 mM NaCl stress compared to the WT (Fig. 13D). HRD-B also showed slightly less total fresh biomass accumulation under no stress situation and higher accumulation (12%) under 150 mM NaCl stress and no difference under 200 mM NaCl stress compared to the WT (Fig. 13D). When the dry weights were measured it appeared that except for HRD-C (39%), no other HRD lines showed higher total dry matter accumulation under no-stress condition, however under 150 mMand 200 mM NaCl stress all the HRD lines showed increased total dry matter accumulation (14-38%; 21-40% respectively) compared to its WT except for HRD-A which under 150 mM NaCl stress showed no difference to the WT (Fig. 13A). HRD-C & HRD-D showed an increase in the shoot dry matter (6-28%; 6-19%; 27-28%) under no stress, 150 mM NaCl and 200 mM NaCl stress condition respectively. HRD-A & HRD-B showed increase (4-16%) in shoot dry weight only under 200 mM NaCl stress condition (Fig. 13B). More prominently the root dry weights were significantly higher (40-150%; 68-76%) under 150 mM and 200 mM NaCl stress respectively in all the HRD lines compared to the WT (Fig. 13C). The increase in the root dry biomass was more evident under stress compared to the shoot dry biomass (Fig. 13B, C).

In the calculation of seed yield for all genotypes, lines HRD-A, HRD-B & HRD-C showed higher seed yield (21.8%, 26.6% & 46.7% respectively) under no-stress condition compared to the WT. Line HRD-D showed no difference in seed yield compared to the WT (Table 6). Under 150 mM NaCl stress treatment all the HRD lines were able to set seeds unlike the WT which failed to set any seeds (Table 6). The percentage reduction observed in the seed yield under 150 mM NaCl in HRD-A, HRD-B, HRD-C and HRD-D was 52.2%, 49.7 %, 52.4% and 56.7% respectively.

From above results it seems that HRD lines overall are able to perform much better especially under salt stress condition compared to the WT. HRD-C & HRD-D especially performed much better in terms of growth and tolerance compared to the WT. After 14 days under salt stress the plants were again maintained on Hoagland's solution for recovery. Three out of four HRD lines recovered much faster and stayed alive to grow stronger and produce more leaves and set seeds under 150 mM NaCl unlike the WT which struggled to recover from the stress, and 14

days later under recovery could not survive. These results were more dramatic under 200 mM NaCl stress, even the HRD lines after 22 days under recovery could not survive the stress compared to its WT which only survived 7 days under recovery. The above results suggests that HRD lines (especially line C & D) are able to grow much better than the WT under no-stress condition and tolerate NaCl stress for much longer periods compared to the WT by developing more roots and thereby minimizing the osmotic shock for the plant. The salt concentration of 200 mM is very toxic for rice, yet HRD is able to survive longer periods under this level of stress compared to the WT. Under 150 mM NaCl HRD lines could tolerate the stress and survive on recovery to continue their growth unlike the WT which did not survive the recovery.

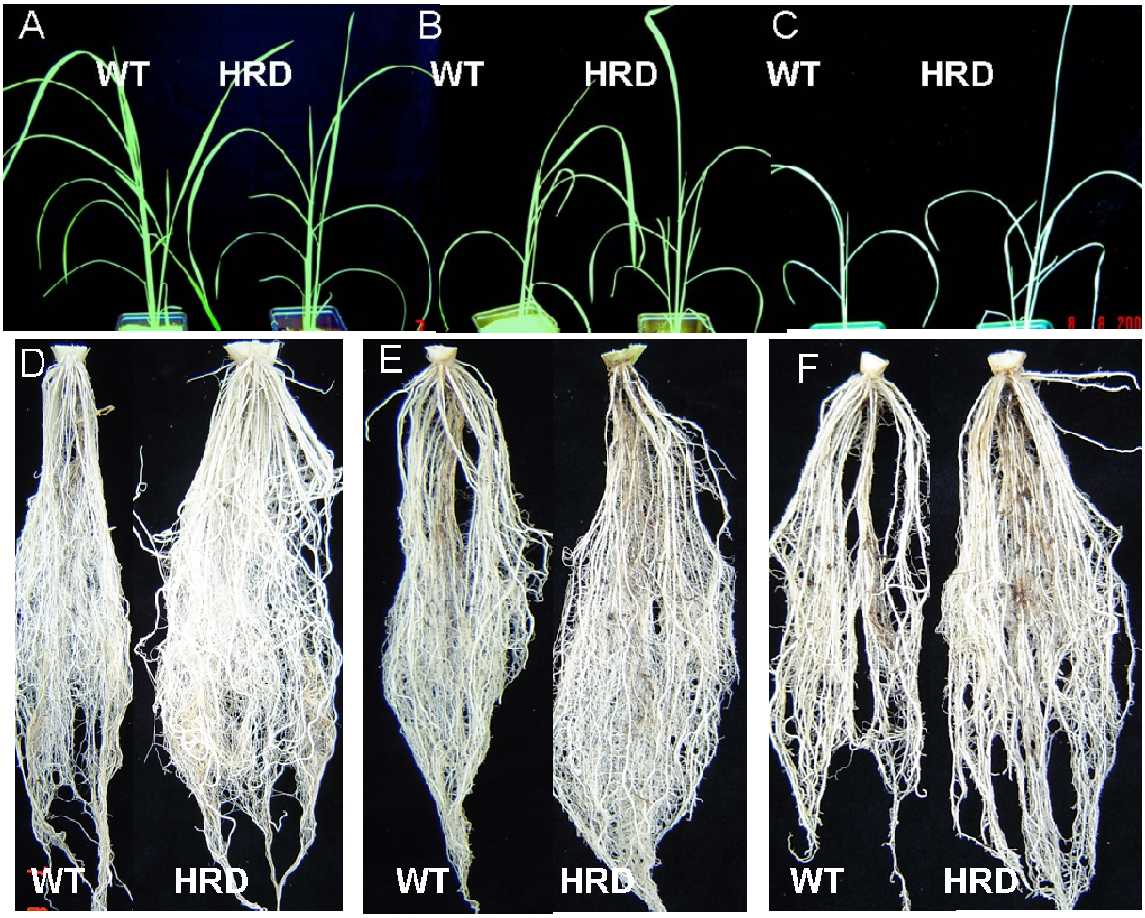

Fig. 12. **HRD vs. WT in rice, under different NaCl concentrations**.
HRD shows profuse shoot and root growth under 0 mM NaCl (A & D); 150 mM (B & E) and 200 mM (C & F).

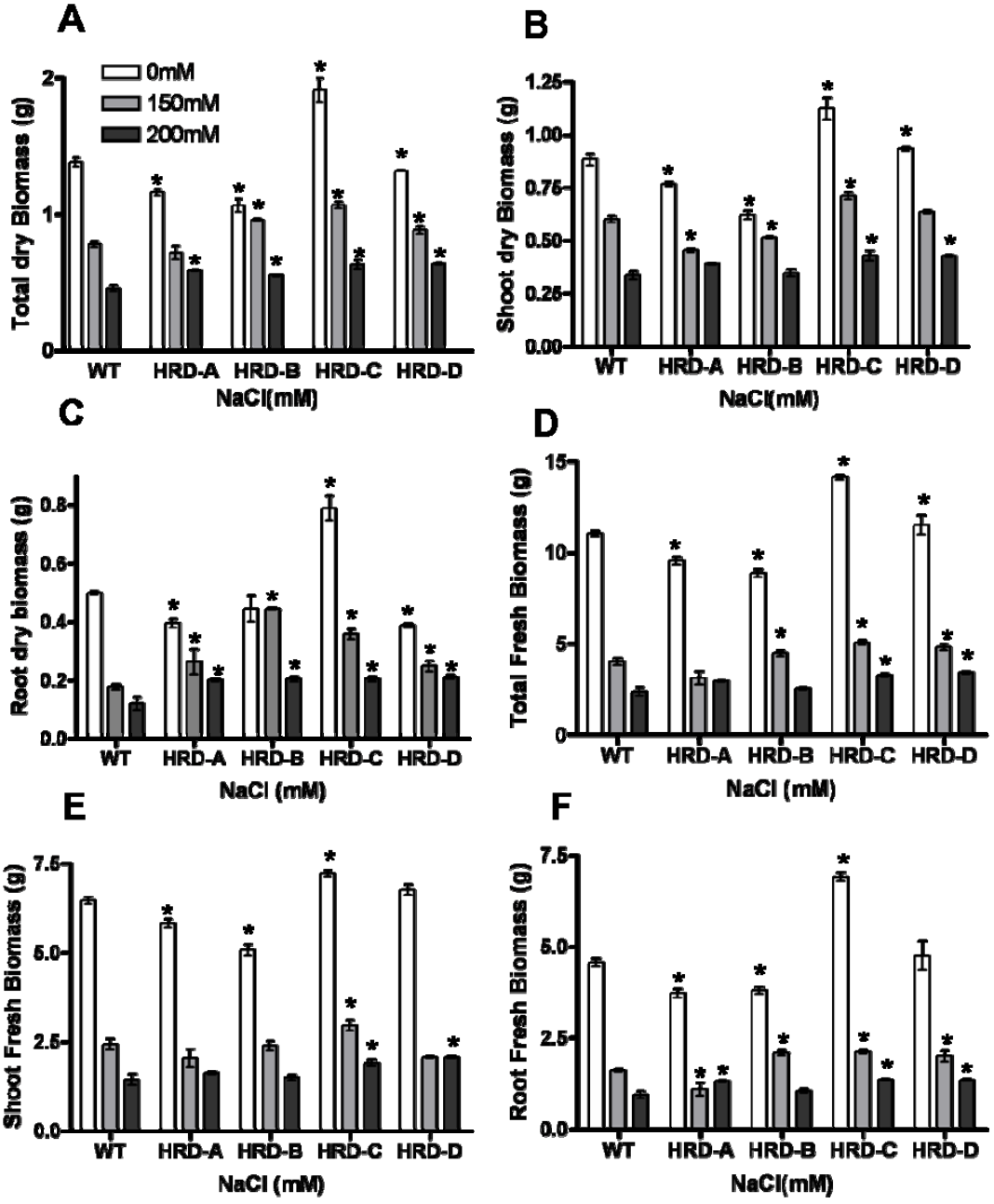

Fig. 13. Physiological analysis of HRD lines (A-D) and WT (Nipponbare) under different NaCl concentrations (0 mM white bar, 150 mM grey bar and 200 mM black bar). Calculated p-values shown for HRD vs. WT. **(A)** total dry biomass, **(B)** shoot dry biomass, **(C)** root dry biomass, **(D)** Total fresh biomass, **(E)** Shoot fresh biomass and **(F)** Root fresh biomass. P-values for A, B, C, D, E &F are (p-value 1e-04). Bars indicate SE ($n \geq 3$); * indicates values significant at ($\alpha < 0.01$) compared to the WT in the same treatment.

Table 6: **Developmental growth measurements of HRD lines vs. WT under NaCl stress (150 mM &200 mM) and no stress (C)**

Plant Line	Height of Plant (cm ± SE)	No. of Leaves (±SE)	No. of Tillers (±SE)	Seed weight (g ± SE)
WT (C)	68.20 ± 1.19	13.40 ± 0.92	3.20 ± 0.20	5.08 ± 0.22
HRD-A (C)	61.50 ± 0.59*	10.67 ± 1.12	4.00 ± 0.00**	6.50 ± 0.16*
HRD-B (C)	57.00 ± 1.78**	12.67 ± 0.25	4.00 ± 0.00**	9.55 ± 0.22**
HRD-C (C)	64.00 ± 1.14	14.00 ± 0.89	4.20 ± 0.37	6.92 ± 0.63**
HRD-D (C)	64.80 ± 1.01	14.00 ± 1.26	4.20 ± 0.20*	5.02 ± 2.17
WT (150 mM)	57.20 ± 0.66	6.40 ± 0.44	3.00 ± 0.00	0.00
HRD-A (150 mM)	55.38 ± 1.68	5.75 ± 0.42	1.75 ± 0.22**	3.10 ± 0.30**
HRD-B (150 mM)	58.75 ± 2.93	6.50 ± 0.57	2.50 ± 0.25	4.80 ± 0.30**
HRD-C (150 mM)	58.80 ± 1.66	11.20 ± 0.37**	3.00 ± 0.00	3.29 ± 0.17**
HRD-D (150 mM)	59.30 ± 1.10	9.40 ± 0.67*	3.00 ± 0.00	2.17 ± 0.38**
WT (200 mM)	42.83 ± 0.81	5.00 ± 0.80	1.67 ± 0.23	0.00
HRD-A (200 mM)	50.67 ± 0.68**	7.33 ± 0.51**	2.33 ± 0.25	0.00
HRD-B (200 mM)	49.83 ± 0.46**	6.00 ± 0.44	1.67 ± 0.25	0.00
HRD-C (200 mM)	50.60 ± 1.88*	7.50 ± 0.67**	2.88 ± 0.15*	0.00
HRD-D (200 mM)	52.40 ± 0.39**	7.80 ± 0.37**	3.00 ± 0.00**	0.00

**** =(p<0.01)**

*** = (p<0.05)**

To get an insight on the mechanism through which HRD lines in rice were able to tolerate salt, we analyzed the ion content of two of the best performing HRD lines in rice (C and D) (which performed much better than the rest of the HRD lines) to determine the ion contents of the cells like the Ca^{2+}, K^{+} and the Na^{+}, which are known to play a key role in salt tolerance mechanism (Zhu, 2002). The results show that under no–stress situation HRD-D accumulated slightly higher amounts of Na^{+}, whereas HRD-C shows no difference in Na^{+} accumulation compared to the WT (Fig. 14A). Under 150 mM NaCl both HRD lines accumulated more Na^{+} compared to the WT, however under 200 mM NaCl both the HRD lines accumulated less Na^{+} compared to the WT (Fig. 14A). The levels of K^{+} and Ca^{2+} were maintained higher in both the HRD lines under no–stress, 0 mM NaCl and 200 mM NaCl conditions compared to the WT (Fig. 14B& C). The K^{+}/Na^{+} ratio showed no difference in both HRD lines under no stress condition

compared to the WT, but were much higher under 150 mM NaCl and 200 mM NaCl conditions in both HRD lines compared to the WT (Fig. 14D-F). A high K^+/Na^+ ratio in the cytosol is essential for normal cellular functions of plants. This result indicates that maintaining higher K^+/Na^+ ratio, a mechanism well known for maintaining cell homeostasis (Zhu, 2001) helps the plant in reducing Na^+ toxicity in the cell. Ca^{2+} plays an important role in providing salt tolerance to plants. Increased Ca^{2+} concentration may contribute to activation of a Ca^{2+}-dependent NaCl stress signaling pathway that resulted in enhanced tolerance of the HRD plants to NaCl stress. These results show that the salt tolerance in HRD lines in rice exhibits a similar mechanism for tolerance as observed for the HRD lines in Arabidopsis showing a conservation of mechanism among different species.

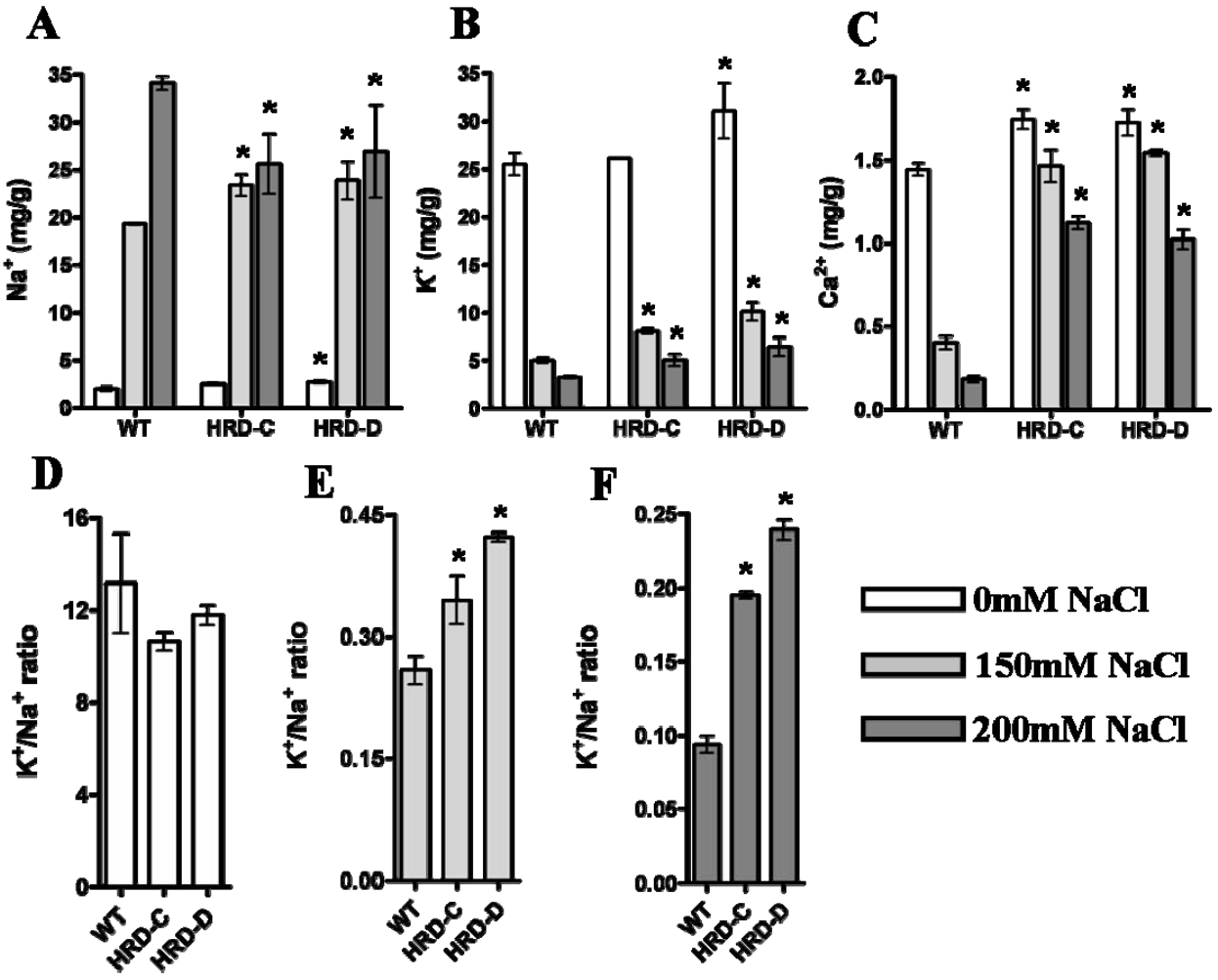

Fig. 14. Ion accumulation in 35S*:HRD* lines vs. WT (Nipponbare) in rice under 0 mM, 150 mM and 200 mM NaCl.

(A) Na^+ accumulation of HRD-C and HRD-D under 0 mM, 150 mM and 200 mM NaCl compared to the WT. **(B)** K^+ accumulation of HRD-C and HRD-D under 0 mM, 150 mM and 200 mM NaCl compared to the WT. **(C)** Ca^{2+} accumulation of HRD-C and HRD-D under 0 mM, 150 mM and 200 mM NaCl compared to the WT. **(D)** K^+/Na^+ ratio of HRD-C and HRD-D under 0 mM NaCl compared to the WT. **(E)** K^+/Na^+ ratio of HRD-C and HRD-D under 150 mM NaCl compared to the WT. **(F)**. K^+/Na^+ ratio of HRD-C and HRD-D under 200 mM NaCl compared to the WT. Bars indicates SE (n=3); * indicates values are significant at (α <0.01) compared to the WT.

Discussion

The *HRD* gene belongs to a class of AP2/ERF-like transcription factors, classified as group IIIb in a recent comprehensive classification of the AP2/ERF family (Nakano et al., 2006). The related *DREB/CBF* genes that provide abiotic stress tolerance on overexpression (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999) belong to the group IIIc, but no phenotypic function has yet been attributed to the four Arabidopsis and six rice group IIIb members. Our analysis of *HRD* promoter expression (Fig. 9) supports the publicly available expression data in Genevestigator (Zimmermann et al., 2004), showing that the *HRD* gene is expressed in inflorescence tissue including petals, inflorescence stem, mature seed and pollen. Therefore, the gene is probably involved in the maturation of the inflorescence, which structure requires a developmental stage protection against desiccation and other stresses. Likewise, overexpression of the *HRD* gene in Arabidopsis confers drought resistance and salt tolerance, two abiotic stress components of desiccation tolerance (Verslues et al., 2006). Also HRD overexpression in Arabidopsis gives resistance to the fungal wilt pathogen Verticillium, a biotic stress component.

Microarray analysis of *HRD* overexpression revealed significant differentially regulated clusters of genes represented as GO (Gene Ontology) terms that correlated with corresponding drought regulated "GO clusters" (Fig. 10). *HRD* overexpression results in the induction of GO clusters normally expressed under drought stress, such as response to water deprivation and osmotic stress, supporting the induction of a drought adaptive mechanism by *HRD*. In addition GO clusters repressed under drought, are upregulated by *HRD*, suggesting a protective influence on essential processes such as protein biosynthesis and carbohydrate metabolism. Overexpression of the *DREB/CBF* group IIIc genes of the AP2/ERF family (Fowler and Thomashow, 2002), showed similarity to *HRD* in their regulation of stress response genes. However, *HRD* displays differences in the differential expression of some GO clusters such as protein biosynthesis and other specific genes, which probably contribute to its different unique functions in Arabidopsis and rice.

Genes for abiotic stress tolerance have often been identified by ectopic expression in vegetative tissue, which can be easily tested in drought/salinity assays. In this way the Arabidopsis *DREB1A* gene, normally induced by cold/salt stress, on overexpression also confers

drought stress resistance (Kasuga et al., 1999). Under natural situations in plants, the expression of stress resistance genes are controlled to respond adaptively to stress, or are expressed at specific developmental stages and tissues providing stress resistance, but by ectopic expression can exhibit the phenotype in another target tissue or condition. Thus, the Arabidopsis *HRD* gene, normally active in inflorescence stage tissue, exhibits by ectopic expression a novel vegetative stage phenotype of enhancement in root and leaf structure. In support of this developmental stage stress resistance function, the role of root and leaf structure has been well recognized as an adaptive mechanism for drought resistance and WUE in crops such as rice (Price et al., 2002).

The overexpression of *HRD* in Arabidopsis gives higher drought tolerance to plants and increases their WUE under drought stress by transpiring less water. The *hrd-D* plants were also able to tolerate and survive under very less soil water content (5.8%) compared to the WT (12%)(Fig. 6A), due to less transpirational water loss, more stable and enhanced cell wall integrity and robust root system harvesting scare water from the soil. Unlike the WT, *hrd-D* also maintained its growth under stress conditions.

HRD expression also conferred increased salt tolerance in Arabidopsis and rice. Many physiological studies have demonstrated that Na^+ toxicity is not only due to toxic effects of Na^+ in the cytosol, but also because K^+ homeostasis is disrupted possibly due to the ability of Na^+ competing for K^+ binding sites. In Arabidopsis, *hrd-D* showed less Na^+ accumulation in the cytoplasm and maintained higher K^+/Na^+ ratio, a mechanism well known for maintaining cell homeostasis and thereby reducing Na^+ toxicity in the cell (Zhu, 2001). High salt concentrations inhibit the activities of most enzymes because of the perturbation of the hydrophobic-electrostatic balance between the forces maintaining protein structure. Many genes involved in protein biosynthesis, cell homeostasis and maintenance of cell integrity are upregulated by HRD, which shows ability of HRD to physiologically tolerate such osmotic stress created by Na^+ toxicity. The overexpression of *HRD* in rice (HRD-C & -D) generates plants with significantly higher biomass, independent of salt stress. Higher biomass in *HRD* rice is attributed to its ability to photosynthesize better under both non stress and osmotic stress conditions due to enhanced mesophyll growth, a characteristic of *HRD* overexpression in Arabidopsis, and an intact PSII system (Karaba et al., 2007). *HRD* overexpression increases root biomass under salt stress

indicating an ability to adapt by inducing roots to avoid or minimize the osmotic stress shock which is created due to higher salt concentration in the soil water, which limits water absorbance. The *HRD* lines show consistent significant differences to the WT under no salt stress and stress conditions (Fig. 13). HRD lines -A & -B showed less biomass accumulation, HRD lines -C & -D showed increased biomass accumulation under no stress condition, however under salt stress all the lines performed better than WT, shown by increased biomass production, especially root biomass (Fig. 13C). *HRD* in rice was tested for salt tolerance under 150 and 200 mM NaCl, which caused the WT to die. HRD lines treated to 150 mM NaCl stress survived and continued growth to set seeds, whereas the HRD lines at 200 mM NaCl survived for longer periods under stress compared to WT but later perished. These results indicate that expression of HRD improves salt tolerance of rice and boosts its performance in terms of biomass accumulation and seed set compared to the WT. Also the biochemical analysis results revealed that rice HRD lines maintain higher K^+ and Ca^{2+} levels in the cytosol and also maintain higher K^+/Na^+ ratio under 150 mM and 200 mM NaCl stress condition compared to the WT. This shows that rice HRD lines also show overlap of salt tolerance mechanism as observed in HRD lines in Arabidopsis.

Our current understanding of *HRD* providing drought and salt tolerance in Arabidopsis and rice indicates that *HRD* might be involved in a conserved mechanism rendering abiotic stress tolerance by maintaining cell membrane stability and cell homeostasis. The enhancement of the plant tissues like the roots and shoots by *HRD* expression is recognized as a common stress adaptive mechanism for drought and salt tolerance. The functional categories of stress associated genes up-regulated in the HRD-overexpressing plants provide supporting evidence for the action of protective mechanisms in drought and salt tolerance of the transgenic plants. How *HRD* is able to accumulate less Na^+ compared to the WT and also maintain higher K^+/Na^+ ratio under salt stress is still to be investigated. Further characterization of *HRD* in relation to osmotic stress, by metabolic analysis of the levels of osmoprotectants like proline in the cells, which is known to increase ability of plants to tolerate high salinity and drought (Delauney et al., 1993), could help to shed some light on *HRD*'s physiological role in providing osmotic stress. Also *HRD* provides biotic stress resistance to the pathogen Verticillium that affects the root of the plants, probably by activating a general stress resistance mechanism. The significant enhanced drought and salt

tolerance in the crop plant rice due to overepxression of *HRD*, suggests that this gene may show great promise and potential for genetic improvement of stress tolerance in rice.

Acknowledgements

We thank Gert van Arkel for help in rice transformation. This work was supported by grants from the Generation NWO-WOTRO grants.

Chapter 3

The Arabidopsis AP2/ERF-transcription factor SHINE improves water use efficiency, drought and salt tolerance

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Part of this work is published in (Aharoni A., Dixit S., et al.) Plant Cell, 2004

The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in Arabidopsis.

Abstract

Drought and salt are major abiotic stress factors that cause water deficit in plants, affecting growth and crop production. Drought stress can lead to increase in epicuticular wax on the plant surface and help reduce transpirational water loss. The *SHINE* (*SHN*) gene, an AP2/ERF transcription factor (TF) from Arabidopsis, increases epicuticular wax and alters epidermal properties in Arabidopsis. We showed that overexpression of the *SHN1* gene in Arabidopsis increases drought resistance and improves water use efficiency (WUE) by reducing transpirational water loss due to lower stomatal density. It also provides enhanced salt tolerance in Arabidopsis by maintaining ion homeostasis in the cell. Overexpression of the Arabidopsis *SHN2* gene in rice conferred salt tolerance accompanied by increased biomass and yield.

Introduction

Plants can sense environmental stress and quickly respond by signal transduction and stress-responsive gene expression. Dehydration or salinity can elicit osmotic stress and alter the expression of a set of genes (Zhu et al., 1998) that are also induced by low temperature stress (Thomashow, 1998). Some of these genes respond to multiple stresses, e.g. dehydration-inducible genes respond to cold stress and vice versa, suggesting parallel induction mechanisms. This also explains cross-tolerance to different stresses as was shown by overexpression of the AP2/ERF factors CBF1, DREB1A and CBF4 that resulted in drought/salt/cold tolerance in Arabidopsis (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Haake et al., 2002). Similarly, overexpression of *DREB* orthologs from rice (*OsDREB1*) resulted in salt, cold, and drought tolerance in Arabidopsis (Dubouzet et al., 2003). These results indicate conservation of the stress tolerance pathways in plants.

Drought and salt stress are major abiotic stresses that limit crop growth and production worldwide and their effect on crop plants can range from minor reductions in yield to the destruction of crops leading to famine (Flowers and Yeo, 1995; Hetherington, 1998). Both drought and salt stress leads to osmotic stress which hinders the uptake of water in plant cells leading to physiological drought. Water conservation and regulation in plant cells is therefore essential. Several plant-adaptive mechanisms which reduce water loss from plants and improve water use efficiency (WUE) have been studied. The plant surface, which acts as a medium between the plant and the environment, plays an important role in regulating water loss from the plant. The cuticle acts as a waxy protective barrier against environmental stress as well as a medium for the exchange of gases and water. Environmental stresses like drought are known to increase the accumulation of waxes in crop plants (Bondada et al., 1996; Jenks et al., 2001; Samdur et al., 2003) which helps in water conservation. Several studies have correlated plant wax characteristics with the transpiration barrier properties of the plant cuticle (Jenks et al., 1994; Vogg et al., 2004; Zhang et al., 2005).

Overexpression of the Arabidopsis *SHINE* (*SHN/WIN*) clade of AP2/ERF transcription factors regulate the expression of a number of wax biosynthesis genes and increases epicuticular wax deposition in Arabidopsis (Aharoni et al., 2004; Broun et al., 2004). In this study we

characterize the *SHN* gene in terms of abiotic stress tolerance. We used the Arabidopsis overexpression lines to test for drought and salt tolerance and additionally for water use efficiency (WUE) a physiological trait for sustainable crop production.

Rice is the staple food for the majority of people around the globe and it is also considered to be a salt sensitive crop, hence more prone to yield losses due to salt stress. Hence improving salt tolerance in rice with improved production could minimize the losses incurred by salt stress. Here we address the mechanisms through which *SHN* is able to confer abiotic stress tolerance to the plants. To test the broad applicability of the *SHN* genes we characterized *SHN2* overexpressors in rice for salt tolerance to complement other studies on drought and WUE (Karaba, 2007).

Materials and Methods

Plant growth and conditions

The Arabidopsis plants used are in ecotype Wassilewskija (Ws-3) and were grown in the greenhouse at around 22°C. The rice plants used were *Oryza sativa* ssp. *japonica* cv. Nipponbare. The rice plants were grown in the greenhouse with short day conditions (12h light, 12h dark) in pots with sand and were maintained on Hoagland's solution (Hoagland, 1950) throughout the experimental period. For growth measurements fresh weight (FW) of plants was determined immediately after harvest and samples were oven-dried at 65°C for one week to obtain dry weight (DW).

Generation of plant transformation constructs and transgenic plants

Fragments encompassing the full-length coding regions were amplified (using pfu DNA polymerase) from flower-bud cDNA for *SHN1/WIN1* (At1g15360) to generate the overexpression construct. The cDNA used for amplification was from the Arabidopsis ecotype Columbia. Oligonucleotides AP35 (59-CGGATCCATGGTACAGACGAAGAGTTCAG-39) and AP36 (59-CGAGCTCGATTTAGTTTGTATTGAGAAGC-39) were used to amplify *SHN1*. The oligonucleotides introduced *Bam*HI and *Sst*I restriction sites to the amplified fragments at their 5' and 3', respectively, which were used to ligate the coding region fragments to the *Bam*HI and *Sst*I

sites in the pBI121 binary vector (Clontech, Palo Alto, CA) in between a 35S CaMV promoter and a nopaline synthase terminator. Fragments were A-tailed and introduced to the pGEM-T Easy vector as described by the manufacturer (Promega, Madison, WI) and subsequently sequenced from both sides before digestion and ligation to the binary vector. PCR, restriction digests, plasmid DNA isolation, and gel electrophoresis were performed using standard protocols. The rd29A-*DREB1A* construct was similar to that described (Kasuga et al., 1999), except that the gene fusion was inserted into the pBinPlus vector. The constructs were introduced into the Arabidopsis plants using the floral dipping transformation method (Clough and Bent, 1998). The seeds were plated on half-strength MS medium (Duchefa, Haarlem, The Netherlands) and 1% sucrose. Seedlings selected on 50 mg/L of kanamycin were subsequently transferred to the greenhouse.

To make an overexpression construct for rice transformation, the Arabidopsis *SHINE2* (*SHN2*) gene (At5g11190) was used. The *SHN2* overexpression construct was assembled by multi-point ligation, in which the individual fragments (promoter, *SHN2* gene, terminator) with appropriate compatible cohesive ends were ligated together to the binary vector in one reaction. A CaMV35S promoter fragment extending from -526 to the transcription start site, was obtained as a 0.55 kb *Hin*dIII-*Bam*HI fragment from a pBS-SK+ derivative of pDH51 (Pietrzak et al., 1986). The full length coding region of *SHN2* (At5g11190) was obtained as *Bam*HI-*Not*I fragment (Aharoni et al., 2004). A CaMV35S terminator fragment was obtained as a 0.21 kb *Not*I-*Eco*RI fragment from a pBS-SK+ derivative of pDH51 (Pietrzak et al., 1986). The construct was made in the binary vector pMOG22 (ZENECA-MOGEN, NL) which contains a chimeric CaMV 35S-hygromycin phosphotransferase-tNos for selection of transformed plants.

Chlorophyll leaching assay, fresh weight, and stomatal analyses

For chlorophyll leaching assays, roots and inflorescence stems of 4-week-old Arabidopsis plants were cut off, and the remaining rosette was rinsed with tap water, weighed, and put in tubes containing 30 ml of 80% ethanol at room temperature (gently agitating in the dark). 400 µl was removed from each sample every 10 min during the first hour and then after 90 and 120 min. Absorbance of each sample was measured at 664 and 647 nm, and the following formula (Lolle et al., 1997) was used to calculate the micromolar concentration of total chlorophyll per gram of

fresh weight of tissue: total µM chlorophyll = 7.93 (A_{664}) + 19.53 (A_{647}). Seed from the wild type and the mutant lines were stratified in cold (4º C) for three days and sown in 9-cm diameter pots, at a density of ~12 seeds/pot. The plants were given nutrition on the 10th day after germination, allowed to grow to 4 weeks, and then used for water-loss analysis. The rosette and emerging stems of plants were detached from the roots and weighed immediately for the fresh weight. All samples were maintained at room temperature (22º C) and weighed at several regular time intervals. Initial observations were taken at short time intervals of 2 min and then later gradually increased to longer intervals of 1 h. The samples were weighed for 7 h or more. Observations were taken from four different plants of wild type and mutants, and the experiment was repeated in three batches at different days. The average fresh weight, average dry weight (samples were kept at 60º C for 2 d and then weighed); average rate of water loss per unit fresh weight, and the standard deviation were calculated. A graph was plotted with average rate of water loss per unit fresh weight against time in minutes.

For stomatal density, pavement cell density and stomatal index measurements, we used similar size and age mature green rosette leaves, derived from 6-week-old plants of the wild type and 35S:*SHN1* line no. 2-2 (*SHN1*-A). Two leaves from four different plants (from each of the two genotypes) were used to generate imprints of their abaxial surface. Impressions of leaf epidermis were done using a commercial nail polish (HEMA, The Netherlands) for Arabidopsis leaves. The nail polish was applied and dried for about 10-15 min. Subsequently, the imprints were detached from the leaf surface, and pieces derived from the region in between the main vein and the leaf blade edge were mounted on glass microscope slides with 50% glycerol and observed under 20x magnification using a light microscope (Zeiss, Jena, Germany). The number of epidermal pavement cells and stomata were counted per mm^2 (two different regions per leaf) and the stomatal index was calculated (Mishra, 1997).

Arabidopsis dry-down drought resistance test

For the drought tolerance experiments, the soil mixture comprised one part of sand and perlite and two parts of compost (a mixture made up of 25% clay and 75% turf with EC = 1 [nitrogen, phosphorous, and potassium]; Hortimea, Elst, The Netherlands). Seeds were sown

(after three days at 4°C) at a density of six plants per 4-cm pot in a tray with 51 pots (Aracon containers; BetaTech, Gent, Belgium). Nutrients (Hydroagri, Rotterdam, The Netherlands; 2.6 EC) were supplied 10 days after germination (DAG), and at 14 DAG the plants were subjected to drought (for 9, 10, 11, or 12 d) by transferring the pots to dry trays (after drying each pot from outside). Every 2 d in drought, the plants were moved within the tray to nullify pot position effects. Subsequently, plants were rehydrated and observed for recovery after 1 week. Experiments comparing drought tolerance between the wild type and *DREB1A*, *shn-D*, and 35S:*SHN1* (no. 2-2) plants were repeated twice and three times with 35S:*SHN1* (no. 2-5)

Arabidopsis gravimetric drought resistance test

Plants were sown in 7x7 cm plastic pots (4 plants per pot) in the greenhouse conditions as described above. Soil mixture used to grow the plants was the same as used in quick drought testing method. The soil was weighed accurately in each pot. The pots were well watered and maintained at 100% field capacity. (FC- the amount of water left in the soil after it has been saturated and allowed to drain by gravity for 24 hours). Two weeks after the germination, the bottom holes of the pots were sealed to avoid water drainage. A control set of pots (≥ 9 pots each line) representing the WT and two 35S:*SHN1* lines (no. 2-2 and 2-5) (here referred as *SHN1*-A and *SHN1*-B) were maintained at 100% field capacity for the rest of the experimental period. A similar set of WT and *SHN1* lines were exposed to drought by withholding water until the WT died (approx. 10-11days of withholding water). Control pots (n=9) with no plants were maintained at 100% FC throughout the experimental period and similar pots with no plants were maintained at 100% field capacity until the beginning of the drought treatment, when water was withheld until the end of the experiment. This allowed the measurement of water loss through evaporation in the control as well as the drought treated set of pots. These data were used as a correction factor for transpiration estimates. During the experimental period, the pots were weighed daily using a portable balance (0.001g accuracy). For control pots the daily difference in the weight on subsequent days was corrected by adding water to maintain the 100% FC. For the drought set of pots the difference in the weight of pots was noted down. At the 15th DAG or the 1st day of dehydration (DOD), the representative plants from WT, *SHN1*-A and *SHN1*-B were

sampled to measure the initial biomass (B_{15}). The WT plants were carefully monitored daily for their appearance and when assessed as having wilted enough to survive for one more day in drought, a sample set of pots ($n \geq 4$) was rehydrated to be checked for recovery. In addition, biomass from each set of pots representing WT, *SHN1*-A and *SHN1*-B were collected and this process of sampling was repeated daily until the WT had passed irreversible wilting point and could not recover the next day. This day of permanent wilting point was noted as day after WT's death (DAWD). In this experiment the WT died on 11^{th} DOD or 25^{th} DAG. The water added during the experimental period in the control set of pots maintained at 100% FC was summed to arrive at the cumulative water lost (CWT) by the plants in the process of transpiration from the control plants. The difference in the weights of pots of the drought set was calculated as the total water lost by transpiration in drought treated plants. Precise percentage of water present in each pot was measured at the beginning of the drought test and when the WT died to determine the minimum threshold level of water beyond which the WT cannot survive. This was calculated using following formula,

Water pot^{-1}(g) = Total wt. of pot – Σ (dry wt. of soil pot^{-1} + wt. of empty pot + Fresh biomass of plant)

% water g^{-1} of soil = ($_{final}$ Water pot^{-1} / dry wt of soil pot^{-1}) x 100

Assuming that the growth was linear during the experimental period, WUE was computed as WUE= (B_{25}-B_{15})/CWT, where B_{25} and B_{15} are the total biomass (g pot^{-1}) measured on 25 and 15 DAG, respectively. CWT is the cumulative water transpired by the plants (g) during the experimental period and is computed as follows: For control pots,

CWT (g) = Σ (water added to the plants each day) - Σ (water lost by evaporation from the soil).

For pots exposed to drought CWT was calculated as follows,

CWT (g) = ($_{initial}$ Water pot^{-1} – $_{final}$ Water pot^{-1}) - Σ(water lost by evaporation).

Mean Transpiration Rate per plant per day were calculated as **MTR**= CWT/11DOD (Days of observation).

All the fresh biomass collected was later oven dried at 60°C for 5 days and the dry weights were measured separately. All experiments were tested for significance of parameters by analysis of variance (ANOVA), comparing the *shn-D, SHN1*-A *and SHN1*-B lines vs. WT and control vs.

drought treatments. Statistical analysis was done using Microsoft Excel 7.0 (Microsoft, 2000) and Graph Pad Prism version 4.00 for Windows, Graph Pad Software, San Diego California USA, www.graphpad.com.

Arabidopsis salt stress tolerance test

To screen for salt tolerance (Cheong et al., 2003) we used seedlings grown at a density of 1-2 plants per 2-cm pot in a tray with potting soil (Hortimea, Elst, The Netherlands). Nutrients (Hydroagri, Rotterdam, Netherlands; 2.6 EC) were supplied at 14 DAG. At 21 DAG the plants were treated three times (at intervals of 3 days) with NaCl (200, 250 and 300 mM) and monitored for the development of chlorosis symptoms in the next 2 weeks. Survival rates were counted on the 10^{th} day after the third application of NaCl. The experiment was repeated three times.

Rice salt stress tolerance test

For the salt tolerance test in rice we used transgenic rice plants constitutively expressing one of the Arabidopsis *SHINE* genes (*SHN2).* Progeny (T2) of four independent lines (T1) of transgenic (*SHN2*-A, *SHN2*-B, *SHN2*-C and *SHN2*-D) plants and untransformed WT (Nipponbare) plants were used for the salt stress tolerance test. Seeds were germinated on wet filter paper in the growth chamber at 28 ºC using a day cycle (12h light, 12h dark) for 12 days. The germinated seeds were then transferred to the greenhouse with long day conditions (16h light, 8h dark) in large pots (12cm X 12cm) with sand and were maintained on Hoagland's solution (Hoagland, 1950) throughout the experimental period. Three weeks after the plants were transferred to the greenhouse, they were exposed to different NaCl concentrations (150 mM and 200 mM NaCl) for 14 days and photographed. Roots of replicate plants (n=3) of each line were separately collected for fresh and dry weight determination. For the dry weight determination, the roots were dried at 70 ºC for 5 days and weighed.

Determination of Na^+ Ca^{2+} and K^+ ion content

In Arabidopsis the seedlings and in rice the roots of the treated and control plants were rinsed with deionized water and dried at 65°C for one week. About 15-50 mg of dry material was

digested with 1 ml of the digestion mixture (sulphuric acid - salicylic acid and selenium) and 2 carborundum beads and swirled carefully until all the plant material was moistened and treated overnight. Temperature was increased gradually in small steps to about 330 °C and later on cooling 0.1 ml of hydrogen peroxide was added and heated again. This step was repeated 3 times until the digest had turned colorless. On cooling down to room temperature 5 ml of demi-water was added to make up to the mark and left overnight. The Na^+ Ca^{2+} and K^+ ion content were determined by using an Atomic Emission Spectrophotometer (Elex, Eppendorf, Hamburg, Germany). Three individual plants were taken as replicate samples for each treatment in Arabidopsis and three dried roots of each line in rice were taken as replicates for this analysis.

Results

Identification of the *shn-D* mutant

By screening a collection of 2000 transposon activation tag lines (Marsch-Martinez et al., 2002), a mutant plant was identified that showed leaf surface alterations (Fig. 1A). Both rosette and cauline leaves of the mutant (termed *shine* or *shn-D* denoting the semi-dominant mutation) had a more brilliant, shiny green color when compared with wild-type plants and often had curved-down edges (Fig. 1A). The stem of mature plants was often bowed down and siliques were slightly smaller than the wild type and also showed a more brilliant surface. Structure of other floral organs and plant fertility did not seem to be affected in *shn-D*. Progeny analysis of the self-pollinated *shn-D* mutant line suggested a dominant mutation (three-quarters of the plants exhibited the *shn-D* phenotype).

Molecular characterization of the mutant revealed that the activation tag insert was between a gene encoding an unknown protein (4025 bp upstream of the promoter) and a gene encoding a member of the plant-specific AP2/ERF family of transcription factors (At1g15360) (620 bp upstream of the promoter) (Fig. 2A). Both genes were overexpressed in the *shn-D* mutant, however the downstream gene (At1g15360) was chosen to be the primary candidate determining the *shn-D* phenotype. Consequently, the coding region of the gene (termed *SHN1* or *SHN1/WIN1*) was cloned and constitutively expressed in Arabidopsis under the control of the 35S promoter of Cauliflower mosaic virus (CaMV). In fact, all the transgenic plants raised (20 individuals) showed

a phenotype resembling the original activation tag line, the characteristic *shn-D* brilliant green leaf and silique surface and downward curling of the leaves. The phenotype of most of the 35S:*SHN1* lines (both primary transformants and subsequent generations) was more severe than the original *shn-D* mutant (Aharoni et al., 2004).

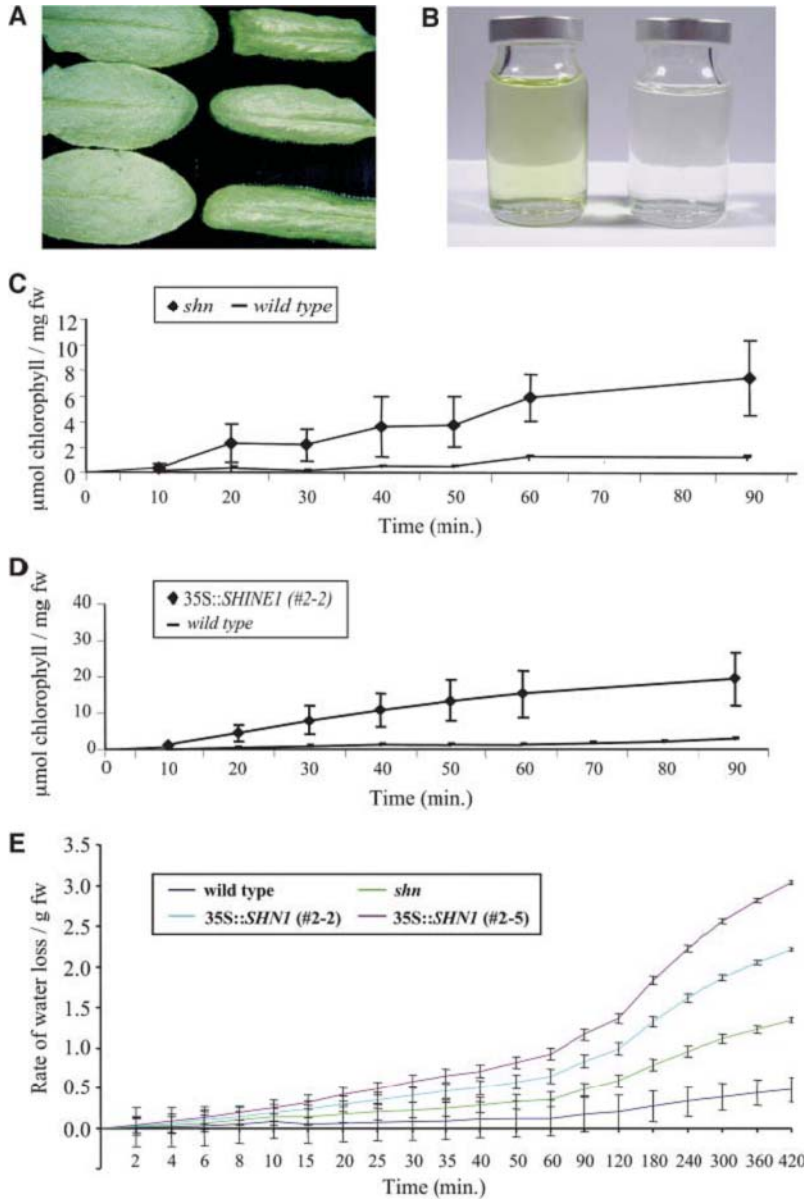

Fig. 1. Analysis of the *shn-D* mutant for water loss and surface permeability of leaves.
(A) Mature rosette leaves of wild-type plants (ecotype Wassilewskija [Ws]) and the *shn-D* activation tag mutant on the left and right, respectively. **(B)** Chlorophyll extracted in 80% ethanol for 1 h from mature rosette leaves of *shn-D* progeny (left container) compared with wild-type leaves (right container). **(C)** Chlorophyll leaching assays with mature rosette leaves of *shn-D* and wild-type immersed in 80% ethanol for different time intervals. The results are derived from three independent experiments and depicted with standard error of the mean for each time point. fw, fresh weight. **(D)** Chlorophyll leaching assays as described above but using mature rosette leaves derived from 35S:*SHN1* (2-2) progeny and wild-type plants. **(E)** Rate of water loss from the progeny of the activation tag *shn-D* mutant, two 35S:*SHN1* primary transformants (2-2 and 2-5), and the wild type. Four rosette explants (root system and inflorescence stem detached) were weighed during the time intervals depicted. The results are derived from three independent experiments and depicted with standard error of the mean for each time point.

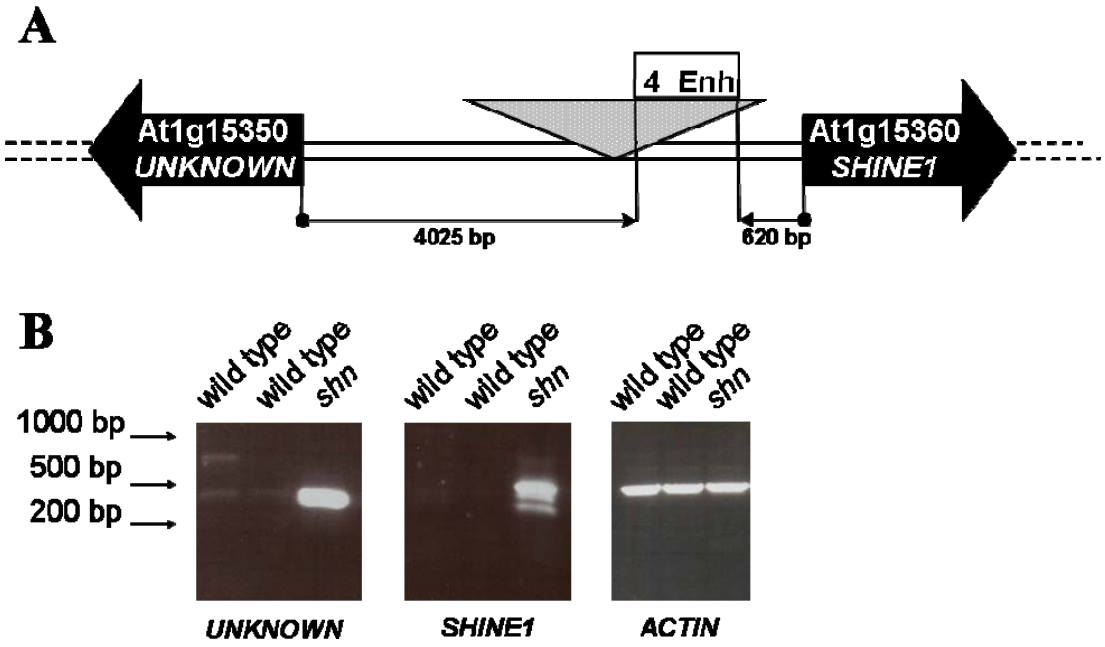

Fig. 2. **Location of the transposon insertion in the *shn-D* mutant and activation of the flanking genes.** **(A)** Location of the transposon insertion (inverted triangle) of *shn-D* on chromosome 1 between a gene with unknown function (At1g15350) and a member of the AP2/ERF transcription factor family (At1g15360, *SHN1*). The distance of the enhancer (Enh) element in base pairs from the predicted ATG of the genes is also depicted. **(B)** RT-PCR experiments using oligonucleotides for amplification of the two genes flanking the transposon insertion in *shn-D*. Expression of both genes is strongly induced in rosette leaves of *shn-D*, whereas hardly any signal could be detected in two different wild-type plants. Amplification of the actin gene was used as a control for presence and levels of cDNA. (Aharoni et al., 2004)

Phenotype of *SHN* overexpression in Arabidopsis

The *shn-D* mutant showed several changes in its epidermal properties such as increase in the leaf epicuticular wax deposition, change in the size and shape of the petal epidermal cells, leaf curling and less number of trichomes (Aharoni et al., 2004). We tested whether two other features of epidermal cell differentiation were also altered by the overexpression of *SHN1*. Both pavement cell density and stomatal density on the abaxial side of the 35S:*SHN1* lines were reduced compared with wild-type leaves (Table 1). Calculating the stomatal index revealed that it was reduced by 41% in the 35S:*SHN1* leaves compared with the wild type (Table 1). Leaching assays with progeny of two 35S:*SHN1* primary transformants (2-2 and 2-5) showed that their cuticle was

more permeable to ethanol because chlorophyll could be extracted more easily (Fig. 1D). In line with the overall stronger phenotype of the 35S:*SHN1* lines, the difference in chlorophyll leaching compared with wild-type leaves was more dramatic than initially observed for the activation tag *shn-D* mutant (Fig. 1C and 1D). The two 35S:*SHN1* primary transformants (2-2 and 2-5) also showed an increased rate of water loss compared with the wild type (Fig. 1E). Overexpression of two other members of the SHN clade results in a similar phenotype (Aharoni et al., 2004).

Table 1: Stomatal Density, Pavement Cell Density, and Stomatal Index of Mature 35S:*SHN1* and Wild-Type Rosette Leaf Blades

Plant line	Stomatal Density (cells/mm^2 ± SE)	Pavement Cell Density (cells/mm^2 ± SE)	Stomatal Index
Wild Type	27.03 ± 2.41	80.16 ± 4.97	25.22 ± 1.12
35S:*SHN1*	8.91 ± 0.94	51.56 ± 3.84	14.73 ± 0.99

The plant AP2/ERF superfamily of transcription factors contains 147 members in Arabidopsis (Nakano et al., 2006). Sequence homology searches and phylogenetic analysis across the entire AP2/ERF family showed that SHN1/WIN1 is part of a small, distinct group of three proteins, 199, 189, and 186 amino acid residues long (SHN1/WIN1, SHN2, and SHN3, respectively) (Aharoni et al., 2004). They contain the highly conserved AP2 domain and share two other conserved motifs in their central portion (mm) and C termini (cm). The two complete motifs outside the AP2 domain are only present in the SHN clade proteins, whereas their next Arabidopsis homolog (At5g25190) contains only part of the mm domain and the cm domain (Aharoni et al., 2004). SHN2 and SHN3 show the highest sequence identity among the three SHN proteins (71%), whereas SHN1 and SHN2 show the minimal homology among the clade members (55%). Interestingly, only a single protein sequence in the public database was identified, from rice (*Oryza sativa*) (OsSHN1, accession number BAD15859), which contains the complete mm and cm regions and is most similar to the SHN clade proteins (showing 65% identity to SHN1). In

addition, the SHN clade members are conserved in gene structure because all three of them contain a single intron positioned 80 bp from the start codon. The *OsSHN1* gene contains a single intron that is located 3 bp further downstream (i.e., 83 bp of the first exon) (Aharoni et al., 2004) .

Expression analysis using promoter–GUS fusions of the 3 *SHN* clade genes revealed expression in distinct tissues or cell-types having a role in plant protective layers that were formed as a result of wounding, abscission, dehiscence, tissue strengthening and the cuticle (Aharoni et al., 2004). Micorarray analysis of drought resistant 35S::*SHINE1* revealed induction of several genes involved in lipid metabolism like *CER1*, *CER2*, beta-ketoacyl-CoA synthase, genes involved in cell wall synthesis, MYB genes, genes involved in signaling like kinases and another set of genes involved in calcium binding and signaling (Trijatmiko, 2005).

Plants overexpressing *SHN1* show enhanced drought tolerance

We examined the effects of the changes in plant epidermal properties, as a result from *SHN1* overexpression, on drought tolerance capacity. For this, a quick high-throughput screen for drought resistance was set up where the 15-day-old seedlings of the original activation tag lines, two of the 35S:*SHN1* transformant lines (lines no. 2-5 and 2-2), and the wild type (ecotype Ws) were exposed to a period of 9 to 11 days of dehydration (DOD) (Fig. 3). Subsequently, seedlings were watered and their recovery monitored for a week. Whereas wild-type plants did not recover from the dehydration treatments longer than 9 days and completely dried out, all seedlings derived from lines expressing the *SHN1* gene recovered to become greener and stronger (Fig. 3). Consistent with the phenotypic characteristics described above, seedlings derived from the activation tagged line were relatively weak in recovery when compared with the two transgenic 35S:*SHN1* lines.

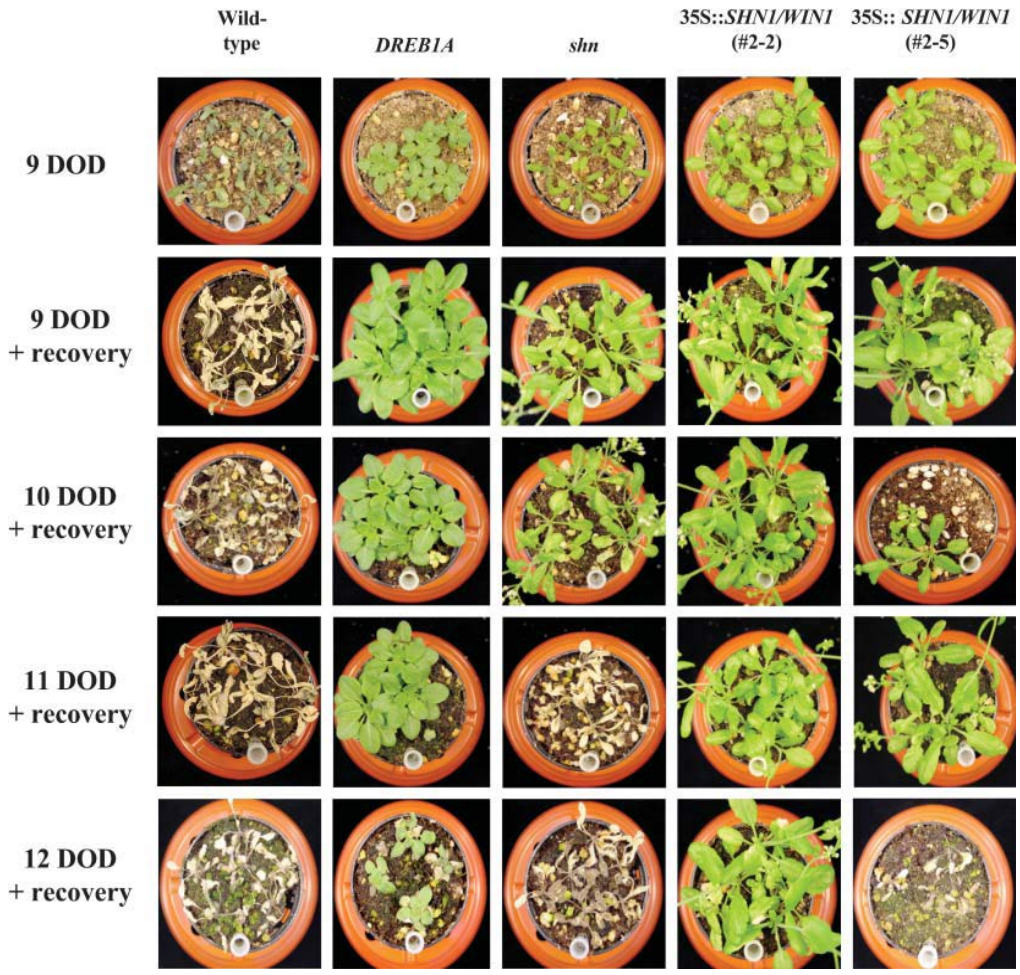

Fig. 3. Drought tolerance of *SHN* overexpressors. Fifteen-day-old seedlings of the wild type, progenies of *shn-D*, two 35S:*SHN1* lines (2-2 and 2-5), and a positive control rd29-*DREB1A* line (providing drought tolerance; Kasuga et al., 1999) were exposed for a period of 9 to 12 (DOD). Subsequently, seedlings were watered, and their appearance after a week (recovery) is presented in the image (apart from the first row at 9 DOD, in which pictures were taken directly at the end of the dehydration period). The 9 DOD results provide a clear difference between the wild type and *shn-D* as well as 35S:*SHN1*and *DREB1A*, in which there is 100% recovery of the overexpression lines and 0% recovery of the wild type.

SHN Overexpression gives Increased Water Use efficiency in Arabidopsis

Since the size of *shn-D* plants and WT had little difference, it was important to know the minimum threshold levels of water to which these plants could survive and below which they did not recover. Hence, a more detailed drought stress test was carried out on WT and two 35S:*SHN1* lines (2-2 and 2-5) named as *SHN1*-A and *SHN1*-B respectively, in which a set of replicate pots

each with 4 plants was subjected to drought stress at 14 DAG by withholding water to the pots. The pots were weighed daily and the water transpired was replenished in the control set of pots maintained at 100% FC. The exact amount of water was calculated in the soil at the beginning of drought stress (14 DAG) and later in the drought period when the WT died (11DOD) and this minimum level of water threshold was compared to the *SHN1*-A and *SHN*1-B lines to see if they could survive at those water levels. In this test the WT could not survive beyond 12.7 % water levels at 11DOD. On this day the *SHN1*-A line contained 17% and *SHN*1-B line 15% water in the soil and survived the drought stress. At 12^{th} and 13^{th} DOD, the *SHN1*-A line contained 15.4% and 10.5 % water respectively in the soil and survived upon rehydration (Fig. 4A), Line *SHN*1-B, which contained 12.3% and 9.0 % water respectively in the soil and also survived on rehydration (Fig. 4A). The amount of water transpired per day both under stress and non-stress condition was measured for WT, *SHN1*-A and *SHN*1-B. *SHN1*-A and *SHN*1-B's rate of transpiration per day (MTR) was much less (50-60%) compared to its WT both under stress and non-stress condition (Fig. 4C). Also, the relative average transpiration water loss to evaporation throughout the experimental period of *SHN1*-A and *SHN*1-B was less compared to the WT, both under control and drought treated conditions (Table 2). Further we measured the WUE of *SHN1*-A and *SHN*1-B compared to the WT using the gravimetric method (see Materials and Methods) where WUE accounts for the ratio between the total gain in the biomass during the experimental period and the cumulative water transpired (CWT). *SHN1*-A and *SHN*1-B both showed increased WUE (2.7 times and 2.2 times respectively), under drought stress compared to the WT, which showed a 25.0% decrease in WUE under drought stress (Fig. 4B). The total dry biomass (shoots + roots) was calculated for WT, *SHN1*-A and *SHN*1-B and all the lines showed a decrease in total dry biomass accumulation under drought stress. However WT showed the highest decrease in total dry biomass accumulation (40.7%) compared to *SHN1*-A and *SHN1*-B in which the biomass decreased with 19.4% and 14.6% respectively (Fig. 4D). This result suggests that *SHN1*-A and *SHN1*-B not only survived below the minimum threshold of water levels required for WT but were also able to prolong the drought stress situation by losing less water through transpiration and delaying the drought stress. The transgenic lines performed better in terms of growth compared to the WT under drought stress situation by showing much less reduction in biomass.

Table 2: Relative transpiration (T) water loss (mg/g fresh weight) to evaporation (E) water loss (mg/g fresh weight), T/E ratio.

	Number of plant(n=4)				Average
Plant line	**1**	**2**	**3**	**4**	**(± SE)**
WT (Control)	0.1791	0.1781	0.1722	0.1781	0.1769 ± 0.0016
***SHN1-A* (Control)**	0.0697	0.0796	0.0697	0.0597	0.0697 ± 0.0041
***SHN1-A* (Control)**	0.0796	0.0896	0.0896	0.0697	0.0821 ± 0.0048
WT (11DOD)	0.0752	0.0754	0.0611	0.0778	0.0724 ± 0.0038
***SHN1-A* (11DOD)**	0.0294	0.0167	0.0221	0.0296	0.0244 ± 0.0031
***SHN1-A* (11DOD)**	0.0230	0.0328	0.0436	0.0236	0.0307 ± 0.0048

***SHN* gives increased salt tolerance in Arabidopsis**

A salt tolerance assay was employed (Cheong et al., 2003) by treating the WT and the 35S:*SHN1* lines (*SHN1*-A and *SHN1*-B) with 300 mM NaCl and then monitoring the plants for chlorosis and survival (Fig. 5). The phenotype of the salt stressed plants was examined and the tolerant percentage was calculated on the basis of the undamaged non-bleached plants, which was higher for the 35S:*SHN1* plants, which could reach full maturity in contrast to the WT (Fig. 4 and Table 3). The percentage reduction in the shoot/leaf dry matter accumulation of *SHN1-A* and *SHN1-B* under salt stress was respectively 54% and 56.6% compared to wild-type that showed 76.3% reduction in growth. Chemical compositional analysis revealed that 35S:*SHN1* and WT accumulates similar amounts of Na^+ both under salt stress and no stress condition (Fig. 6A & B). However, *35S:SHN1* under salt stress maintains a higher K^+ / Na^+ ratio (Fig. 6C), which is a well known plant tolerance mechanism by maintaining osmotic balance of the cells. The K^+ levels are reduced less in 35S:*SHN1* compared to WT (Fig. 6E). Ca^{2+} levels are maintained in the 35S:*SHN1* whereas they are reduced in WT (Fig. 6D). This result indicates that the maintenance of the Ca^{+2+}levels under salt stress conditions in 35S:*SHN1* increases the selectivity of the K^+ influx over the Na^+ probably through the Na^+/K^+ transporter at the cell membrane, which helps in maintaining the balance of K^+ in the cell and reduces the toxic effect of higher Na^+ in the cell.

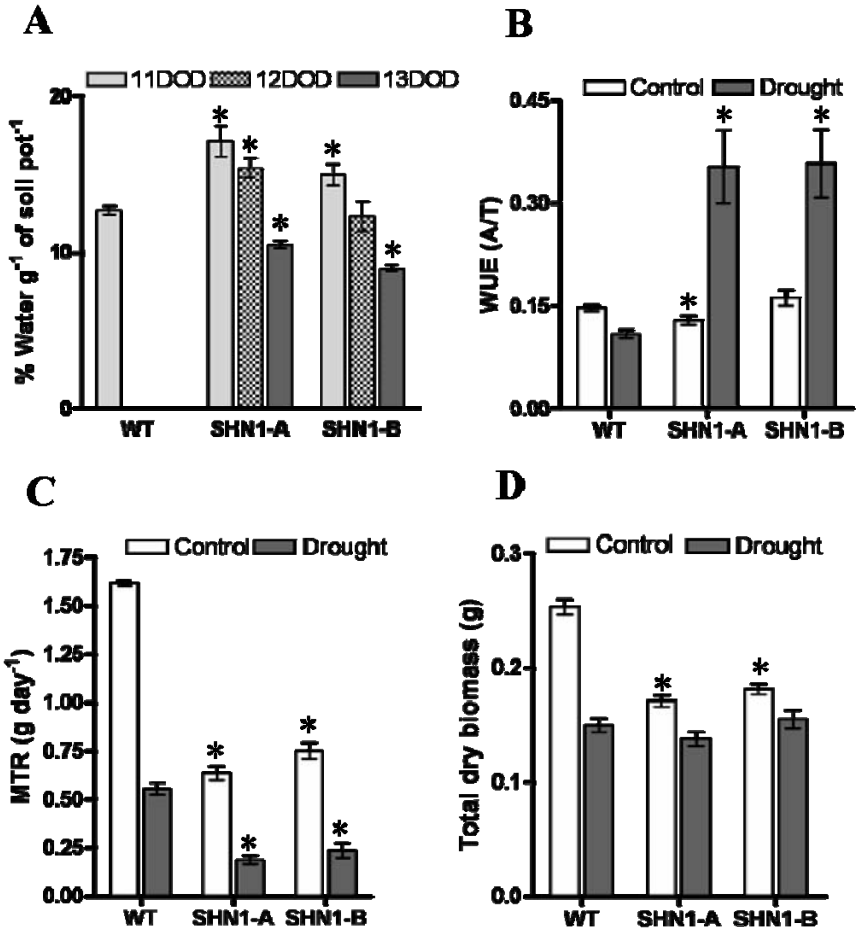

Fig. 4. *SHN* overexpression improves WUE in Arabidopsis.
Two 35S:*SHN1* lines, *SHN1*-A (# 2-2) and *SHN1*-B (# 2-5) and WT plants tested under well-watered and drought stress conditions, bars indicate SE (n≥4), p-values shown for *SHN1*-A, *SHN1*-B compared to the WT. **(A)** Levels of soil water shown as percentage of water per gram of soil per pot. **(B)** Water use efficiency (WUE) (p-value 8e-04). **(C)** Mean Transpiration rate (MTR) (p-value 1e-04). **(D)** Total dry Biomass (p-value 1e-04). * indicates values are significant at (α <0.01) compared to the WT in the same treatment.

Table 3: Tolerance of *SHN* to 300 mM NaCl stress

Genotype	Total Plants	Non chlorotic	% Tolerant
Ws	20	03	15
35s-SHN1	20	17*	85*

*values of 35S SHN1 were significantly different from WT using chi-square test at (α<0.01)

Fig. 5. Salt tolerance by overexpression of *SHN* in Arabidopsis.
(A) WT and **(B**) 35S-*SHN1* Arabidopsis treated with 300 mM NaCl concentrations showing chlorotic/dead plants (A) and surviving 35S-*SHN* plants (B).

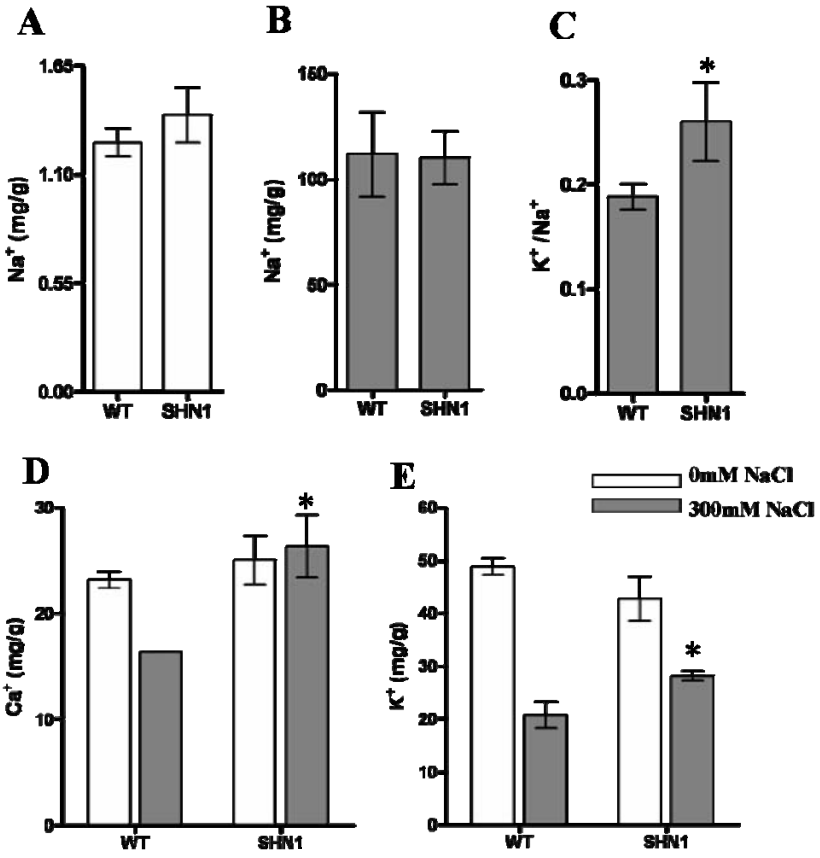

Fig. 6. Ion accumulation in *35S:SHN* vs. WT under no stress and 300 mM NaCl.
(A) Na^+ accumulation of 35S:*SHN* and WT under 0 mM NaCl treatment. **(B)** Na^+ accumulation of 35S:*SHN* and WT under 300 mM NaCl. **(C)** K^+/Na^+ ratio of 35S:*SHN* and WT under 300 mM NaCl. **(D)** Ca^{2+} levels of 35S:*SHN* and WT both under 0 mM and 300 mM NaCl. **(E)**. K^+ levels of 35S:*SHN* and WT both under 0 mM and 300 mM NaCl. * indicates values are significant at (α <0.01) compared to the WT in the same treatment.

Transformation of an Arabidopsis *SHN2* gene overexpression construct in rice

To analyze the effect of overexpression of the Arabidopsis *SHINE* genes in rice, *SHN2* was introduced into rice (*Oryza sativa*, subspecies *japonica*) cultivar Nipponbare, controlled by the CaMV 35s promoter, by means of Agrobacterium-mediated transformation of embryogenic callus. Fifteen independent lines were generated. The overexpression of *SHN2* in rice (referred to as rice *SHN2* lines) did not exhibit any obvious phenotype such as displayed in Arabidopsis. However, semi-quantitative RT-PCR analysis confirmed high levels expression of *SHN2* (Karaba et al., 2007). Four transformants with enough seeds were chosen for further experiments. The transformants did not show any significant reduction in growth, seed yield or germination when grown under normal greenhouse conditions. *SHN2* overexpressors in rice did not reveal an increase in cuticle permeability or reduction in the stomatal density on the abaxial side of the rice leaves (Trijatmiko, 2005), unlike Arabidopsis. Two independent *SHN2* overexpressors in rice tested for drought tolerance, exhibited a significant higher water use efficiency (WUE), with or without drought stress, exhibiting 29-40% higher WUE under stress condition (Karaba et al., 2007). These lines also showed a reduction in mean transpiration rate (MTR) (Karaba et al., 2007).

SHN2 **overexpression in rice improves salt tolerance**

In a replicated pot experiment for salt tolerance in rice, T3 generation lines for 4 independent transgenic plants (T2) tested for the presence of insert using oligonucleotides specific for the marker gene *HPT*, were subjected to different NaCl concentrations under greenhouse conditions. The plants were grown on sand and maintained throughout the experimental period on Hoagland's solution (Hoagland, 1950) and during salt stress the NaCl was given to plants by adding it in the Hoagland's solution. Three week old plants were given different concentrations of NaCl (0 mM, 150 mM and 200 mM) for 14 days and were later tested for their tolerance level based on their recovery, growth and survival. At the 10^{th} day under NaCl stress various growth data were collected such as the height of the plant, number of leaves, number of tillers (Table 4), fresh and dry weights of shoots & roots (Fig. 8) and documented by photographs (Fig. 7). At the end of the experiment when the plants had completed there lifecycle, the seed yield per plant was

determined (Table 4). All *SHN2* lines, except for *SHN2*-B, showed slightly reduced plant height compared to the WT under no stress condition (Fig. 7A and Table 4). Under 150 mM NaCl stress, all *SHN2* lines showed no difference in height and number of tillers except for *SHN2*-A which was little shorter and produced less number of tillers compared to the WT. Under treatment of 200 mM NaCl stress *SHN2*-A and *SHN2*-C showed no difference in height whereas *SHN2*-B and *SHN2*-D were taller compared to the WT (Table 4). All *SHN2* lines showed no difference in the total number of leaves under no stress and 150 mM NaCl stress condition except for *SHN2*-A and *SHN2*-C which had fewer leaves under no-stress compared to the WT (Fig. 7A,B and Table 4). Under no-stress conditions all *SHN2* lines had the same number of tillers compared to WT. Under 200 mM NaCl all *SHN2* lines showed an increase in the total number of leaves and tillers compared to the WT, except for *SHN2*-A which did not differ in its number of tillers compared to the WT (Fig. 7C and Table 4). *SHN2*-B and *SHN2*-D showed an increase in the shoot (38-42%) and total fresh biomass (shoot & root) (25-28%) under 200 mM NaCl conditions (Fig. 8A, B & Fig. 7C,F) compared to the WT. When the dry weights were measured it appeared that all the *SHN2* lines had a higher total dry biomass accumulation (13-20%) under 200 mM NaCl stress compared to WT (Fig. 8D). Shoot dry weight was 10-19% increased in *SHN2*-B, *SHN2*-C and *SHN2*-D. *SHN2*-A & *SHN2*-C showed an increase in root dry biomass (47-57%) only under 200 mM NaCl stress condition compared to the WT (Fig. 8E, F). Seed yield in *SHN2-B* and *SHN2-D* was respectively 29.3% and 21.9% higher without stress compared to the WT, whereas *SHN2-A* showed no difference in total seed weight. Seed yield of *SHN2*-C, however was 38% less under no-stress condition compared to the WT (Table 4). Under 150 mM NaCl stress treatment all the *SHN2* lines were able to set seeds unlike the WT (Table 4). The percentage reduction observed in the seed yield under 150 mM NaCl in *SHN2-A, SHN2-B, SHN2-C* and *SHN2-D* was 60.7%, 55 %, 76.5% and 53.1% respectively. From above results it seems that *SHN2* plants are able to perform much better, especially under salt stress condition compared to the WT. *SHN2*-B and *SHN2*-D especially performed much better in terms of growth and tolerance. After 14 days under salt stress the plants were again maintained on Hoagland's solution for recovery. Two out of four *SHN2* lines (B & D) recovered much faster and stayed alive to grow stronger and produce more leaves and set seeds under 150 mM NaCl unlike the WT, which could not survive. These results were

more dramatic under 200 mM NaCl stress, when even the *SHN2* lines after 16 days under recovery did not survive. The above results suggests that *SHN2* lines (especially line B and D) were able to grow much better than the WT under salt stress conditions and could tolerate NaCl stress for much longer periods compared to the WT by developing more roots as an adaptive trait to minimize the osmotic shock for the plant.

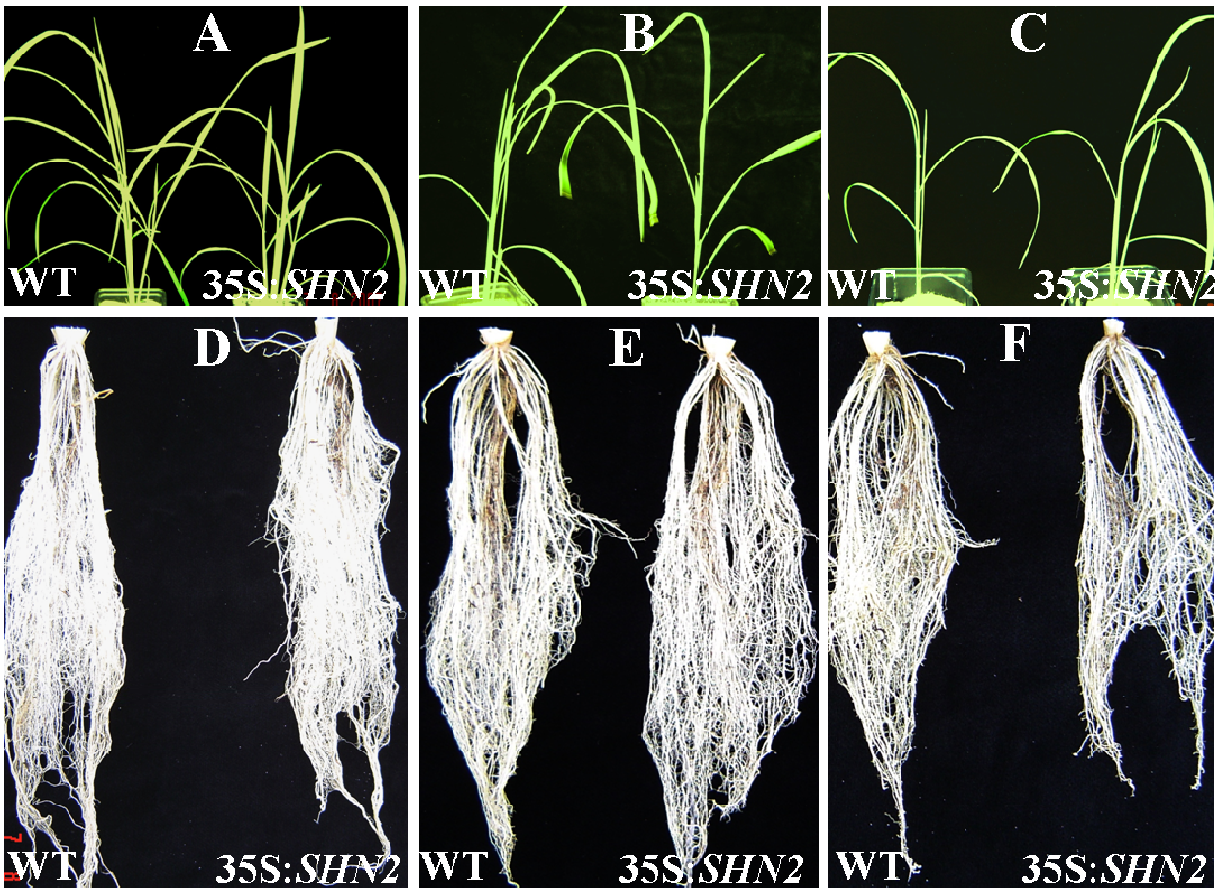

Fig. 7. **Phenotype of *35S:SHN2* vs. WT in rice, under different NaCl concentrations.**
35S:SHN2 shows no difference in shoot and root growth under 0 mM NaCl (**A** and **D**); and shows more shoot and root growth under 150 mM (**B** and **E**) and 200 mM NaCl (**C** and **F)**.

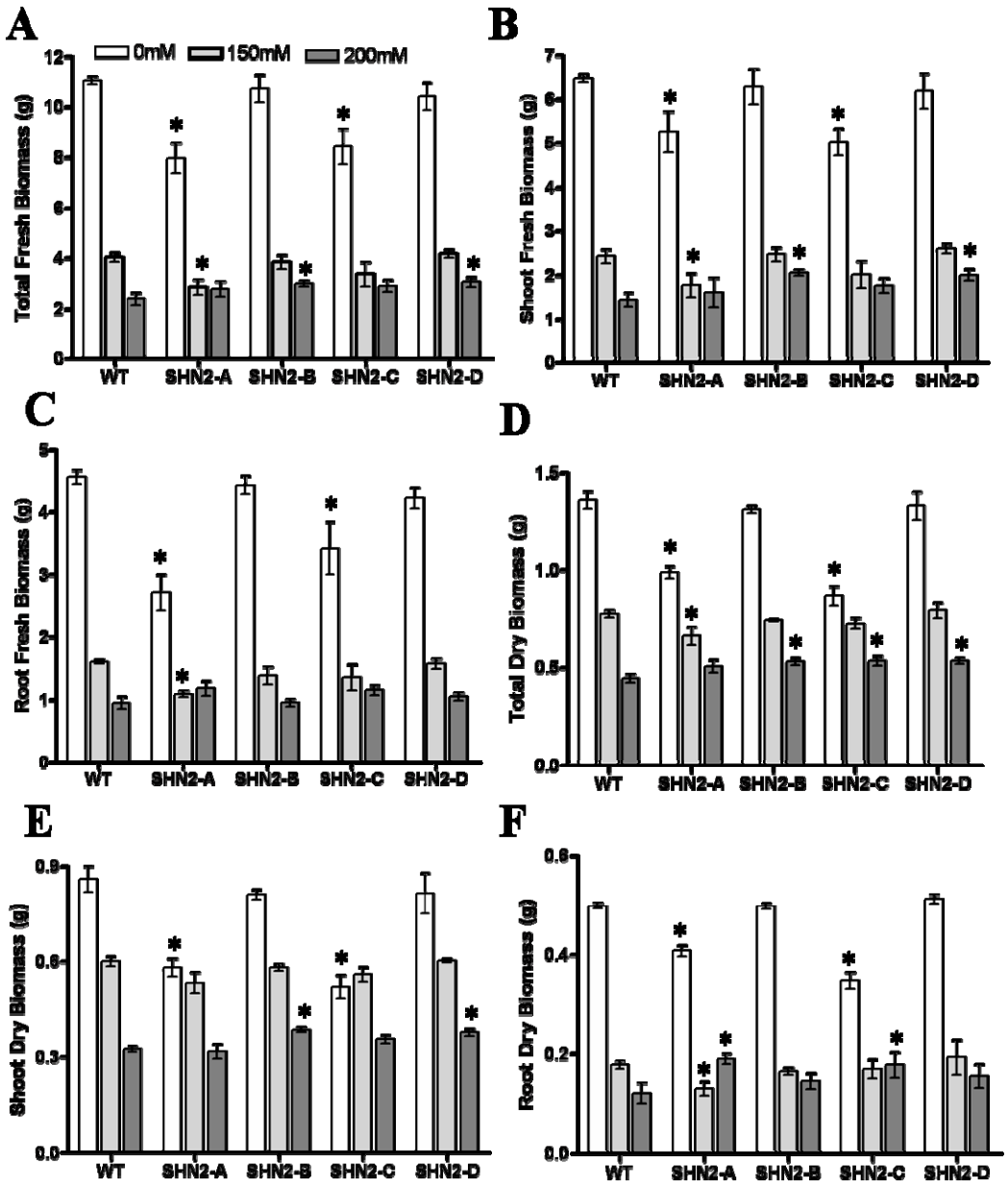

Fig. 8. Physiological analysis of 35S:*SHN2* lines (A-D) and WT (Nipponbare) under different NaCl concentrations. (0 mM white bar, 150 mM grey bar and 200 mM dark grey bar). Bars indicate SE (n>4), calculated p-values shown for 35S:*SHN2* vs. WT. **(A)** Total fresh biomass **(B)** Shoot fresh biomass (p-value 1.1e-03)., **(C)** Root fresh biomass, **(D)**, total dry biomass, **(E)** shoot dry biomass and **(F)** root dry biomass (p-value 3e-03). P-values for A, C,D and E are (p-value 1e-04). * indicates values are significant at (α <0.01) compared to the WT in the same treatment.

Table 4. Growth parameters of 35S:*SHN2* lines vs. WT under NaCl stress (150 mM and 200 mM) and no stress (C)

Plant Line	Height of Plant (cm ± SE)	No. of Leaves (± SE)	No. of Tillers (± SE)	Seed yield (g ± SE)
WT (C)	68.20 ± 1.19	13.40 ± 0.92	3.20 ± 0.20	5.08 ± 0.22
SHN2-A (C)	59.60 ± 1.75*	8.40 ± 0.39**	2.60 ± 0.24	4.68 ± 0.48
SHN2-B (C)	62.20 ± 1.49	10.20 ± 0.66	3.00 ± 0.00	7.19 ± 0.92**
SHN2-C (C)	55.00 ± 2.07*	8.40 ± 0.59**	2.40 ± 0.24	3.68 ± 0.39*
SHN2-D (C)	55.60 ± 2.03*	10.60 ± 0.50	3.40 ± 0.24	6.51 ± 0.67**
WT (150 mM)	57.20 ± 0.66	6.40 ± 0.39	3.00 ± 0.00	0.00
SHN2-A (150 mM)	50.40 ± 1.50*	5.40 ± 0.24	2.40 ± 0.24*	2.10 ± 0.17**
SHN2-B (150 mM)	55.80 ± 0.58	6.00 ± 0.44	2.60 ± 0.24	2.83 ± 0.35**
SHN2-C (150 mM)	54.40 ± 0.67	5.20 ± 0.20	2.60 ± 0.24	1.73 ± 0.15**
SHN2-D (150 mM)	57.60 ± 0.92	6.00 ± 0.31	3.00 ± 0.00	1.50 ± 0.17**
WT (200 mM)	42.83 ± 0.87	5.00 ± 0.31	2.00 ± 0.00	0.00
SHN2-A (200 mM)	47.80 ± 1.64	6.40 ± 0.24*	2.00 ± 0.00	0.00
SHN2-B (200 mM)	47.90 ± 1.18**	6.40 ± 0.24*	2.40 ± 0.24	0.00
SHN2-C (200 mM)	47.60 ± 1.93	6.80 ± 0.37*	2.20 ± 0.20	0.00
SHN2-D (200 mM)	49.24 ± 0.83**	7.40 ± 0.24**	2.60 ± 0.24**	0.00

**** =(p<0.01)** from WT in the same treatment.
*** = (p<0.05)** from WT in same treatment.

To get an insight in the mechanism through which *SHN2* lines in rice were able to tolerate salt, we analyzed the ion content of two of the best performing *SHN2* lines in rice (B and D) to determine the ion contents of the cells like the Ca^{2+}, K^{+} and the Na^{+}, which are known to play a key role in salt tolerance mechanism (Zhu, 2002). The results show that under no–stress situation all the lines accumulated similar amounts of Na^{+} (Fig. 9A). Under 150 mM NaCl *SHN2-B* accumulated more Na^{+} and *SHN2-D* accumulated less Na^{+} compared to the WT. However, under 200 mM NaCl both the *SHN2* lines accumulated less Na^{+} compared to the WT (Fig. 9A). The levels of K^{+} under no–stress situation was higher in *SHN2-B* and lower in *SHN2-D* compared to the WT, however under 150 mM and 200 mM NaCl both the lines maintained higher K^{+} levels compared to the WT (Fig. 9B). The levels of Ca^{2+} were always higher under no stress, 150 mM NaCl and 200 mM NaCl conditions in both *SHN2* lines compared to the WT (Fig. 9C). The

K^+/Na^+ ratio was also higher under no stress condition but was dramatically increased under 150 mM NaCl and 200 mM NaCl conditions in both *SHN2* lines compared to the WT (Fig. 9D-F). This result indicates that maintaining higher K^+/Na^+ ratio, a mechanism well known for maintaining cell homeostasis (Zhu, 2001a) leads to reduced Na^+ toxicity in the cell. Additionally, the increase in cytosolic Ca^{2+} initiates the stress signal transduction leading to salt adaptation.

These results show that the salt tolerance in *SHN2* lines in rice is exhibiting a similar mechanism for tolerance as observed for the *SHN1* lines in Arabidopsis showing a conservation of mechanism among different species.

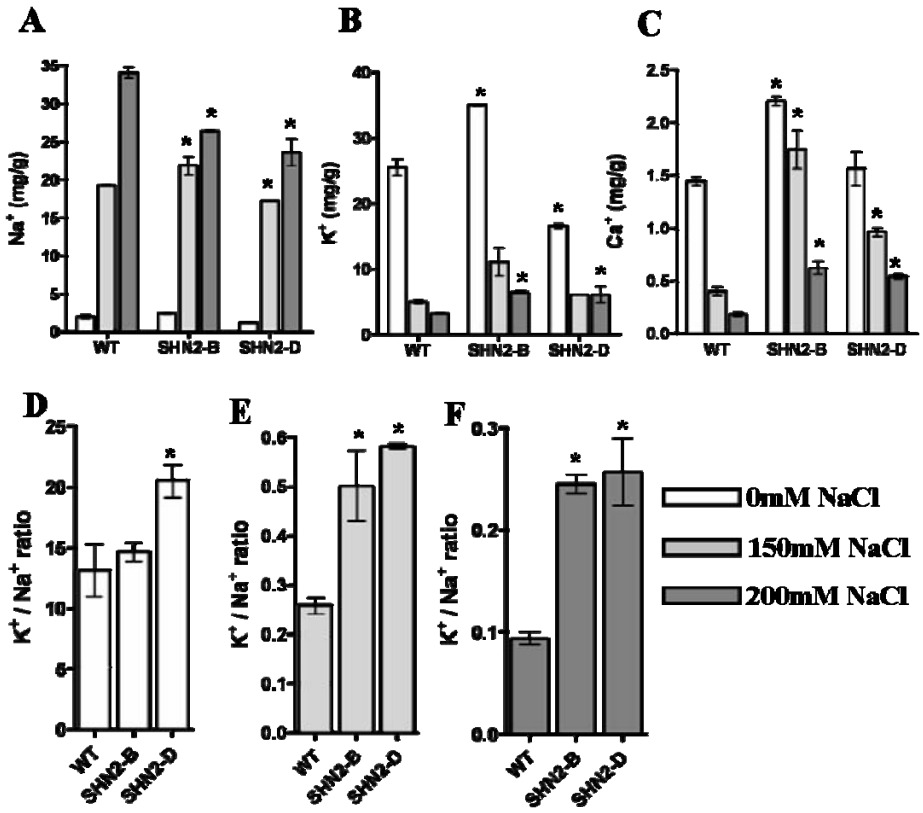

Fig. 9. Ion accumulation in 35S*:SHN2* lines vs. WT (Nipponbare) in rice under 0 mM, 150 mM and 200 mM NaCl.

(A) Na^+ accumulation of *SHN2*-B and *SHN2*-D under 0 mM, 150 mM and 200 mM NaCl compared to the WT. **(B)** K^+ accumulation of *SHN2*-B and *SHN2*-D under 0 mM, 150 mM and 200 mM NaCl compared to the WT. **(C)** Ca^{2+} accumulation of *SHN2*-B and *SHN2*-D under 0 mM, 150 mM and 200 mM NaCl compared to the WT. **(D)** K^+/Na^+ ratio of *SHN2*-B and *SHN2*-D under 0 mM NaCl compared to the WT. **(E)** K^+/Na^+ ratio of *SHN2*-B and *SHN2*-D under 150 mM NaCl compared to the WT. **(F)**. K^+/Na^+ ratio of

SHN2-B and *SHN2*-D under 200 mM NaCl compared to the WT. Bars indicate SE (n=3); * indicates values are significant at (α <0.01) compared to the WT in the same treatment.

Discussion

The plant AP2/ERF superfamily of transcription factors that have been shown to act either in developmental programs, such as flower development, or as mediators in the plant responses to various environmental stresses (Okamuro et al., 1997; Liu et al., 1998; Riechmann and Meyerowitz, 1998; Singh et al., 2002), contains 147 members in Arabidopsis (Alonso et al., 2003; Nakano et al., 2006). Different members of the AP2/ERF family regulate genes involved in response to pathogenesis, cold, drought, ethylene, and jasmonates (Memelink et al., 2001). Such response cascades could be executed by direct regulation of metabolic pathways, which lead to the production of metabolites essential for plant survival. Sequence homology searches and phylogenetic analysis across the entire AP2/ERF family showed that SHN1/WIN1 is part of a small, distinct group of three proteins, 199, 189, and 186 amino acid residues long (SHN1, SHN2, and SHN3, respectively) (Aharoni et al., 2004). They contain the highly conserved AP2 domain and share two other conserved motifs in their central portion (mm) and C termini (cm). The two complete motifs outside the AP2 domain are only present in the SHN clade proteins, whereas their next Arabidopsis homolog (At5g25190) contains only part of the mm domain and the cm domain (Aharoni et al., 2004). SHN2 and SHN3 show the highest sequence identity among the three SHN proteins (71%), whereas SHN1 and SHN2 show the lowest homology among the clade members (55%). Interestingly, only a single protein sequence, from rice (*Oryza sativa*) (OsSHN1, accession number BAD15859 was identified in the public database,, which contains the complete mm and cm regions and is most similar to the SHN clade proteins (showing 65% identity to SHN1) (Aharoni et al., 2004). In addition, the SHN clade members are conserved in gene structure because all three of them contain a single intron positioned 80 bp from the start codon. The *OsSHN1* gene contains a single intron that is located 3 bp further downstream (i.e., 83 bp of the first exon) (Aharoni et al., 2004) .

Overexpression of *SHN* in Arabidopsis increases cuticular wax composition and/or altered the epidermal cells and overall surface structure, which resulted in the *shn-D* glossy leaf surface

phenotype (Aharoni et al., 2004). Adding to this, the decrease in numbers of trichomes, changes in trichome branching patterns, and the alteration of stomatal indices emphasize that *SHN* genes strongly affect epidermal cell differentiation. *SHN* overexpressing lines showed a dramatic increase in chlorophyll leaching when immersed in ethanol. This indicates that overexpression of *SHN* genes causes not only changes in epidermal differentiation but also structural defects in the cuticle. Another point of evidence that plants overexpressing *SHN1* are altered in the cuticular membrane was our fresh weight loss experiment. Rosette explants (without roots or inflorescence stem), derived either from *shn-D* or plants overexpressing *SHN1*, showed a clear increase in water loss as compared with wild-type explants.

Plants loose water in the process of transpiration either through the stomata (allowing movement of gases and water vapor) or the cuticle (a hydrophobic heterogeneous layer comprising of lipids, cutin and intra-cuticular wax). Although stomatal water loss accounts for most of the transpiration loss, the amount of water lost through the cuticle (cuticular transpiration) cannot be ignored. The cuticular water loss can range from 0.05% (xerophytes) to 32% (herbaceous species) of the total water lost in transpiration by the plant (Larcher, 1975). Drought stress increases wax deposition on the plant surface (Sanchez et al., 2001; Samdur et al., 2003; Cameron et al., 2006; Kim et al., 2007) and decreases stomatal conductance to minimize stomatal water loss (Chaves and Oliveira, 2004) Water conservation by the plant is also dependent on the cuticular properties, which play a major role by reducing cuticular transpiration (Kerstiens, 1996).

The molecular dissection of WUE has been initiated in the model plant Arabidopsis where the *erecta* mutant was found critical in altering transpiration efficiency by modifying leaf diffusive properties due to altered stomatal density mostly through a role in epidermal pavement cell expansion and mesophyll capacity for photosynthesis (Masle et al., 2005). Expression analysis using promoter–GUS fusions of the 3 *SHN* clade genes revealed expression in distinct tissues or cell-types having a role in plant protective layers that were formed as a result of wounding, abscission, dehiscence, tissue strengthening and the cuticle (Aharoni et al., 2004). The expression patterns of the *SHN* genes emphasize a role in the interface between the outermost plant surfaces and the environment (wounding sites, root cap cells, and in some organs at the epidermal layers) and at the interface between cells and cell layers either above ground (e.g., the

dehiscence and abscission zones) or below ground (e.g., the endodermis). Cuticle metabolism and the structure of the epidermal surfaces are crucial factors in determining plant water management. To examine the effect of the different phenotypes we observed on drought tolerance, we conducted a set of experiments that demonstrated that overexpression of the *SHN* gene enhances the drought tolerance and increases the WUE of the plant and its recovery after a period of water deficiency. Because a change in stomatal index was detected in plants overexpressing *SHN1,* it is probable that the reduction in the number of stomata leads to reduced transpiration both during the non-stress and drought period and helping the plant to conserve more water and delay the drought situation, following the drought avoidance mechanism. This characteristic might be the reason for achieving higher WUE in Arabidopsis. On the other hand, it cannot be ruled out that changes in the structure of the root system caused the increased efficiency for uptake of water under water restriction. in the overexpression lines. Preliminary visual observations performed on root systems of the wild type and overexpressors indicated a change in root structure with longer lateral roots in the overexpression lines (data not shown). In this respect, it has previously been shown that plant stress and root growth conditions can alter root structure and components like suberin (Zimmermann et al., 2000). The two *SHN* overexpressors were also able to tolerate and survive under lower soil water content (10.5%-9.0%) compared to the WT (12%) (Fig. 4A), due to less transpirational water loss, and probably more stable and strong cell wall integrity.

Microarray analysis of drought resistant *SHN1* revealed induction of several genes involved in lipid metabolism like *CER1*, *CER2, KCS1*, beta-ketoacyl-CoA synthase, genes involved in cell wall synthesis, stress induced genes like RD22 and *MYB* genes, genes involved in signaling like kinases and a set of genes involved in calcium binding and signaling (Trijatmiko, 2005), all of them supporting the biological role proposed in plant protective layers. Due to similarities in expression of these genes no loss-of-function phenotypes for any of the three *SHN* genes was obtained, when attempting to obtain plants silenced for *SHN1, SHN2*, and *SHN3* using the RNA interference approach (as no T-DNA knockout lines could be obtained to any of the genes in the public databases) (Aharoni et al., 2004).

SHN also showed increased salt tolerance in Arabidopsis and rice. Many physiological studies have demonstrated that Na^+ toxicity is not only due to toxic effects of Na^+ in the cytosol,

but also because K^+ homeostasis is disrupted possibly due to the ability of Na^+ competing for K^+ binding sites. In Arabidopsis, 35S:*SHN1* lines (*SHN1*-A and *SHN1*-B) showed similar Na^+ accumulation in the cytoplasm compared to the WT, yet the overexpressor lines were much more tolerant to 300 mM NaCl. They were also able to maintain higher Ca^{2+} levels both under no stress and salt stress situation and showed less reduction in their K^+ levels compared to the WT. They also maintained higher K^+/Na^+ ratio, a mechanism well known by maintaining cell homeostasis and thereby reducing Na^+ toxicity in the cell (Zhu, 2001b). For ionic aspect of salt stress, a signaling pathway based on the *SOS* (Salt Overly Sensitive) genes has been well established. The input of the SOS pathway is likely excess intracellular Na^+, which somehow triggers a cytoplasmic Ca^{2+} signal (Zhu, 2000). How this calcium signal is different from the one triggered by drought, cold, or other stimuli remains a mystery. The outputs are expression and activity changes of transporters for ions such as Na^+, K^+, and H^+. Salt stress induced calcium binding proteins are known to induce specific kinases, which are known to give salt tolerance in plants (Cunningham and Fink, 1996; Pandey et al., 2002) when overexpressed. Induction of Ca^{2+} in the cytoplasm activates genes involved in regulating the selectivity of K^+/Na^+ and Na^+/H^+ transporters at the cell membrane, increasing the selectivity of K^+ over Na^+ in the cell and effluxing or compartmentalizing Na^+ into the vacuoles of the cell, thereby reducing the Na^+ toxicity and maintaining the ion homeostasis condition of the cell and essential cell enzymatic activities which are controlled by the K^+ levels in the cell. Many genes involved in cell wall synthesis, kinase signaling and another set of genes involved in calcium binding and signaling are upregulated by *SHN*, which shows ability of *SHN* to tolerate such osmotic stress created by Na^+ toxicity.

In a drought stress experiment transgenic rice plants constitutively expressing the Arabidopsis *SHN2* gene showed increased WUE and drought resistance (Karaba et al., 2007) compared to the WT plants, similar to that seen in Arabidopsis. These results show that the SHN1 and SHN2 functions are overlapping and similar in rice in terms of drought resistance. Since overexpression of *SHN1* gave salt tolerance in Arabidopsis we tested if it behaves the same way in rice. Hence we analysed four independent *SHN2* overexpressor lines in rice for salt tolerance at 150 mM and 200 mM NaCl, where they showed no difference on an average in the total biomass in the 150 mM NaCl and even more total biomass in the 200 mM NaCl compared to the WT in

same treatment. The analysis of ion content revealed that rice *SHN2* lines accumulate less Na^+, maintain higher K^+ and Ca^+ levels in the cytosol and also maintain higher K^+/Na^+ ratio under 150 mM and 200 mM NaCl stress condition compared to the WT. This shows that rice *SHN2* lines also show overlap of salt tolerance mechanism as observed in *SHN1* lines in Arabidopsis.

In this study we showed that the SHN clade of transcription factors has conserved functions in affecting the epidermal properties and providing drought resistance and salt tolerance when overexpressed. As the genes function both in Arabidopsis and rice, it suggests that they will function between dicots and monocots and be applicable to wide range of crops for providing drought and salt tolerance, the two major abiotic stress factors. Overexpression of *SHN* in Arabidopsis increases drought resistance and WUE, probably due to decreased transpiration related to lower stomatal density and accompanying increase in epicuticular wax. However in contrast to Arabidopsis, overexpression of the *SHN* gene in rice does not give a visual increase in leaf epicuticular wax, but still gave increased drought resistance and WUE to the plants by exhibiting reduced transpiration water loss (Karaba et al., 2007). This observation in the rice drought experiment demonstrates that the altered epidermal properties are more important in the drought resistance phenotype, which seems independent of the leaf epicuticular wax. Therefore epicuticular wax and the leaf phenotype does not need to be modified in order to provide drought resistance in crop plants. However, the *SHN* gene may provide a change in wax components (intra- or epi-cuticular), which might affect the wax complex conferred by the predominant aldehyde component (Haas, 2001). This alternative explanation is being tested further (Jetter and Pereira, pers. commun.)

The SHN clade provides an additional gene and mechanism for improving drought resistance and can be used along with other genes having complementary resistance mechanism. Overexpression of *SHN* in Arabidopsis also gives enhanced salt tolerance to the plants, by maintaining higher K^+/Na^+ ratio and probably activates the NaCl-stress signal transduction pathway where several calcium binding proteins and specific kinases are induced which are known to render salt tolerance to the plant. It has been suggested that claims for salt tolerance in crops should be at least based on quantitative estimates of growth of advanced progenies of transgenic lines (Flowers, 2004). Many efforts have been made in developing transgenic rice lines

tolerant to salt stress, but few have shown strong quantitative evidence of salt tolerance in the crop. Overexpression of genes encoding enzymes responsible for the accumulation of some compatible solutes such as proline and trehalose enhanced salt tolerance in rice (Zhu et al., 1998; Garg et al., 2002; Jang et al., 2003). These compounds serve as osmoprotectants and in some cases, stabilize biomolecules under stress conditions (Yancey et al., 1982). Improved tolerance of rice to salt stress has also been demonstrated by introducing specific genes encoding late embryogenesis abundant (LEA) proteins from barley (Xu et al., 1996; Rohila et al., 2002). These proteins were thought to improve the performance of rice plants by protecting cell membranes from injury under abiotic stress.

Salt and drought tolerance are complex traits that involve multiple physiological, biochemical and environmental factors and regulation of numerous genes. Engineering a single pathway or mechanism may not be adequate to confer drought or salt tolerance in the field. Overexpression of genes encoding regulatory proteins that control the expression of many stress-resistance genes involved in different pathways or mechanisms might be more promising to get plants tolerant to these abiotic stresses in field conditions. Overexpression of a rice gene encoding a calcium-dependent protein kinase (OsCDPK) conferred drought and salt tolerance on rice plants (Saijo et al., 2000). Only under stress conditions, overproduction of this protein could trigger the downstream signaling and induce the expression of some stress-responsive genes. Overexpression of Arabidopsis genes encoding DREB1A/CBF3 and ABF3 proteins enhanced drought and salt tolerance in transgenic rice (Oh et al., 2005). These proteins represent two of the characterized transcription factors that are related to the ABA-independent and ABA-dependent pathways, respectively. This can be seen as overexpression of *DREB1A*/*CBF3* and *ABF3* activates different groups of stress-regulated genes. Microarray analysis of the *SHN* overexpressor genotypes in Arabidopsis also reveals expression of a different class of genes than from that of *DREB1A* (Trijatmiko, 2005), suggesting the potential to activate complementary stress resistance pathways. Overexpression of Arabidopsis genes, 35S:*CBF3* and the 35S:*ABF3*, in transgenic Arabidopsis resulted in various levels of growth inhibition under normal conditions (Kasuga et al., 1999; Kang et al., 2002), presumably because of the constitutive high-level expression of stress-inducible genes. However, rice plants overexpressing *OsCDPK*, *DREB1A*/*CBF3* and *ABF3* do not

exhibit growth inhibition (Saijo, 2000; Oh et al., 2005). In our study, rice plants overexpressing *SHN* also showed normal growth, supporting the idea that rice is evolutionarily more tolerant to the expression of stress regulated genes than dicots like Arabidopsis (Oh et al., 2005).

Realizing the importance of losses through drought and salt, the two major abiotic stresses in crop plants, the need for developing varieties tolerant to such abiotic stresses will grow. Transcription factors like *SHN*, that gives enhanced drought and salt tolerance to the plant, can be useful to increase expression of a set if downstream genes that alter cuticular properties and result in reduced transpiration without compromising the biomass of the plant. The mechanism through which it renders drought and salt tolerance might be through osmotic adjustment and cell membrane stability, protection of important macromolecules from degradation, and maintenance of redox homeostasis and detoxification. The functional categories of genes up-regulated in the *SHN* overexpressing plants may provide supporting evidence for the actions of such mechanism in drought and salt tolerance of the transgenic plants. Engineering approaches using TF's and enzymatic function genes involved in wax biosynthesis, which not only increases the wax production in the overexpressors but also leads to several epidermal changes, can prove to be promising in crop improvement for stress tolerance.

Chapter 4

BOUNTIFUL, an Arabidopsis AT-hook DNA-binding protein, confers a robust plant phenotype, disease resistance and salt tolerance

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Abstract

We describe here the functional analysis of the Arabidopsis *BOUNTIFUL (BFL)* gene, an AT-hook DNA binding transcription co-factor, identified via a gain-of-function Arabidopsis mutant *bfl-D. bfl-D* shows an erect robust growth habit, increased lateral roots and more rosette leaves and siliques. Analysis of the leaf structure shows an increase in the size of cells that is also exhibited as longer lateral roots. *BFL* is normally expressed in the root and ectopically in all tissues in the mutant, thus displaying a hypermorphic phenotype throughout the plant. *bfl-D* also exhibits resistance to the fungal pathogen *Verticillium dahliae*. In addition, overexpression of *BFL* significantly improves salt tolerance in Arabidopsis and leads to higher biomass production compared to wild-type, both under non-stress and stress conditions, accompanied by an enhancement in the expression of abiotic and biotic stress associated genes in Arabidopsis. The results suggest that overexpression of *BFL* in crops could be useful for improvement of biomass, disease resistance and salt tolerance.

Introduction

The beginning of the 21st century has seen an increased interest in biomass for use in the large-scale production of biofuels. Biomass is a renewable, carbon-neutral energy that in combination with more efficient use of energy and increased energy conservation will allow us to reduce our dependency on the diminishing reserves of fossil fuel and to diminish the environmental effects e.g. on global warming. High-biomass yielding crops will be an important component of sustainable, biomass-based energy systems. Developing these crops requires a shift from breeding for seed yield to breeding of crops for increased biomass or vegetative yield. Moreover, for breeding programs to be most effective they should be designed to assure that varieties are high-yielding in both present and future environments. This means many ecological and genetic factors that control plant biomass production should be taken into account in crop improvement programmes. Ecological factors can be imposed by many biotic and abiotic challenges like pathogen and pest infestation, drought, salinity, cold and heat stress, whereas genetic factors can intrinsically affect many processes in plants. Hence, an increase in biomass in plants depends on a multitude of environmental factors (sunlight, the availability of water and nutrients, pests etc) and on the plant's capacity to use its biochemical processes and its own internal 'energy reserves' in an optimal manner to bridge periods of environmental stress. The current need to produce bioenergy crops with increased biomass is all the more compulsive, since it is likely to compete with agricultural land use, predominantly used for food/feed crops.

In order to meet the demand for food from the growing world population there will have to be significant increases in yield of the major crops. Increasing the maximum yield potential is viewed as an important, if not vital, part of any strategy for achieving this increase in yield (Khush and Peng, 1996). Since the harvest index for many field crops, such as rice, is approaching a ceiling value, an increase in yield potential will have to involve an increase in crop biomass, i.e. there will have to be more net photosynthesis (Cassman, 1994; Ying et al., 1998; Mann, 1999a, b). This may be achieved by an increase in leaf area index (*LAI*) or an increase in net photosynthesis per unit leaf area. Since *LAI* is generally already high in most crops, the increased assimilate production must come from improved photosynthesis.

To search for genes involved in plant biomass production, we screened an activation tag library in Arabidopsis for mutants showing increased leaf biomass. We identified a gain-of-function mutant called *bountiful* (*bfl-D*), which shows a robust plant phenotype caused by ectopic overexpression of an AT-hook protein domain containing gene named *BOUNTIFUL (BFL)*. Here the *bfl-D* mutant phenotype is studied in relation to crop trait improvement.

The *BFL* gene belongs to a multigene family of single AT-hook domain containing proteins. Unlike other well-characterized DNA-binding motifs, the AT-hook is a small motif which has a typical sequence pattern centered on a glycine-arginine-proline (GRP) tri-peptide that is sufficient to bind DNA (Aravind and Landsman, 1998). Typical AT-hook proteins, like the non-histone chromosomal HMG-I(Y) proteins, contain 1-15 AT-hook motifs, play important roles in chromatin structure and act as transcription factor co-factors. Proteins with AT-hook domains often also have several transcription factor domains (Aravind and Landsman, 1998), suggesting that the AT-hook motifs may serve as accessory DNA-binding domains for transcription factors, presumably to anchor them to particular DNA structures (e.g. AT-rich DNA). Apart from this biochemical function, a phenotypic function of AT-hook domain genes in plants was described for a *BFL* related family member *ESCAROLA (ESC)* which was identified from a T-DNA activation tag population (Weigel et al., 2000) with a leaf phenotype similar to *BFL*. More recently, an AT-hook motif-containing transcription factor CaATL1 from chili pepper, was shown to exhibit pathogen resistance when overexpressed in tomato (Kim et al., 2007). The *BFL* gene and many family members are expressed in roots and probably have redundant functions, thus probably remaining undiscovered by classical mutagenesis methods.

Materials and Methods

Pant material and stress tolerance analyses

The Arabidopsis plant genotypes used are in ecotype Wassilewskija (Ws-3) and were grown in the greenhouse at around 22°C. For growth measurements, fresh weight (FW) of plants was determined immediately after harvesting, and samples were oven-dried at 65°C for one week to obtain dry weight (DW) values. The *bfl-D* mutant was tested for abiotic and biotic stress tolerance. To evaluate the biotic stress phenotype of the mutant, resistance to *Verticillium dahliae*

was tested as an example of a non host specific resistance. For this seedlings were grown in the growth chamber at 22-24 ^{0}C with 12 hour light, in 10 cm diameter pots containing a mixture of compost:sand:perlite (2:1:1). At two weeks after germination the seedlings were gently uprooted and dipped briefly in a saturated conidial suspension of Verticillium culture, and then placed back into the pots with soil covering the roots. The plants were watered a few days after treatment and subsequently at regular intervals and examined at various stages for disease symptoms. The plants were scored in 5-pot replicates per genotype and the disease scores were taken as the frequency of plants that survived to maturity. The wild-type (WT) ecotype Ws showed no survival in this assay, while potential resistant candidate genotypes showed survival ranging from 20-100%.

For salt tolerance assays, plants were grown in potting soil (Hortimea, Elst, The Netherlands). Seeds were sown (after three days at 4°C) at density of 1 plant per 2.5-cm pot in a tray. Nutrients (Hydroagri, Rotterdam, The Netherlands; 2.6 EC) were supplied 2 weeks after germination, and one week later the plants were subjected to three applications of 300 mM/L NaCl solution applied at a three day interval. The plants were subsequently monitored for chlorosis for the next 2 weeks. Photographs were taken and survival rates were determined on the 21st day after the start of the NaCl application of (Cheong et al., 2003). The experiment was repeated three times.

Determination of Na^{+} Ca^{2+} and K^{+} ion content

The harvested seedlings of treated and control plants were rinsed with deionized water and dried at 65°C for one week. About 15 to 50 mg of dry material was treated with 1 ml of the digestion mixture (sulphuric acid - salicylic acid and selenium) and 2 carborundum beads, and swirled carefully until all the plant material was moistened and kept for digestion overnight. The temperature was increased gradually in small steps to about 330 °C and later after cooling 0.1 ml of hydrogen peroxide was added and then heated again. This step was repeated 3 times until the digest had turned colorless. On cooling down to room temperature 5 ml of demi-water was added to make up to the mark and left overnight. The Na^{+} Ca^{2+} and K^{+} Ion Content were determined by using an Atomic Emission Spectrophotometer (Elex, Eppendorf, Hamburg, Germany).

Sequence alignment

Multiple sequence alignments and phylogenetic analysis were performed using CLUSTAL-X 1.81 with default settings (Thompson et al., 1997). We used the neighbor joining method for calculating the phylogenetic tree and 1000 bootstrap replicates (recommended by the program). The GENEDOC (Nicholas, 1997) and Tree View (Page, 1996) programs were used for editing the alignment and drawing the phylogenetic tree, respectively.

DNA analysis and plant transformation

Plant DNA was isolated and 10 ng of genomic DNA was used for TAIL PCR (Marsch-Martinez et al., 2002) and sequencing of the flanking DNA to reveal the insert position in the Arabidopsis genome using BlastN (Altschul et al., 1990). Fragments encompassing the full length coding regions of At3g04570 were amplified (using *pfu* DNA polymerase) from Arabidopsis ecotype Columbia genomic DNA to generate the overexpression and promoter constructs. For the recapitulation construct with 35S::BFL cDNA, a BFL cDNA fragment was amplified using the oligonucleotides BFL-F (5'-GG-TCTAGA-GGGCTTTCATGGCGAATC -3') and BFL-R (5'-CAGAGCTC-GCACGTTAAAATCCTGACCTA-3') that introduced *Xba*I and *Sst*I restriction sites at the 5' and 3' respectively, which were used to ligate the 960-bp coding region fragment to compatible sites in the pNEW1 binary vector (Marsch Martinez and Pereira, unpublished), in between a CaMV35S promoter (Pietrzak et al., 1986) and the nopaline synthase terminator (tNOS). For the recapitulation construct with 35S enhancer tetramer: 1828 bp upstream of the ATG was taken (primers *Xba*I- 5'-ATTCTAGA-CCGAAACTGGATTTGTGTG-3' and *Sst*I - 5'-CAGAGCTC-GCACGTTAAAATCCTGACCTA-3'), to give a total of 2781 bp gene sequence. Constructs were made in pBI121 with multiple fragment ligation of: 1) tetramer of 35S enhancer *Hin*d-*Xba*I; 2) DNA binding protein promoter-cDNA sequence *Xba*I-*Sst*I. Orientation of the 35S enhancer tetramer is opposite with respect to the BFL promoter than in the original mutant.

For the BFL promoter-GUS fusion construct 1837 bp upstream of the ATG was taken (*Hin*dIII- 5'-ATAAGCTT-AAGACGGAGCCGAAACTGGA-3' and 5'-ATTCGCCATGGAAGCCCTAGA-3' with a nucleotide exchange to create a *Nco*I site -1846 bps)

and fused with GUS-nos terminator *Nco*I-*Eco*RI in pBinPlus (9,469 of the BAC clone F7018-GenBank ATAC011437).

In all cases fragments were A-tailed and introduced to the pGEM-T Easy vector as described by the manufacturer (Promega) and subsequently sequenced from both sides before digestion and ligation to the binary vector. PCR, restriction digests, plasmid DNA isolation and gel electrophoresis were performed using standard protocols. The constructs were introduced into the plants using the floral dip transformation method (Clough and Bent, 1998). The seeds were plated on one-half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) containing 15 g/L sucrose. Seedlings were selected on 50 mg/L kanamycin and subsequently transferred to the greenhouse.

Microarray analysis

Gene expression of the *bfl-D* mutant overexpressing the *BFL* gene and of the Ws-3 wild type in plant growing under well-watered conditions was compared using microarray analysis. Leaf material was harvested at vegetative stage just before bolting, with two biological replicates for each experimental treatment for hybridization. Total RNA was isolated using TRIZOL reagent (Life Technologies, Inc.) and purified using RNeasy Minielute Kit (Qiagen, Carlsbad, CA, USA). The Affymetrix Arabidopsis ATH1 GeneChips were used for microarray analysis carried out at the Virginia Bioinformatics Institute Core laboratory facilities using standard protocols recommended by Affymetrix.

The RNA was tested for quality control using an Agilent 2100 Bioanalyzer to quantify the RNA and estimate the quality seen as sharpness of the major ribosomal bands. For labeling of target for hybridization, the one-cycle eukaryotic target labeling protocol was used as described in the Affymetrix manual. Total RNA (2μg) is first reverse transcribed using a T7-Oligo (dT) Promoter Primer in the first-strand cDNA synthesis reaction. Following RNase H-mediated second-strand cDNA synthesis, the double-stranded cDNA is purified and serves as a template in the subsequent in vitro transcription (IVT) reaction. The IVT reaction is carried out in the presence of T7 RNA Polymerase and a biotinylated nucleotide analog/ribonucleotide mix for complementary RNA (cRNA) amplification and biotin labeling. The biotinylated cRNA targets

are then cleaned up, fragmented, and hybridized to GeneChip expression arrays. The Fluidics Station 400 is used to wash and stain the probe arrays, which is operated using the GCOS/Microarray Suite. After the wash protocols are complete, the probe arrays are scanned using the scanner also controlled by Affymetrix® Microarray Suite or GCOS.

For analysis of the expression data obtained from the scanned probe arrays, the arrays were normalized using RMA and a moderated t-test (LIMMA) was used to identify differentially expressed genes. The *p*-values from the test were converted to *q*-values (FDR) to correct for multiple hypothesis testing (Storey and Tibshirani, 2003) using the Q-Value software (http://faculty.washington.edu/jstorey/qvalue/). Genes with $q<0.01$ were declared as differentially expressed and used for further analysis, CLENCH (Shah and Fedoroff, 2004) was used for the enrichment analysis of GO terms in the set of differentially expressed genes in the *bfl-D* and the drought experiment.

Histological analysis

Impressions of the leaf epidermis of Arabidopsis leaves were obtained by using a commercial nail polish (HEMA, The Netherlands). The nail polish was applied on the adaxial surface of leaves, dried for about 10-15 minutes and the layer of imprint was removed and observed under a light microscope at 40x magnification. Mature 3^{rd}, 4^{th}, 5^{th} and 6^{th} leaves of *bfl-D* and WT were used. Observations were conducted either under the binocular (WILD M3Z of Heerbrugg Switzerland, type-S), or with a light microscope (Zeiss). An RS Photometric CoolSNAP camera (MediaCybernetics®) was used for taking digital images, processed with the corresponding CoolSNAP software.

Results

Identification of *bountiful* (*BFL*) gene in Arabidopsis

In a phenotypic screen of an Arabidopsis transposon-based activation tag library (Marsch-Martinez et al., 2002), a robust mutant plant was identified that had an erect vigorous growth habit, with leaves of increased size (Fig.1B) and profuse number of siliques, and was hence named *bountiful (bfl*) . Molecular characterization of the mutant revealed that the activation tag

insert was at position 1,231,122 on chromosome 3 (according to TAIR sequence), located between two outwardly transcribing open reading frames. Adjacent to the transposon tag right border was located a gene At3g04560 encoding an expressed protein with unknown function, whose translation starts 1778 bp from the transposon insert. On the other side and 105 bp away from the left border, was located the translation start of At3g04570, encoding a putative AT-hook DNA binding protein. The insertion tag is in the 5'untranslated region of At3g04570. RT-PCR experiments revealed enhanced ectopic expression of At3g04570 in the mutant in comparison to WT leaves in which the transcript could not be detected (data not shown), suggesting that altered expression of At3g04570 most likely caused the *bountiful* phenotype and the gene was therefore named *BOUNTIFUL* (*BFL*).

Overexpression constructs were made with the predicted coding region of the At3g04570 gene under control of the CaMV35S promoter (35S-*BFL*) and of the *BFL* gene including its endogenous promoter flanked upstream by the 35S enhancer tetramer (4Enh-*BFL*). For the 4Enh-*BFL* construct, a genomic region of about 1,830 bp upstream of the ATG was arbitrarily chosen as *BFL* regulatory sequence upstream of the *BFL* coding sequence. The 35S-*BFL* primary transformants (n=5) displayed a phenotype that was reminiscent of *bfl-D* but was overall more severe than that of the original activation tag line. However, primary transformants obtained with the 4Enh-*BFL* (n=12) resembled the activation tag *bfl* mutant with various degrees of severity (data not shown). Northern and RT-PCR analysis on rosette leaves of 35S-*BFL* T_1 progeny plants and 4Enh-*BFL* primary transformants revealed a correlation between the severity of the phenotype and the amount of *BFL* transcript, with the 35S-*BFL* transgenic plants displaying the highest expression levels (data not shown).

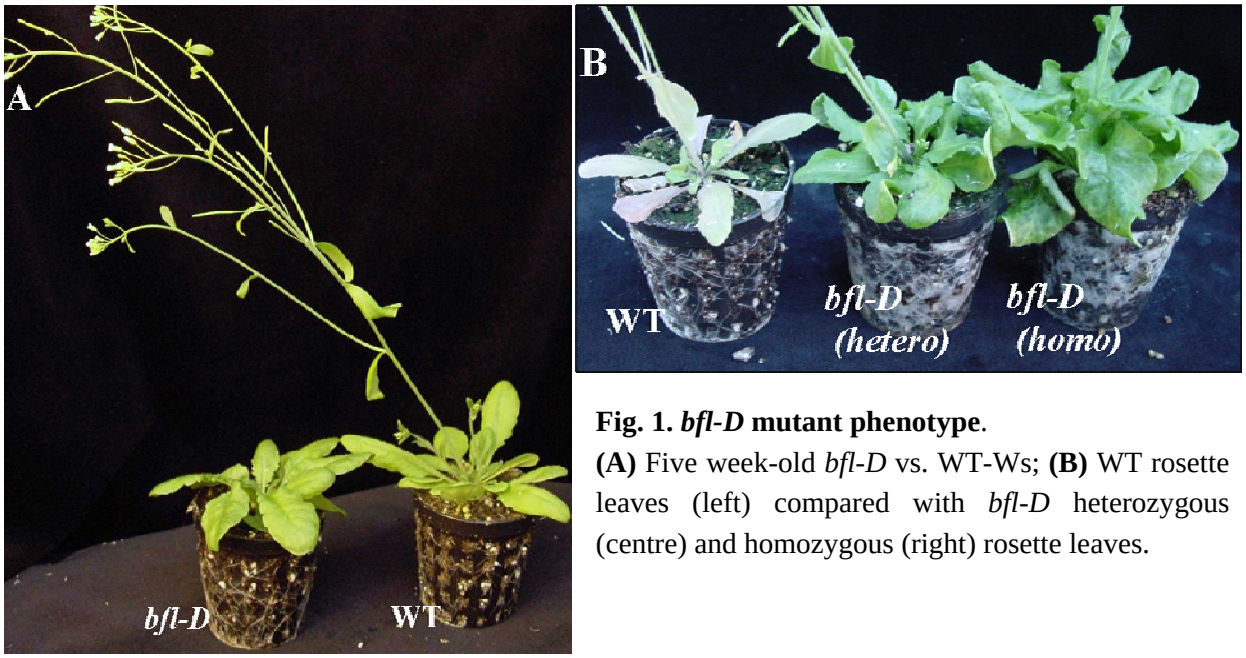

Fig. 1. *bfl-D* mutant phenotype.
(A) Five week-old *bfl-D* vs. WT-Ws; **(B)** WT rosette leaves (left) compared with *bfl-D* heterozygous (centre) and homozygous (right) rosette leaves.

Phenotype of *BFL* overexpression in Arabidopsis

Segregation analysis of progeny plants indicated *bfl* as a semi-dominant mutant and the allele was named *bfl-D*. Homozygote *bfl-D* plants were characterized by more rosette leaves that were larger in size and more curly and wrinkled than wild type. Moreover, they flowered later than WT and the *bfl-D* heterozygote plants, which resulted in the formation of extra leaves (Fig. 1B). Mature homozygote plants had increased branching but reduced overall height, and displayed partial sterility.

Growth stage measurements like onset of bud formation, flower formation and silique formation in terms of number of days after germination (DAG) were taken on a set of WT (n>15) plants in comparison with homozygote and heterozygote *bfl-D* plants (Table 1). The life cycle of *bfl-D* mutants was prolonged as shown by the longer time it took to flower (Fig. 2D & Table 1) and seed set (silique formation) with a more significant difference in case of homozygote plants (Table 1). However, the number of rosette leaves at 14 DAG both in WT and *bfl-D* heterozygote plants still in the vegetative stage (no flower bud formation) was the same (Table 1) unlike in the *bfl-D* homozygote which showed less number of rosette leaves compared to the WT. Here the emergence of the rosette leaf is defined according to Boyes et al. (2001). Additionally, the size of

the *bfl-D* heterozygote leaves visually appeared to be slightly larger than the WT at this stage, whereas the *bfl-D* homozygote showed smaller size compared to the WT (data not shown). The number of rosette leaves measured at 21 DAG in *bfl-D* heterozygote plants revealed a similar number as the WT (Table 1), but were much larger in size (data not shown), although at this stage the WT had just entered the reproductive stage (flower bud formation). This result indicates that although *bfl-D* heterozygote is a late flowering mutant, it does not show lateness in the growth of rosette formation and even shows larger rosette leaf size compared to the WT at the vegetative stage (Table 1). The prolonged vegetative stage of the *bfl-D* heterozygote enables it to further expand its leaf size contributing more towards biomass production (Fig.1A). The results also indicate that the rate of the cell cycle in *bfl-D* does not slow down but is extended with the prolonged vegetative stage.

Late flowering resulted in a higher average number of rosette leaves at the time of emergence of the first bud (significantly in *bfl-D* homozygote plants), as well as in more primary lateral shoots (PLS =shoots that develop on the main meristem of the plant) (significantly in both homozygotes and heterozygotes) (Fig.2 A, B and Appendix, Table 1). However no significant difference was observed in the number of secondary lateral shoot (SLS) formation among the *bfl-D* and WT (Fig. 2C). In agreement with visual observations, heterozygote *bfl-D* plants had about 30% more siliques at maturity than WT plants (Fig. 2E), although the average seed weight did not differ significantly (Fig. 2F). The total number of seeds per silique was counted and showed that *bfl-D* heterozygotes had on average about 36 seeds per silique compared to 47 seeds in the WT plants (Fig. 2H). However, there was no difference observed in the size of the seeds of *bfl-D* and it's WT. This suggests that the decrease in the total number of seeds per silique in *bfl-D* heterozygote plants was compensated by the increase in its number of siliques, under the limited growth conditions of a small Aracon pot. On the other hand, homozygote *bfl-D* plants produced slightly less siliques, having seeds with significantly lower weight than WT seeds (data not shown). The higher number of siliques that was observed is due to formation of more PLS. The *bfl-D* heterozygote, homozygote and 4Enh-*BFL* plants also had a higher percentage of dry weight compared to the WT (Fig. 2G).

Table 1. Growth stage development of *bfl-D* vs. WT

Plant type	No. of Rosette leaves		Reproductive stage						End of life cycle
	14 DAG	21 DAG	Flower bud formation (DAG)		Flower formation (DAG)		Silique formation (DAG)		(DAG)
			>50%	100%	>50%	100%	>50%	100%	
WT	5-6	7-8	-	23	-	28	-	32	68
***bfl-D* (hetero)**	5-6	8-10	26	34 *	34	47	47	68*	78*
***bfl-D* (homo)**	3-5	5-6*	38	43 *	47	68 *	63	73*	83*

* indicates values are significant at (α<0.01) compared to the WT.

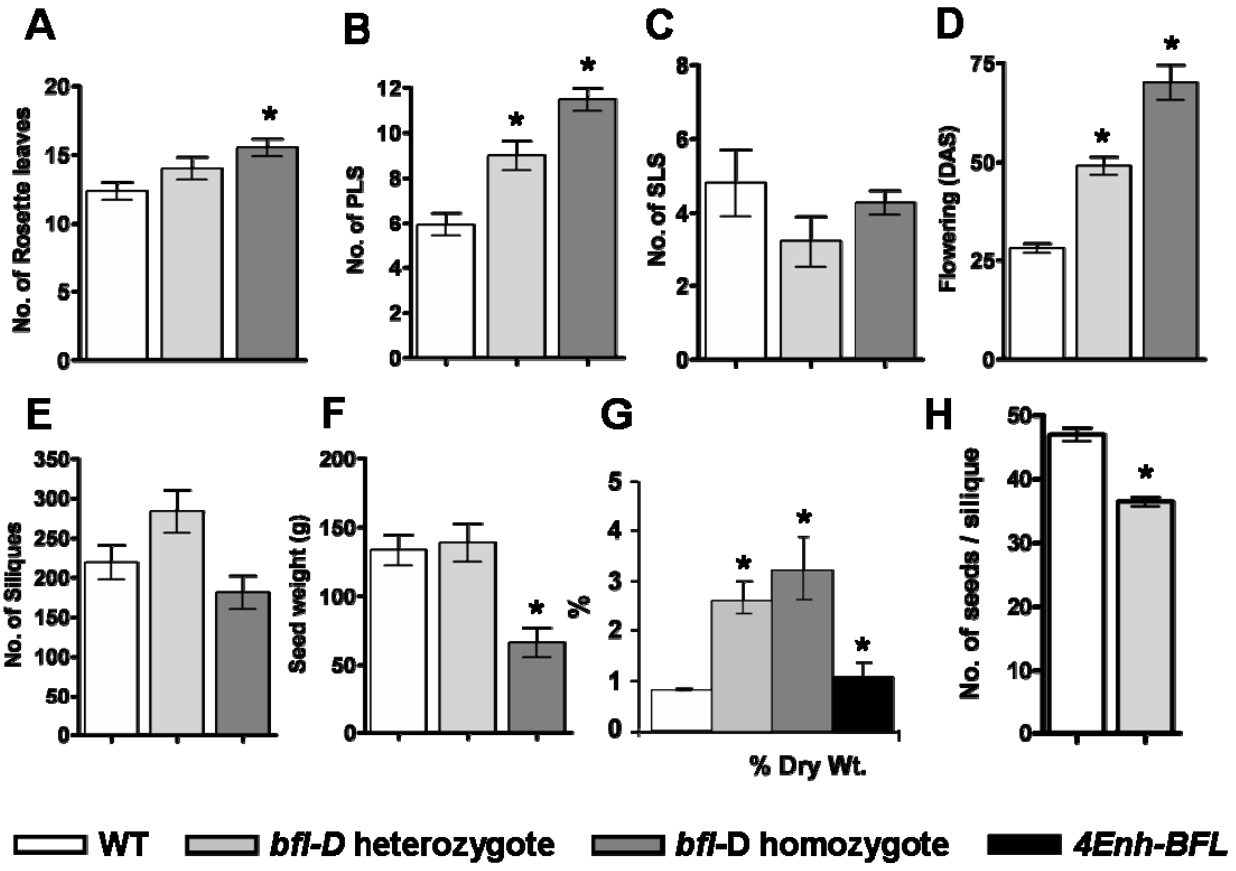

Fig. 2. Developmental differences of the *bfl-D* compared to the WT and the heterozygote. (A) Total number of rosette leaves in *bfl-D* compared to the WT at maturity**. (B)** Number of primary lateral shoots (PLS) **(C)** Number of secondary lateral shoots (SLS). **(D)** Number of days after sowing (DAS) at which 100% of the flowers were opened. **(E)** Number of siliques. **(F)** Seed weight (g). **(G)** Percent dry weight of

bfl-D heterozygote, homozygote and 4Enhancer-*BFL*promotor-*BFL* transformant line. **(H)** Number of seeds per silique. Bars represent standard error of the mean. * indicates all values are significant at ($\alpha<0.01$) compared to the WT. ** indicates values are significant at ($\alpha<0.05$) compared to the WT

bfl-D shows increase in cell size in leaves

bfl-D has larger leaves compared to the WT. As the size of the leaf depends both on cell size and cell number, both parameters were analyzed in *bfl-D* leaves. First, to determine the size of the cells, imprints of the epidermis of fully grown (when rosette growth is complete) 3rd, 4th, 5th and 6th rosette leaves were made and observed under a light microscope. It appeared that *bfl-D* cells are larger than WT leaf cells (Fig 3 and Table 2). However the total number of cells was the same for WT and mutant (Table 2). Next, the number of cells per leaf was determined in WT and *bfl-D* plants. The *bfl-D* leaf area was almost 1.5 times larger than the WT leaf area (Table 2).

Table 2. Cell density, size and number in mature leaves.

Plant line (Arabidopsis)	Pavement cell density (cells/mm2 ± SE)	Average cell size (µm2 ± SE)	Cell number Per leaf (±SE)	Average total leaf area (cm^2 ± SE)
WT	320 ± 37	3160 ± 214	117356 ± 13587	3.67 ± 1.14
bfl-D	221 ± 26*	4574 ± 335*	124349 ± 14834	5.58 ± 0.88*
Ratio *bfl-D*:Ws	0.692	1.45	1.05	1.52

* indicates that values are significant at ($\alpha<0.01$) compared to the WT.

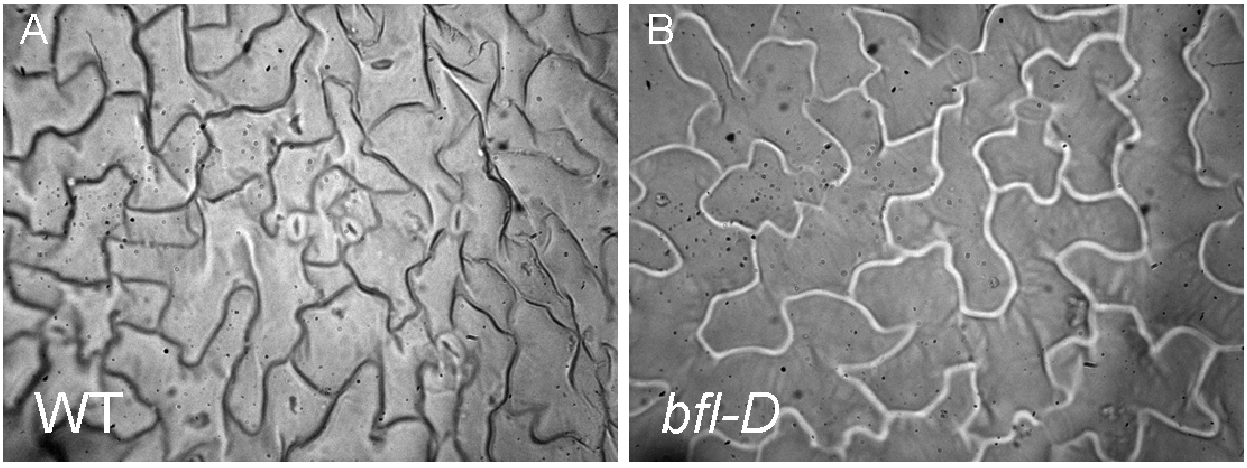

Fig. 3. Pavement cell density of mature Arabidopsis leaves in WT and *bfl-D*.
(A) WT leaf epidermal cells. **(B)** *bfl-D* leaf epidermal cells.

bfl-D displays enhanced root development

Visual observation of *bfl-D* plants grown in soil indicated that they had a more developed root system than WT plants. To gain further insight into the changes that occurred in this mutant, homozygote and heterozygote *bfl-D* genotypes, overexpression lines and WT seedlings were grown *in vitro* in vertically oriented plates and root growth monitored at regular intervals. In agreement with previous observations, *bfl-D* seedlings displayed shorter roots compared to WT, and a more developed lateral roots system (Fig. 4 and Appendix I - Table 2). In addition, the length of the primary lateral roots in mutant and overexpression seedlings appeared to be increased compared to WT seedlings. This increase in the total root length could be a result of cell expansion in the root cells as seen in the leaves.

Fig. 4. Root length (RL) and number of primary lateral roots (PLR) in *bfl-D* and WT plants grown *in vitro*.

bfl-D displays enhanced pathogen resistance

We tested the *bfl-D* mutant for resistance to the non race-specific pathogen *Verticillium dahliae*. In replicated infection experiments with Verticillium, *bfl-D* mutants showed a high level of resistance displaying none of the wilting symptoms visible in wild-type shown at maturity (Fig. 5). To confirm the effect of the *BFL* gene, the resistance tests were repeated in the overexpression lines. No wilting symptoms or evidence of an effect of the pathogen was visible at maturity suggesting a resistance phenotype. In screens for drought resistance the mutant lines did not show significant resistance, suggesting that the Verticillium resistance observed was not due to a general resistance to wilting.

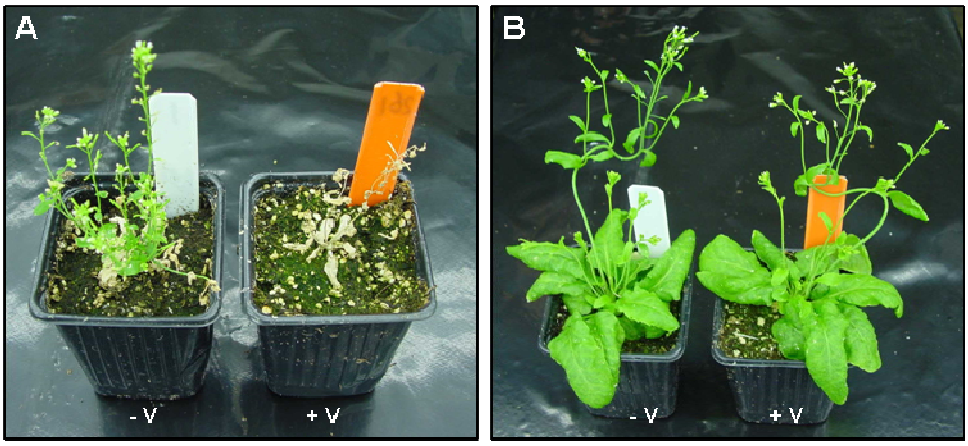

Fig. 5. The effect of inoculation with Verticillium on *bfl-D* and WT plants. Two weeks old *bfl-D* and WT plants were exposed to *Verticillium dahlia* pathogen (+V) and monitored until maturity for wilting and survival symptoms. **(A)** Effect of Verticillium infection on WT-Ws. **(B)** Effect of Verticillium infection on *bfl-D*.

***bfl-D* displays enhanced salt tolerance**

In a salt tolerance assay the WT and two *bfl-D* heterozygote lines (BFL-A & BFL-B) were treated with 250 and 300 mM NaCl and the plants were monitored for chlorosis and survival (Fig. 6A). The percentage tolerance was calculated on the basis of the undamaged non-chlorotic plants, which is higher at both NaCl concentrations for the *bfl-D* plants (Table 3). The percentage reduction in the shoot/leaf dry matter accumulation of both *bfl-D* lines under 250-300 mM salt stress ranged from 38-50% compared to WT that showed 59-67% reduction in growth. Chemical compositional analysis revealed that *bfl-D* accumulates lower amounts of Na^+ compared to the WT under salt stress (Fig. 6C). The potassium (K^+) levels decreased under stress conditions in WT whereas in *bfl-D* lines they are similar in both conditions (Fig. 6E). Ca^{2+} levels however are higher in both *bfl-D* lines (BFL-A and BFL-B), both under stressed and non-stressed conditions compared to WT (Fig.6D)

Table 3: Tolerance of *bfl-D* to NaCl stress

Genotype	NaCl treatment	Total plants	Non chlorotic	% Tolerant
WT	300mM	20	0	00
BFL-A	300mM	20	16	80 *
BFL-B	300mM	20	12	60 *
WT	250mM	20	1	5
BFL-A	250mM	20	18	90 *
BFL-B	250mM	20	14	70 *

*All the values comparing BFL-A and BFL-B with the WT were significant at ($\alpha<0.01$) using chi-square test.

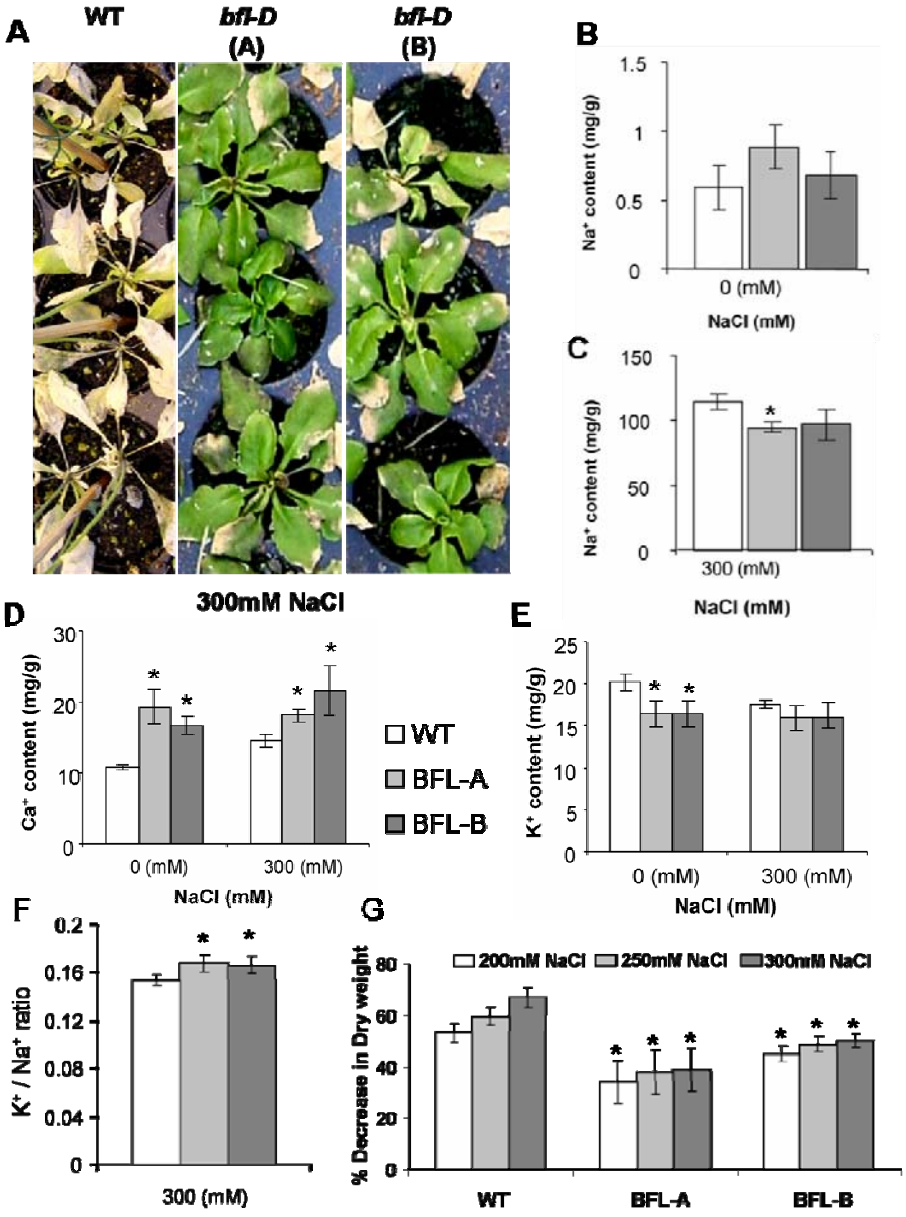

Fig. 6. **Ion accumulation under non-stress and 300mM NaCl stress of *bfl-D* vs. WT.**

(**A**) WT and *bfl-D* plants treated with 300mM NaCl for 21 days. Concentration of ions in the leaves and stems of the plants grown in soil and treated with and without high concentrations (300mM) of NaCl (B-E). **(B)** Na^+ content in non-treated plants**; (C)** Na^+ content under 300mM NaCl treatment**; (D)** Ca^{2+} levels both under treated and non-treated plants. **(E)** K^+ levels both under treated and non-treated plants**. (F)** K^+/Na^+ under 300mM NaCl**; (G)** Percentage decrease in the dry weight accumulation under different salt concentrations; bar in white indicates 200mM NaCl concentration, bar in light gray indicates 250mM NaCl and bar in dark grey indicates 300mM NaCl. (Error bars indicate standard error of mean. * indicates values significant at (α<0.01) compared to the WT.

Effects of *BFL* activation on the global gene expression profile

To investigate the effect of *BFL* overexpression on the expression of other genes that might explain the *BFL* phenotype and its possible function, global gene expression patterns in WT were compared with *bfl-D* homozygote and heterozygote genotypes. Genes with q<0.01 were declared as differentially expressed which revealed 3822 and 1084 genes upregulated in the hetero- and homozygote mutants compared to the wildtype. The higher number of differentially expressed genes and the variety of functional categories (Table 4) expressed in the *bfl-D* heterozygote probably explains the robust phenotype. The differentially expressed genes were compared to publicly available microarray data available in AtGenExpress (http://www.arabidopsis.org/info/expression/ATGenExpress.jsp). To identify significant biological pathways regulated by the *BFL* gene we examined the differentially expressed genes in terms of the gene ontology (GO) functional categories. The significant differentially regulated genes due to *BFL* overexpression are given with corresponding significant GO terms (Table 4). Several genes which were found to be upregulated by *BFL*, are known to be induced in response to salt stress, ethylene and abscisic acid. Others are known to relate to ER golgi vesicle-mediated transport, vacuole organization and biogenesis, chromatin assembly or disassembly, chromosome organization and biogenesis, chloroplast organization and biogenesis, protein transport, leaf morphogenesis and seed germination. This result suggests that *BFL* promotes the expression of categories of genes that are involved in rendering salt tolerance and pathogen resistance to plants, thereby promoting a salt tolerance and pathogen resistance mechanism.

Table 4: Genes (annotated in GO terms) regulated by BFL expression

Up-regulated	GO functional categories	Down-regulated	GO functional categories
GO: 0006412	translation	GO: 0009624	response to nematode
GO: 0042254	ribosome biogenesis and assembly	GO: 0019915	sequestering of lipid
GO: 0015031	protein transport		
GO: 0009651	response to salt stress		
GO: 0009723	response to ethylene stimulus		
GO: 0046686	response to cadmium ion		
GO: 0009738	abscisic acid mediated signaling		
GO: 0009744	response to sucrose stimulus		
GO: 0019303	D-ribose catabolic process		
GO: 0006888	ER to Golgi vesicle-mediated transport		
GO: 0019430	removal of superoxide radicals		
GO: 0006633	fatty acid biosynthetic process		
GO: 0006541	glutamine metabolic process		
GO: 0006086	acetyl-CoA biosynthetic process from pyruvate		
GO: 0008652	amino acid biosynthetic process		
GO: 0006085	acetyl-CoA biosynthetic process		
GO: 0007033	vacuole organization and biogenesis		
GO: 0007001	chromosome organization and biogenesis (sensu Eukaryota)		
GO: 0006333	chromatin assembly or disassembly		
GO: 0006334	nucleosome assembly		
GO: 0009658	chloroplast organization and biogenesis		
GO: 0010020	chloroplast fission		
GO: 0007010	cytoskeleton organization and biogenesis		
GO: 0007017	microtubule-based process		
GO: 0009853	photorespiration		
GO: 0009965	leaf morphogenesis		
GO: 0009845	seed germination		
GO: 0009793	embryonic development ending in seed dormancy		

Discussion

In this study we have identified a gain-of-function mutant named *bountiful (bfl-D)* which displays distinct plant architecture and robust plant phenotype by producing larger and more rosette leaves, lateral roots and siliques. This provides a model to analyze the gene-phenotype

relationship underlying the expression of a robust plant phenotype. Molecular analysis showed that the mutant allele was caused by overexpression of the *BFL* gene (At3g04570), encoding an AT-hook DNA binding protein, due to the proximity of the 35S enhancer tetramer on the endogenous *BFL* promoter. Confirmatory evidence came from the expression of the 4Enh-*BFL* construct in transgenic plants that could mimic the *BFL* expression level and the phenotype in the activation tag line. Overexpression with the 35S promoter led to very high levels of *BFL* expression that gave a very severe *bfl-D* mutant phenotype. This supports the hypothesis that the *bfl-D* mutant is a hypermorphic allele, the phenotype due to a moderate level of ectopic expression of *BFL*.

In the Arabidopsis genome, approximately 30 BFL paralogs were identified based on amino acid similarity. Fourteen paralogs showed very high similarity to BFL (Appendix II-B). BFL proteins also have a number of homologs in rice. BFL consists of two characteristic domains, an AT-hook motif and a domain of unknown function, DUF296. The AT-hook motif is a short DNA binding protein motif, first described in the high mobility group non-histone chromosomal proteins named HMG-I/Y (Reeves and Nissen, 1990). The AT-hook motif is known to interact with the minor groove of AT-rich sequences (Huth et al., 1997) and is present in nuclear localized proteins. These proteins are known to play an important role in chromatin structure and act as transcription factor cofactors (Onate et al., 1994; Falvo et al., 1995). In Arabidopsis, AHL1 an AT-hook DNA binding protein (Fujimoto et al., 2004) has been proposed to function as a MAR (matrix attachment region) DNA binding protein, localized to the surface of chromosomes during mitosis. A recent study shows the possible function of plant AT-hook motifs in pathogen defense response (Kim et al., 2007). The BFL protein may therefore be involved in determining functional chromatin structure affecting adjacent gene expression and defense related responses in plants.

The endogenous expression pattern of BFL seems to be restricted to the roots, as revealed by RT-PCR and Northern analysis performed on different WT tissues (data not shown). A screen of available microarray data in TAIR (http://www.arabidopsis.org/) shows a number of the AT-hook containing genes including *ESC* having expression in the roots. In agreement with these observations, MPSS-derived expression pattern for 20bp signatures in the *BFL* gene (retrieved from the MPSS database at http://mpss.udel.edu/at/) revealed that expression of this gene is

mainly restricted to roots; in addition it is expressed in actively growing calli (a few in germinated seedlings). Further, *BFL* promotor-*GUS* fusion analysis in six transformants showed activity of the *BFL* promoter early in the emerging radicle, already at one day after germination (data not shown). These results suggest a number of similar AT-hook domain genes expressed in the root, and probably having a partially redundant function. Therefore, no knockout mutants of single genes have revealed mutant phenotypes (Fujimoto et al., 2004). This is probably why we also did not observe a knockout phenotype with two insertion mutant lines analyzed (data not shown). RNAi silencing of multiple genes would probably be necessary to examine a phenotypic effect.

BFL affects cell size through cell expansion

The *bfl-D* mutant exhibits larger rosette leaf area, almost 1.5 times more than the WT and the cell size is also approximately 1.5 times larger than the WT. However, the number of the cells per leaf area remains the same suggesting that the cell division process in *bfl-D* remains unaffected and cell expansion takes place which results in larger rosette leaves and ultimately higher biomass accumulated over a longer vegetative period (late flowering) than the WT. Also the *bfl-D* heterozygote does not show a significant increase in rosette leaves at maturity, unlike many late flowering mutants. The late flowering and higher number of rosette leaves are found with late flowering mutants as well as natural variants for flowering in Arabidopsis (Koornneef et al., 1991; El-Lithy et al., 2004). Our observation however, indicates that *bfl-D* shows larger rosette leaves at the same vegetative stages compared to the WT (14DAG), suggesting that irrespective of the lateness *bfl-D* shows no difference in time of rosette leaf formation and already displays a higher biomass compared to the WT at 14 DAG (Table 1). Quantitative analysis on the leaf size and dry matter accumulation at 14 DAG of *bfl-D* compared to the WT would validate this observation. Additionally the prolonged vegetative stage in *bfl-D* not only helps the plant to produce more rosette leaves but also expand it to even larger leaves through cell expansion.

bfl-D also shows an increase in the number and length of lateral roots, which could be due to enhanced root cell expansion as seen in the leaves. Overall *bfl-D* exhibits a much robust, tall and strong looking plant with thick stems, larger flower buds and flowers, which could also be related to the overall enhanced cell expansion in the plant tissues. Endogenous *BFL* expression is

found mainly in the lateral roots and adjacent root cells, implicating its function might be involved in lateral root formation and adjacent root cell expansion. The root structure appears more branched, with lateral roots more elongated probably through root expansion in the *bfl-D* mutant then the WT (Fig. 4A).

Lateral root formation plays a crucial role in plant development by permitting the growth of branched root systems. The lateral root initiation is known to be regulated by auxin and it also has been reported to promote lateral root initiation by cell cycle stimulation at the G1-to-S transition (Himanen et al., 2002). Auxins are also involved in cell expansion (Mockaitis and Estelle, 2004). Genevestigator expression data analysis (https://www.genevestigator.ethz.ch/; Zimmermann et al., 2004) show that auxins induce the expression of BFL by 1.5-3 times. BFL is thus an auxin responsive gene, which might respond to local concentrations in a specific manner triggering responses such as lateral root formation. The energy in main root elongation might be redistributed towards the elongation of lateral roots. Further auxin-regulated experiments in this mutant can add more to the knowledge of this gene.

***BFL* confers disease resistance**

The robust growth phenotype is also accompanied as resistance to the wilt pathogen *Verticillium dahliae*. This fungal pathogen infects plants through the roots in infected soil and causes wilt symptoms in infected plants. There has been an analysis of genetic variation in Arabidopsis for resistance to Verticillium (Veronese et al., 2003) and the screens for resistance are reproducible, thus supporting its use as a test for pathogen interaction. The *bfl-D* mutant and overexpression lines show significant resistance to Verticillium with no wilting symptoms and very healthy plants in repeated tests. The resistance is probably due to the capacity of the *bfl-D* lines to produce more lateral roots and withstand the fungal growth, providing continual water and nutrients to the plant, compared to wild-type where the roots get infected and unable to support further plant growth. This hypothesis is supported by observation of another mutant line *hrd-D* (Chapter 2) which also displays a higher lateral root growth and Verticillium resistance. Recently the role of an AT-hook motif gene from pepper has been shown in providing resistance against pathogens in tomato (Kim et al., 2007). In addition the analysis of the *bfl-D* microarray shows that

a number of disease related genes get induced in the overexpression mutant, such as the ABA (abscisic acid) and ethylene responsive genes, providing an induced resistance pathway and exhibiting pathogen resistance. The involvement of BFL in disease response pathways is seen in the analysis of BFL expression patterns in response to pathogens (by searching eFP browser available in TAIR; http://www.arabidopsis.org/index.jsp,) where BFL responds by induction to Botrytis and Pseudomonas infection,

***BFL* expression provides salt tolerance**

Molecular genetics studies done with Arabidopsis have shown many stress responses at the molecular level which have been successfully used for genetic dissection of stress response pathways (Zhu, 2002; Shinozaki et al., 2003). Drought and salt signaling pathways, which very often trigger many common reactions in plants, appeared to be very complex. Both stresses lead to cellular dehydration, which causes osmotic stress and removal of water from the cytoplasm into the extracellular space resulting in reduction of the cytosolic and vacuolar volumes. Another effect is the production of reactive oxygen species which in turn affects the cellular structures and metabolism negatively. Early response to water and salt stress are largely identical except for the ionic component. High intracellular concentrations of sodium and chloride ions are an additional problem to salinity stress (Bartels, 2005). Maintaining higher K^+/Na^+ ratio in the cytosol is essential for the normal functioning of the plants. Na^+ competes with K^+ through Na^+-K^+ co-transporters and may also block the K^+ specific transporters of root cells under salinity (Zhu, 2003). This results into Na^+ toxicity and scarcity of K^+ for enzymatic activity and osmotic adjustment in the cell. Overexpression of *BFL* confers salinity tolerance in Arabidopsis by maintaining ion homeostasis in the cell. It is able to maintain higher Ca^{2+} levels both in stress and non-stress conditions and also maintain the K^+ levels under salt stress condition. At the same time it accumulates less Na^+ in the cells unlike the WT which accumulates more amount of Na^+ and both its Ca^{2+} and K^+ levels drops under salt stress (Fig. 6C, D, E). These factors play a crucial role in determining cell death due to Na^+ toxicity in the cell (Chinnusamy et al., 2005). Efflux of Na^+ and uptake of K^+ in the cell, and utilization of Na^+ for osmotic adjustment are strategies used by plants to maintain desirable K^+/Na^+ ratios in the cytosol. Osmotic homeostasis can be achieved

either by compartmentation of Na^+ into the vacuole or by biosynthesis and accumulation of compatible solutes (Zhu, 2002). *BFL* overexpressors were able to maintain higher K^+/Na^+ ratio under salt stress by accumulating less Na^+ in the cell (Fig.6A, F). *BFL* overexpressors also exhibit larger cell size, meaning larger cellular components including the vacuolar space, where it might be able to efflux Na^+ which helps avoiding the salt stress situation in the cell. *BFL* overexpression also provided salt tolerance in terms of plant growth and its ability to bolt and flower at increased salt concentrations (200-300mM NaCl). It shows less percentage decrease in the total dry weight under different salt concentrations compared to the WT (Fig. 6G). Probably the SOS signaling pathway (Shi and Zhu, 2002) is activated in *bfl-D* under salt stress, which helps maintain the homeostasis in plant cell.

A particular gene expression pattern is often associated with the stress tolerant phenotype and it is unknown how this is achieved. This may involve other molecular aspects, like chromatin organization, which have not been well researched (Bartels, 2005). The stress responsive genes are often activated more slowly whereas the “early responsive genes” typically encode transcription factors that activate downstream stress-responsive genes. Therefore BFL might be playing a direct or indirect role in perceiving the stress signal and activating other stress responsive genes upon sensing stress in the plants.

The salt stress tolerance, pathogen resistance and root/leaf structure phenotype signifies a coordinated mechanism of induced resistance conferred by the *BFL* gene, with potential adaptive value to crop plants. Mild ectopic expression of the *BFL* gene provides a very robust plant phenotype with enhanced leaves, siliques and lateral roots. All these traits are very important for crops for increased biomass, yield and adaptive stronger root network. However when *BFL* was strongly overexpressed, it gave a very severe *bfl-D* phenotype which was very small and late flowering. Hence it is important to find the optimal expression levels of *BFL* gene for conferring robust and resistant plant phenotype. Microarray results showed induction of genes in response to salt stress and pathogen resistance (ABA, ethylene), which supports the salt tolerance and pathogen resistance of BFL in plants. Hence BFL expression can benefit the plant in acquiring salt stress tolerance and higher biomass, giving general induced stress resistance to the plant. Its applicability in other plant systems would definitely open new possibilities to improve crops.

Chapter 5

Screening for abiotic stress resistance in Arabidopsis using activation tagging

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Abstract

Crop production faces many challenges from abiotic stress factors such as drought, salinity, cold and high temperature. The changing environment, reducing the availability of freshwater makes drought and salinity an emerging problem and an essential trait for crop improvement. Since drought and salinity are complex traits to breed for, a molecular understanding of the genes and mechanisms would be very useful. To identify genes for drought and salt tolerance we have used an Arabidopsis *En-I* transposon-based activation tagged population to screen for stress resistant mutants. In our strategy, we first selected lines which were compromised in fitness (e.g. low seed set, poor growth) probably due to overexpression of induced stress tolerance pathways. A selected sub-population of 51 lines were screened for drought resistance, where the plants were put under water deficit beyond wilting point and allowed to recover after rehydration. The drought screening revealed eight mutants that were found to be significantly resistant. These mutants were characterized further for their specific phenotypic parameters and the putative tagged genes identified by isolation of the DNA flanking the activation tag insertions. Two of these mutants named *atal* and *vajra* were further studied by identifying adjacent overexpressed genes and overexpressing the candidate tagged genes in Arabidopsis under control of the constitutive CaMV35S promoter. Also, these mutants were characterized for water use efficiency (WUE) and salt tolerance and their mechanism for resistance was investigated. Expressing the candidate genes in Arabidopsis would confirm their role in drought/salt tolerance and render them as candidates for further molecular breeding studies in different cultivated crops.

Introduction

The looming worldwide water crisis requires alternate solutions for agricultural production that at present use most of available water resources to keep up with the needs of population growth. Water shortages cause drought and improper usage also salinity, which together cause dehydration and make up the major abiotic stresses that reduce plant yield. The abiotic stress factors such as drought, excessive water, salinity or cold have been estimated to cause depreciation in crop yield up to 70% when compared to the yield under favorable conditions (Boyer, 1982). Amidst the complexities of environmental stress reactions in crop plants the use of the simple model Arabidopsis, offers an opportunity for the precise genetic analysis of stress reaction pathways common to most plants. The relevance of the Arabidopsis model is evident since the first remarkable examples of improving drought, salt and freezing tolerance (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999) using genes identified in Arabidopsis. These genes are transcription factors (TF) of the AP2/ERF family that regulate the expression of a battery of downstream genes conferring stress resistance in a number of heterologous plants.

The early responses of plants to stress are the sensing and subsequent signal transduction leading to stress-responsive gene expression. In response to osmotic stress elicited by water deficit or high salt the expression of a set of genes is altered (Zhu et al., 1997), some of which are also induced by low temperature stress (Thomashow, 1998). The stress induced genes have been systematically termed *RD* (responsive to dehydration), *ERD* (early responsive to dehydration), *COR* (cold-regulated), *LTI* (low-temperature induced) and *kin* (cold inducible). Environmental stresses can influence abscisic acid (ABA) levels and ABA can also induce many stress responsive genes (Zhu et al., 1997). Detailed genetic analysis has revealed the presence of ABA-dependent and ABA-independent signal transduction pathways in response to different stresses (Shinozaki and Yamaguchi-Shinozaki, 2000). There is a common signal transduction pathway between dehydration and cold stress involving the DRE/CRT cis-acting elements, and two additional signal transduction pathways function solely in dehydration or cold response. These pathways might not necessarily act in a parallel manner but they can interact and converge to activate stress genes (Ishitani et al., 1997). Further elucidation of other genes and pathways is a goal of many studies on abiotic stress tolerance genes.

The sequence of the Arabidopsis genome reveals that two-thirds of the genome is duplicated (The AGI consortium, 2000), with about 15% known genes involved in plant defense. Most signal transduction pathways in plant stress responses involve hundreds of interacting genes with redundant functions that are intractable through classical genetic analysis. A significant limitation of classical loss-of function screens designed to dissect genetic pathways is that they rarely uncover genes that function redundantly. This is because the effects of the mutations are compensated by alternative metabolic or regulatory circuits. Mutants are sometimes also not detected due to lethality because they have an additional role in early embryo or gametophyte development. To overcome these problems to genetically identify genes with functions useful for agriculture, a strategy to overexpress plant genes in a high-throughput way followed by selection for stress tolerant lines, would therefore provide a direct way of identifying genes that can confer stress tolerance through diverse mechanisms.

Activation tagging is a high throughput method to overexpress genes in the plant genome generating gain-of-function dominant phenotypes that can be positively selected for phenotypes of interest. We have developed an efficient transposon-based activation tag library of around 13,000 lines in Arabidopsis that generate a high frequency of gain-of-function mutants (Marsch-Martinez et al., 2002). These 'Activation Tag' (**ATag**) transposon inserts contain a multiple enhancer of the CaMV35S promoter that can act on adjacent plant promoters (~10 kb) and enhance or activate their expression. Results show that about 3 % of the insertions cause an obvious dominant gain-of-function phenotype. The phenotypes for most of these tagged genes were not identified before, suggesting that this transposon-based activation tagging approach promises the disclosure of new gene functions. In contrast the commonly used T-DNA-based activation tagging displays $1/10^{th}$ this frequency of phenotypes (Weigel et al., 2000), probably due to multiple copy based gene silencing.

Our aim is to use this activation tagged population to screen for abiotic stress resistance e.g. drought, salinity and cold. We first selected lines which were compromised for fitness (e.g. lower seed set, poorer growth), which could be a trade off in plants that overexpress induced stress tolerance pathways (Heil and Baldwin, 2002). These selected lines were then screened for drought resistance in a high-throughput assay, where the plants were grown under water deficit

beyond wilting point and allowed to recover after rehydration. The drought screen revealed 8 significantly drought resistant mutants that were characterized further.

In this chapter we describe the selection and analysis of eight drought resistant mutants and the identification of the activated genes and their role in drought and other abiotic stress resistance. Two of this eight identified drought resistant mutants called *atal* and *vajra* are analyzed further for water use efficiency (WUE) and salt tolerance.

Materials and Methods

Arabidopsis plant growth and phenotype

The Arabidopsis plants used are in ecotype Wassilewskija (Ws-3) and were grown in the greenhouse at around 22°C. For growth measurements, fresh weight (FW) of plants was determined immediately after harvesting, and samples were oven-dried at 65°C for one week to obtain dry weight (DW). Cross sections of the Arabidopsis roots from 5-week-old plants were made by free-hand sections with a razor blade, stained with phloroglucinol-HCl (1% [w/v] phloroglucinol in 6 N HCl) and then observed under dark field microscope at 20x magnification (Zhong et al., 2000).

DNA analysis and plant transformation

Plant DNA was isolated and 10 ng used for TAIL PCR (Marsch-Martinez et al., 2002), followed by sequencing of the flanking DNA to reveal the insert position in the Arabidopsis genome using BlastN (Altschul et al., 1990). An overexpression construct was made for Arabidopsis transformation with the candidate gene At3g03350 for the mutant *atal*, encoding a short chain dehydrogenase reductase (SDR) family gene. A fragment of 1877 bp of the gene was amplified from Arabidopsis ecotype Columbia genomic DNA using *pfu* DNA polymerase with primers M13-50-F (5'-CACCATGGTTAGGGATCAGAGGAA-3') and M13-50-R (5'-TTAACCAACCTCATGAGACT-3'), cloned into the pENTR TOPO vector (Invitrogen), and sequenced before ligation into the gateway binary vector pH7WG2 in-between a CaMV35S promoter and a CaMV35S terminator (Fig. 1A) (Karimi, 2002). The binary vector contains a

chimeric CaMV 35S-hygromycin phosphotransferase for hygromycin selection during plant transformation.

For making an overexpression construct with the candidate gene At1g09490 from the mutant *vajra*, encoding a cinnamyl-alcohol dehydrogenase (CAD) like gene of 1648 bp, the gene was amplified from Arabidopsis ecotype Columbia genomic DNA using *pfu* DNA polymerase with primers M14-90-F (5'-CACCATGAACTGCGGAGGAAAGG-3') and M14-90-R (5'-TCATAAAAGACATTTCTCCTTGAGGC-3'), cloned into pENTR TOPO vector (Invitrogen), and sequenced before ligation into the gateway binary vector pH7WG2 in between a CaMV35S promoter and a CaMV35S terminator (Fig. 1B) (Karimi, 2002).

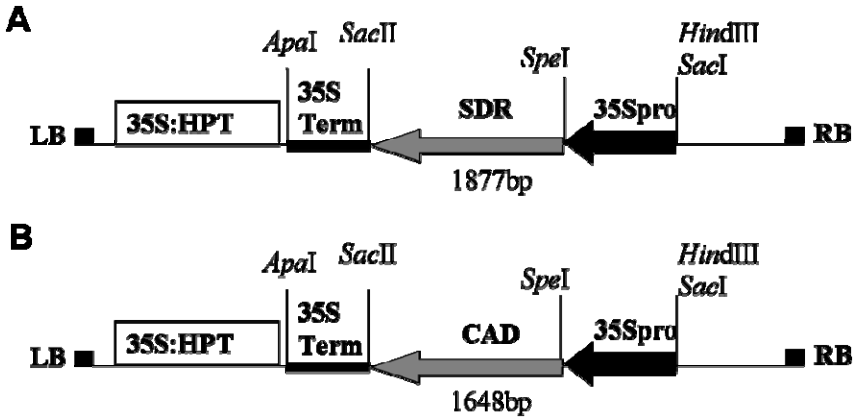

Fig. 1. Overexpression constructs of SDR and CAD gene identified in *atal* and *vajra* mutant.
(**A**) The 35S:SRD_HPT construct contains the *SDR* gene (At3g03350) cloned in the pH7WG2 gateway binary vector (Karimi, 2002) in between a CaMV35S promoter and terminator, with the hygromycin gene for selection in plants. The fragment size in bp (base pairs) is shown below the gene. **(B)** The 35S:CAD-like_HPT construct contains the *CAD*-like gene (At1g09490) cloned in the pH7WG2 gateway binary vector (Karimi, 2002) in between the CaMV35S promoter and terminator, with the hygromycin gene for selection in plants. The fragment size in bp (base pairs) is shown below the gene.

Gene Expression Analyses

Total RNA for Reverse Transcriptase-PCR (RT-PCR) was isolated from mature, green, rosette leaves derived from 4 weeks old activation tag mutants and wild type (WT) (ecotype Ws) plants using the Trizol reagent as described by the manufacturer (Invitrogen, Life technologies). Approximately 1 µg of total RNA was used for DNase I treatment and cDNA synthesis (using SuperScriptII reverse transcriptase) as described by the supplier (Invitrogen, Carlsbad, CA). The

cDNA was diluted 50 times and used for amplification using specific oligonucleotides for the actin gene (Actin-F, 5' ACAGCAGAGCGGGAAATTGT -3' and Actin-R, 5'-AGCTTCCATTCCCACAAACG -3') to equalize the concentrations of the cDNA samples. Subsequently the diluted cDNA was utilized to perform a PCR reaction using specific oligonucleotides designed to amplify the genes flanking the insertion site in the *atal* and *vajra* mutants: oligonucleotides 5'-AGGCATCGGATGCAAGTGAG-3' and 3'-TTGGACCAGGAATAACGACG-5' to amplify the (At3g03350) short chain dehydrogenase (SDR) gene; oligonucleotides 5'-AGCGGACTCTCCAATAACAC-3' and 3'-ACAGACGTCGGATAGGCCAC-5' to amplify the (At3g03370) similar to dehydration responsive gene; oligonucleotides 5'-TGAAGGATGTGACGCTGTCT-3' and 3'-CTGCATAGACTTGGATCGGA-5' to amplify the (At1g09480) cinnamyl-alcohol dehydrogenase like (CAD) gene; oligonucleotides 5'-TCTCGTCAGCCTCCTATTGG-3' and 3'-CCAAGGCGACATCTCTTACG-5' to amplify the (At1g09490) cinnamyl-alcohol dehydrogenase like (CAD) gene; and oligonucleotides 5'-TGGCCGAAGATGCAGCATGG-3' and 3'-GCTGAAGGAGTCTCGAGTGC-5' to amplify the (At1g09500) cinnamyl-alcohol dehydrogenase like (CAD) gene. The reaction conditions for Q-PCR included a denaturing step of 95°C for 3 min, followed by 40 cycles of 10 sec at 95°C, 45 sec 60°C, followed by 61 cycles of elongation at 65°C-95°C for 10 sec.

Arabidopsis dry-down drought resistance test

For the drought tolerance experiments, the soil mixture comprised one part of sand and perlite and two parts of compost (a mixture made up of 25% clay and 75% turf with EC = 1 [nitrogen, phosphorous, and potassium]; Hortimea, Elst, The Netherlands). Seeds were sown (after three nights at 4°C) at density of six plants per 4-cm pot in a tray with 51 pots (Aracon containers; BetaTech, Gent, Belgium). Nutrients (Hydroagri, Rotterdam, The Netherlands; 2.6 EC) were supplied 10 days after germination (DAG), and at 14 DAG the plants were subjected to drought (for 9 and 10 d) by transferring the pots to dry trays (after drying each pot from outside). Every 2 d in drought, the plants were moved within the tray to nullify pot position effects.

Subsequently, plants were rehydrated and observed for recovery after 1 week. Experiments comparing drought tolerance between the WT and the mutants were repeated three times.

Arabidopsis gravimetric drought resistance test

Plants were sown in 7x7 cm plastic pots (4 plants per pot) in the greenhouse conditions as described above. Soil mixture used to grow the plants was the same as used in dry-down drought testing method. The soil was weighed accurately in each pot. The pots were well watered and maintained at 100% field capacity. (FC- the amount of water left in the soil after it has been saturated and allowed to drain by gravity for 24 hours). Two weeks after the germination, the bottom holes of the pots were sealed to avoid water drainage. A control set of pots (≥ 9 pots each line) representing the WT, *atal* and *vajra* were maintained at 100% field capacity for the rest of the experimental period. A similar set of WT, *atal* and *vajra* lines were exposed to drought by withholding water until the WT died (approx. 10-11days of withholding water). Control pots (n=9) with no plants were maintained at 100% FC throughout the experimental period and similar pots with no plants were maintained at 100% field capacity until the beginning of the drought treatment, when water was withheld until the end of the experiment. This allowed the measurement of water loss through evaporation in the control as well as the drought treated set of pots. These data were used as a correction factor for transpiration estimates. During the experimental period, the pots were weighed daily using a portable balance (0.001g accuracy). For control pots the daily difference in the weight on subsequent days was corrected by adding water to maintain the 100% FC. For the drought set of pots the difference in the weight of pots was noted down. At the 15^{th} DAG or the 1^{st} day of dehydration (DOD), the representative plants from WT, *atal* and *vajra* were sampled to measure the initial biomass (B_{15}). The WT plants were carefully monitored daily for their appearance and when assessed as having wilted enough to survive for one more day in drought, a sample set of pots (n≥4) was rehydrated to be checked for recovery. In addition, biomass from each set of pots representing WT, *atal* and *vajra* were collected and this process of sampling was repeated daily until the WT had passed irreversible wilting point and could not recover the next day. This day of permanent wilting point was noted as day after WT's death (DAWD). In this experiment the WT died on 11^{th} DOD or 25^{th} DAG. The

water added during the experimental period in the control set of pots maintained at 100% FC was summed to arrive at the cumulative water lost (CWT) by the plants in the process of transpiration from the control plants. The difference in the weights of pots of the drought set was calculated as the total water lost by transpiration in drought treated plants. Precise percentage of water present in each pot was measured at the beginning of the drought test and when the WT died to determine the minimum threshold level of water beyond which the WT cannot survive. This was calculated using following formula,

Water pot^{-1}(g) = Total wt. of pot – Σ (dry wt. of soil pot^{-1} + wt. of empty pot + Fresh biomass of plant)

% water g^{-1} of soil = ($_{final}$ Water pot^{-1} / dry wt of soil pot^{-1}) x 100

Assuming that the growth was linear during the experimental period, WUE was computed as WUE= (B_{25}-B_{15})/CWT, where B_{25} and B_{15} are the total biomass (g pot^{-1}) measured on 25 and 15 DAG, respectively. CWT is the cumulative water transpired by the plants (g) during the experimental period and is computed as follows: For control pots,

CWT (g) = Σ (water added to the plants each day) - Σ (water lost by evaporation from the soil).

For pots exposed to drought CWT was calculated as follows,

CWT (g) = ($_{initial}$ Water pot^{-1} – $_{final}$ Water pot^{-1}) - Σ(water lost by evaporation).

Mean Transpiration Rate per plant per day were calculated as **MTR**= CWT/11DOD (Days of observation).

All the fresh biomass collected was later oven dried at 60°C for 5 days and the dry weights were measured separately. All experiments were tested for significance of parameters by analysis of variance (ANOVA), comparing the *atal* and *vajra* lines vs. WT and control vs. drought treatments. Statistical analysis was done using Microsoft Excel 7.0 (Microsoft, 2000) and Graph Pad Prism version 4.00 for Windows, Graph Pad Software, San Diego California USA, www.graphpad.com.

Arabidopsis salt stress tolerance test

To screen for salinity tolerance (Cheong et al., 2003) we used seedlings grown at a density of 1-2 plants per 2-cm pot in a tray with potting soil (Hortimea, Elst, The Netherlands). Nutrients

(Hydroagri, Rotterdam, Netherlands; 2.6 EC) were supplied at 14 DAG. At 21 DAG the plants were treated with NaCl (200, 250 and 300 mM) at intervals of 3 days for three applications and monitored for bleaching in the next 2 weeks. Survival rates were counted on the 10^{th} day after the third application of NaCl. The experiment was repeated three times.

Determination of Na^+ Ca^{2+} and K^+ ion content

The harvested seedlings of treated and control plants were rinsed with deionized water and dried at 65°C for one week. About 15 to 50 mg of dry material was treated with 1 ml of the digestion mixture (sulphuric acid - salicylic acid and selenium) and 2 carborundum beads, and swirled carefully until all the plant material was moistened and kept for digestion overnight. The temperature was increased gradually in small steps to about 330 °C and later after cooling 0.1 ml of hydrogen peroxide was added and then heated again. This step was repeated 3 times until the digest had turned colorless. On cooling down to room temperature 5 ml of demi-water was added to make up to the mark and left overnight. The Na^+, Ca^{2+} and K^+ Ion Content were determined by using an Atomic Emission Spectrophotometer (Elex, Eppendorf, Hamburg, Germany).

Results

Screening of activation tag mutants in Arabidopsis for drought resistance

To screen a large population of activation tagged (ATag) mutant lines for drought resistance, we developed a strategy to test preselected candidate stress resistant mutants based on the phenotype of reduced growth or reproductive fitness that might comprise plants that overexpressed stress tolerance pathways. We therefore examined a subset of the activation tag population of 1250 insertion lines containing around 600 ATag insertions (Marsch-Martinez et al., 2002), and selected about 279 ATag lines, which were putatively compromised for fitness (e.g. low seed set, poor growth). On re-examination of these lines in the greenhouse, only 51 lines qualified to the criteria of reduced fitness or growth. A quick high-throughput screen for drought resistance was then conducted with these 51 lines to reveal 8 ATag mutants that survived longer periods of drought stress (2 days longer) than WT control plants (Fig. 2) and recovered well to reach maturity and set seeds after rehydration. These mutant lines were named as *AT1*, *AT2*, *AT3*,

AT4, *AT5*, *AT6*, *atal* (a Sanskrit word meaning immovable/indestructible) and *vajra* (a Sanskrit word meaning tough like a diamond).

Fig. 2. Screening for drought resistant ATag mutants in Arabidopsis.

High-throughput drought resistance tests of Arabidopsis WT and the ATag mutant lines, exposed for a period of 9 to 10 days of dehydration (DOD). Subsequently, plants were watered, and their appearance after a week (recovery) is presented in the image on the right column for 10 DOD plants. Left column of the image shows plants at 9 DOD.

Phenotype of drought resistant mutants

Segregation analysis of progeny plants of these mutants indicated a semi-dominant behaviour for *AT3, AT4* and *vajra*. The phenotypes of the other mutants *AT1, AT2, AT5, AT6* and *atal* were dominant. For the semi-dominant mutants most of the homozygote plants flowered later than the heterozygote plants. However, their plant height remained smaller than WT.

Quantitative measurements on a set of WT plants in comparison with the drought resistant mutant lines confirmed a growth delay in the mutants. The life cycle of the drought resistant mutants was overall longer, and so were the different growth phases monitored as flowering time (bud formation), actual flowering (opening of the flowers) and silique formation (seed setting) (Table 1).

Table 1: Developmental phase (in DAG) of drought resistant mutants compared to the WT

Plant line	Reproductive stage						End of life cycle
	Flower Bud formation (DAG)		Flower formation (DAG)		Silique formation (DAG)		
	>50%	100%	>50%	100%	>50%	100%	
WT	-	15	-	24	-	28	50
AT1	15	18	33	35	33	35	60
AT2	15	18	28	33	33	35	60
AT3	12	15	21	26	30	33	60
AT4	19	22	26	28	35	40	65
AT5	18	22	28	32	35	40	60
AT6	17	20	26	28	30	38	60
atal	16	24	28	45	33	51	69
vajra	15	22	26	37	31	46	61

As shown in Table 1 for the different stages of maturity, the mutants *AT4, AT5, AT6, atal* and *vajra* were later in bud formation (~1 week later) and silique production (~2-3 weeks late) compared to the WT. The *atal* and *vajra* were very late in flower formation (~3 and 2 weeks late respectively) compared to the WT. The WT completed its life cycle in about 50 days after germination (DAG), whereas other mutants completed there life cycle in the range of 60-69 DAG

(10-20 days later than the WT). Overall the *atal* and *vajra* mutants extended their flowering much longer than the other mutants.

AT1 phenotype

The *AT1* mutant line displayed a non-erect growth habit, with rosette leaves of reduced size and small silique length compared to the WT. The height of *AT1* was slightly less that the WT, and the color of the rosette leaves was dark green. *AT1* displayed a delay of 3 days in bud formation compared to the WT; however its flowering time, silique formation period and end of life cycle were prolonged by almost 10 days compared to the WT (Table 1). Rosette leaves were oval shaped, smaller in size and slightly curled at the tips compared to the WT. The total number of the rosette leaves was the same as in WT even though later in flowering. The axillary meristem formation showed a typical pattern of one-sided growth on the main meristem (Fig. 3). The non-erect appearance seems to be due to the unbalanced growth of the axillary meristem. Flower buds and mature flowers showed no difference in size or shape compared to the WT and the mutant produced fertile flowers.

AT2 phenotype

The *AT2* mutant displayed darker green colored plants, reduced height and a delay of different growth stages compared to the WT. *AT2* showed a delay of 3 days in bud formation compared to the WT. However, its flowering time, silique formation period and end of life cycle were prolonged by almost 7-10 days compared to the WT (Table 1). Rosette leaves were oval shaped, smaller and thicker compared to the WT and formed an upward protruding wedge in the middle of the leaf and slightly curled inwards at the tips. The cauline leaves were also smaller in size and curled slightly inwardly at the tips. The number of the rosette leaves was the same as the WT; however it produced a profuse number of flowers and lateral stems (Fig. 3). Flower buds and mature flowers were smaller in size compared to the WT. Silique length was a little smaller compared to the WT and full of seeds.

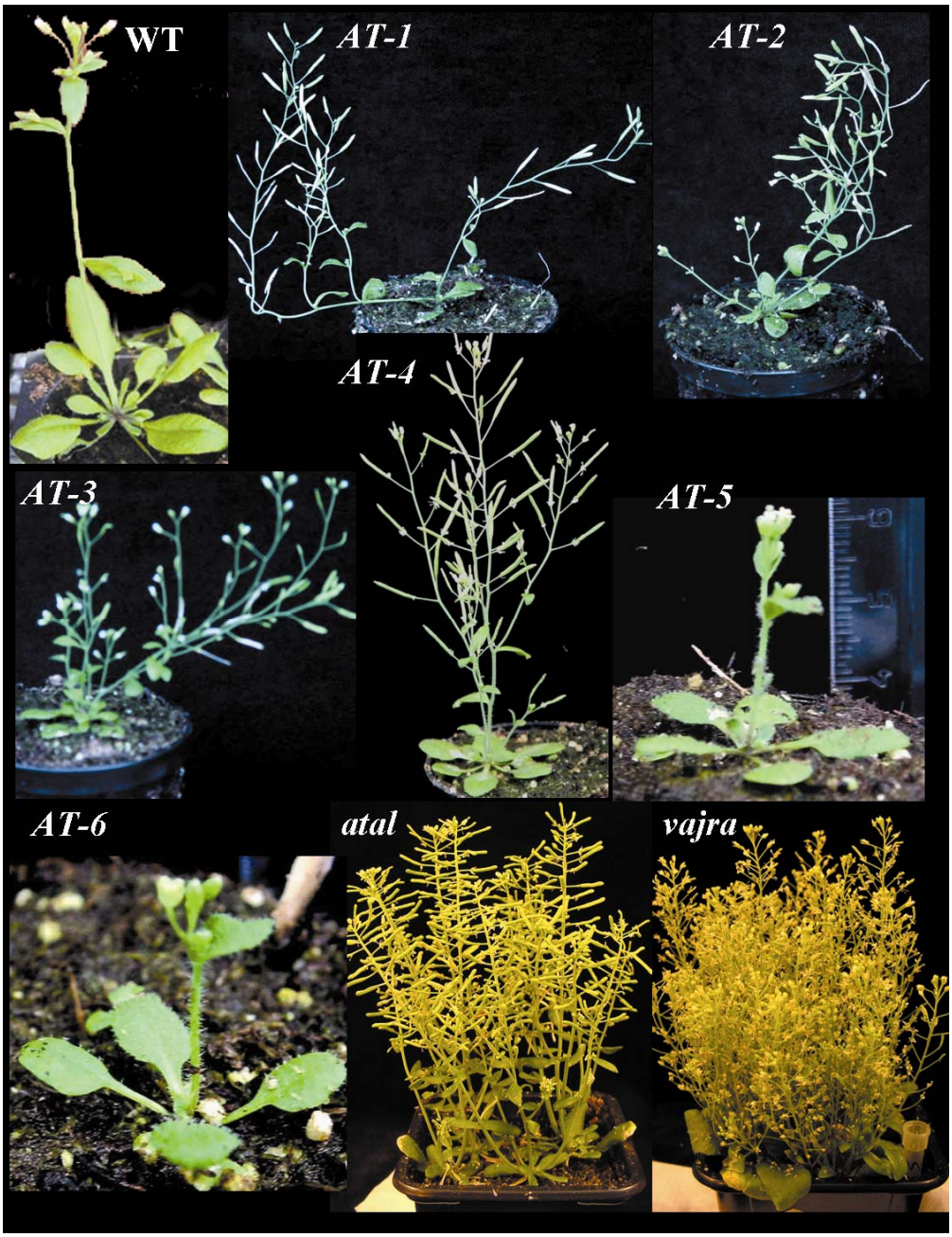

Fig. 3. Phenotypes of the drought resistant mutants
Growth stage evaluated: WT plant 14 DAG, *AT1* 33 DAG, *AT2* 33 DAG, *AT3* 28 DAG, *AT4* 38 DAG, *AT5* 26 DAG, *AT6* 18 DAG, *atal* 45 DAG and *vajra* 40 DAG

AT3 phenotype

The *AT3* mutant phenotype was very similar to that of *AT2*, displaying a dark-green colored plant with reduced height and a slight delay in growth phases compared to the WT. However *AT3* showed a semi-dominant inheritance and was not delay in bud formation compared to the WT. It's flowering period and silique formation period and the end of its life cycle were prolonged for almost 2, 5 and 10 days respectively compared to the WT (Table 1). Rosette leaves were oval shaped, smaller and thicker compared to the WT and formed a wedge in the middle of the leaf and slightly curled inwards at the tips. The cauline leaves were also smaller in size and slightly curled inwardly at the tips. The total number of the rosette leaves was the same as the WT, however it produced profuse number of flowers and lateral meristem (Fig. 3). Flower buds and mature flowers were smaller in size compared to the WT. Silique length was little smaller compared to the WT and siliques were fully fertile.

AT4 phenotype

The *AT4* mutant displayed an erect growth habit phenotype with many lateral branches and profuse flowering (Fig. 3), with dark green rosette and cauline leaves. The plant looked taller compared to the other mutants but smaller compared to the WT (Fig. 3). It also exhibited a delay of its growth phases compared to the WT. *AT4* inherited semi-dominantly and displayed a delay of 7 days in bud formation compared to the WT. The flowering time, silique formation period and end of life cycle was prolonged for almost 4, 12 and 15 days respectively compared to the WT (Table 1). Rosette leaves were thicker and almond or convex shaped, and pointed at the tips compared to the WT. The total number of the rosette leaves was the same as the WT, with flower buds and mature flowers not differing in size or shape. The siliques had same length as WT and were fully fertile.

AT5 and *AT6* phenotype

Visual observations of *AT5* phenotype showed that it was very similar to *AT6*. Both displayed a dark green color plant with a more reduced height, than all the other drought resistant mutants described here (Fig. 3). The number of rosette leaves (4-5) was less compared to the WT.

The mutants also displayed a large delay in the length of their growth phases compared to the WT. Bud formation was delayed for 7 and 5 days compared to the WT. Their flowering time, silique formation period and end of life cycle, were prolonged for almost 3-4, 12-10 and 10-10 days respectively compared to the WT (Table 1). The rosette leaves were oval shaped, smaller, dark green and thicker compared to the WT (Fig. 3). The cauline leaves were also smaller in size. They mainly produced a single thin main inflorescence and with hardly any lateral shoots. The number and size of siliques was smaller compared to the WT. Flower buds and mature flowers showed no difference in size and shape compared to the WT.

***atal* phenotype**

The *atal* mutant has an erect growth habit with profuse lateral branching, profuse flowering and silique formation (Fig. 3). The plants displayed a dark green rosette and cauline leaves, looking like a shrub (Fig. 3). It showed dominant inheritance in segregation analysis of the progeny. The mutant also exhibited a delay in its growth phases compared to the WT, with a delay of 9 days in bud formation. The flowering time, silique formation period and end of life cycle was prolonged for almost 21, 23 and 19 days respectively compared to the WT (Table 1). Rosette leaves were small, thick, lacked petiole and oblong shaped, curled inwards at the edges, with the total number of rosette leaves same as the WT. The main stem was thicker compared to the WT and its elongation was reduced, reducing the height of the plant. Flower buds and mature flowers showed reduced size compared to the WT, the flower buds emerging in compact clusters at the floral axis and appearing as a bunch. The siliques were shorter but thicker in shape compared to the WT, producing a higher number of fully fertile siliques compared to the WT (Fig. 3).

***vajra* phenotype**

The *vajra* phenotype was distinguished by an erect and bushy growth habit with profuse lateral branching, flowering and silique formation (Fig. 3). The plant displayed a dark green rosette with a lighter green color around the leaf vein, giving the leaf a very patchy look. The rosette leaves were serrated and pointed at the tips with a leaf number similar as WT. It showed a dominant inheritance in segregation analysis. This mutant also exhibited extended growth phases

compared to the WT, with a delay of 7 days in bud formation. It's flowering time, silique formation period and end of life cycle were prolonged for almost 13, 18 and 11 days respectively compared to the WT (Table 1). The stem elongation was reduced, and the mutant produced many thin lateral branches, with profuse flower formation, giving the whole plant a very bushy and compact appearance (Fig. 3). It displayed a semi-sterile phenotype as not all the flowers produced seeds. Flower buds and mature flowers were reduced in size compared to the WT. Siliques were shorter and more numerous compared to the WT, due to the increased lateral stem formation.

Identification of insert-flanking genes in drought resistant mutants

In order to find out the gene that was overexpressed and responsible for the mutant phenotypes, we performed TAIL-PCR (Marsch-Martinez et al., 2002) using genomic DNA of the mutants, followed by sequencing of the flanking DNA to reveal the insert positions in the Arabidopsis genome using BlastN (Altschul et al., 1990).

Molecular characterization of the *AT1* mutant revealed that there were two transposon activation tag (ATag) inserts in the line. One insert is located on chromosome 5 at position 25,004,530 (according to the TAIR website; http://www.arabidopsis.org/), located in a gene encoding an embryo specific protein (At5g62210) (Fig.4A). Adjacent to the ATag right border is another gene encoding an embryo specific protein (At5g62200), with the translation start site 1630 bp from the transposon end and the ATag enhancer located another 2 kb away. On the other side of the transposon and 2705 bp away from the left border, is the translation start of At5g62220, encoding an exostosin family protein. The second ATag insert was detected on chromosome 2 at position 19,141,083 located inside a gene encoding a DAG2 protein containing zinc finger motifs (At2g46590) (Fig.4B). Adjacent to the transposon tag right border is a gene encoding a putative calcium binding protein (At2g46600), whose translation starts 2058 bp from the transposon and the ATag enhancer a further 2 kb away. On the other side of the transposon and 3498 bp away from the left border, is the translation start of At2g46580 encoding a pyridoxine 5'-phosphate oxidase-related protein.

For *AT2*, the ATag insert was found at position 23,162,771 of chromosome 5 located inside a gene, encoding a protein with similarity to heat shock proteins (At5g57130) (Fig.4C).

Adjacent to the transposon tag right border is a gene with similarity to the copia-like retrotransposon family protein (At5g57126), whose translation starts 6139 bp from the transposon. On the other side of the transposon and 4380 bp away from the left border, is the translation start of At5g57140, encoding the protein ATPAP28/PAP28 (purple acid phosphatase 28), and even further at 6816 bp away a gene encoding a protein with a basic helix-loop-helix domain.

In the *AT3* mutant the ATag insert was located at position 10,063,431 of chromosome 3, located inside a gene, encoding an expressed protein of unknown function (At3g27250) (Fig.4D). Adjacent to the ATag right border is a gene encoding a kinase like protein with similarity to yeast BDF1 and human RING3 protein (At3g27260), whose translation starts 6217 bp from the transposon and the enhancer of the ATag located 2 kb further. On the other side of the transposon at the left border and 3824 bp away, is the translation start of At3g27240 encoding a putative cytochrome c1, heme protein, which is a mitochondrial precursor.

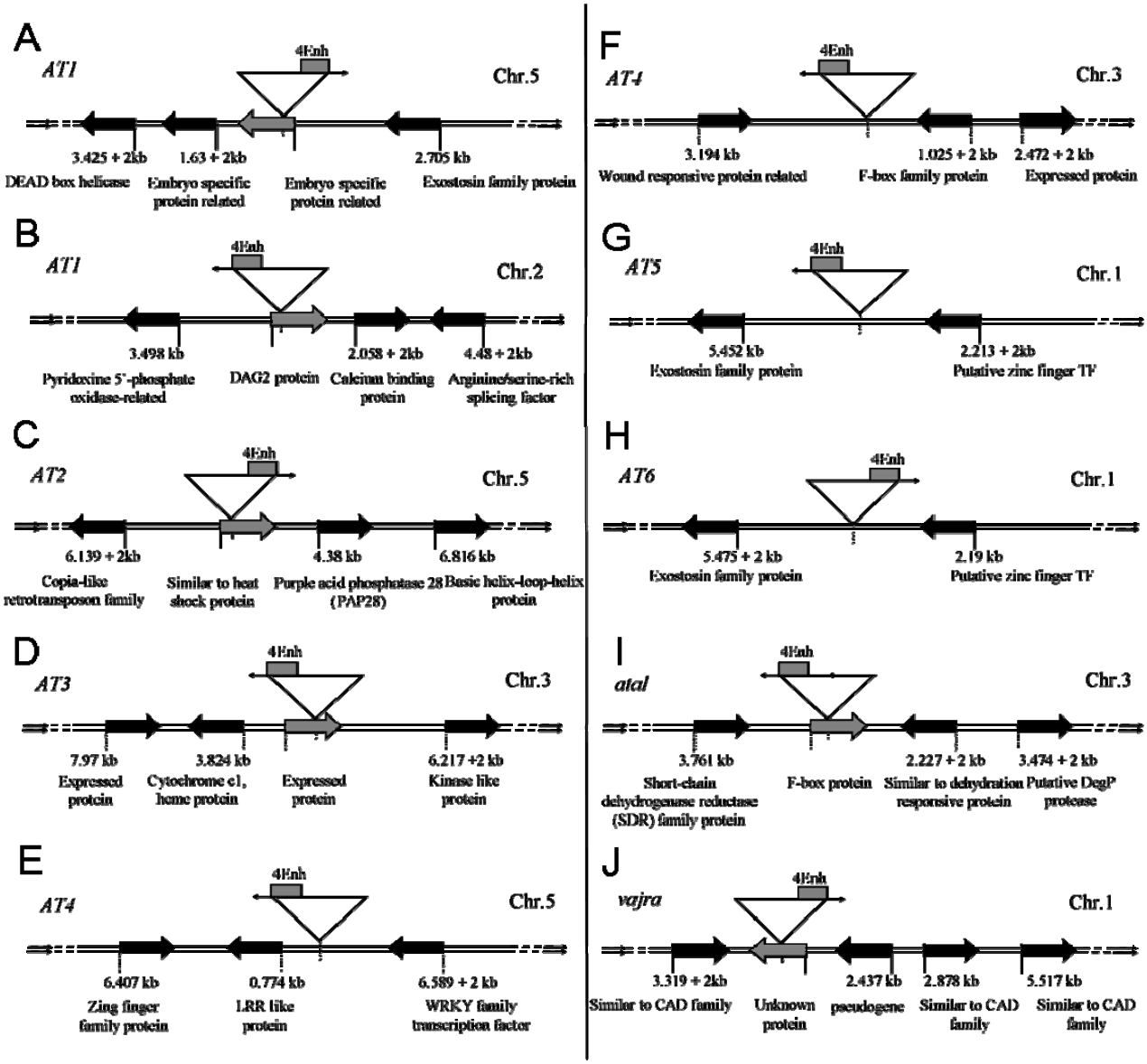

Fig. 4. Map of transposon insert-adjacent genes of drought resistant mutants

(A) *AT1* shows two ATag transposon inserts in its genome, one on chromosome 5 and the other **(B)** on chromosome 2. **(C)** *AT2* has an ATag insert on chromosome 5; **(D)** *AT3* has an insert on chromosome 3. **(E)** *AT2* has two ATag inserts, one on chromosome 5 and other **(F)** on chromosome 3. **(G & H)** *AT5* and *AT6* both show ATag transposon inserts on chromosome 1, also the insert position was very close to each other in both mutants, the only difference being the position of the insert, which is inserted in opposite direction on the genome sequence. **(I)** *atal* has an ATag insert on chromosome 3 and **(J)** *vajra* has an ATag insert on chromosome 1.

In mutant *AT4*, the ATag insert was found at two positions in the genome. One insert is at position 345,421 of chromosome 5 (TAIR sequence), located between two oppositely transcribing open reading frames. Adjacent to the transposon tag right border is a gene encoding a WRKY family transcription factor (At5g01900), whose translation starts 6589 bp from the transposon (Fig. 4E). On the other side and 774 bp away from the left border, is the translation start of At5g01890 encoding a leucine-rich repeat (LRR) transmembrane protein kinase. Adjacent to the LRR gene is a zinc finger protein (At5g01880) 6407 bp away from the left border of the transposon (Fig. 4E). The second ATag insert was found on chromosome 3 at position 3,445,976, located between two inwardly transcribing open reading frames (Fig. 4F). Adjacent to the ATag right border is a gene encoding an F-box family protein (At3g10990), whose translation starts 1025 bp from the transposon. On the other side and 3194 bp away from the left border, is the translation start of At3g10985, encoding a wound-responsive protein.

In *AT5* and *AT6* the ATag inserts were found positioned very close at positions 25,687,382 and 25,687,405 respectively on chromosome 1, located between two transcribing open reading frames. The ATag transposon in *AT5* is inserted in an opposite orientation to that in *AT6*. Adjacent to the ATag right border for *AT5* and left border for *AT6* is a gene encoding a putative zinc finger transcription (At1g68480), with translation start at 2213 bp and 2190 bp from the ATag insert in *AT5* and *AT6* respectively (Fig. 4G, H). Adjacent to the ATag left border for *AT5* and right border for *AT6* is a gene encoding an exostosin family protein (At1g68470), whose translation starts 5452 bp and 5475 bp from the ATag insert in *AT5* and *AT6* respectively (Fig. 4G, H).

In mutant *atal*, the ATag insert was located inside a gene encoding an F-box protein (At3g03360) (Fig.4I), at position 796,253 of chromosome 3. Adjacent to the ATag right border is a gene encoding a dehydration responsive protein (At3g03370), with translation start 2227 bp from the transposon. On the other side and 3761 bp away from the left border, is the translation start of At3g03350 encoding a short-chain dehydrogenase reductase (SDR) family protein.

In the *vajra* mutant the ATag insert was found located inside a gene encoding an unknown protein (At1g09483) (Fig.4J), at position 3,061,296 of chromosome 1. Adjacent to the ATag right border is a gene encoding similarity to *Eucalyptus gunnii* alcohol dehydrogenase of unknown

physiological function or similar to cinnamyl alcohol dehydrogenase (CAD) family (At1g09480), whose translation starts 3319 bp from the transposon. On the other side and 2437 bp away from the left border, is the translation start of At1g09486, encoding a pseudogene. Adjacent to it is another gene, 2878 bp away from the left border of transposon, encoding another gene similar to CAD family (At1g09490).

Expression analysis of flanking genes

To determine if the 35S enhancer tetramer in the ATag transposon was influencing the expression of one or more of the flanking genes in the above mentioned mutants, we performed Q-PCR experiments on leaves of wild type and the mutant plants. Unfortunately, our experiments with all the mutants except for *atal* and *vajra* did not succeed due to technical failures. In *atal,* our candidate genes were At3g03350 and At3g03370 as they are in close proximity to the enhancer sequence. No transcriptional changes could be detected for either of the genes (data not shown). However, we decided to overexpress the At3g03350 gene since it was well annotated as a short chain dehydrogenase reductase (SDR) family protein and the other gene (At3g03370) until recently was annotated as expressed protein only but is now annotated as a dehydration-responsive protein-related. A construct was made with the predicted coding region of the At3g03350 gene under control of the CaMV35S promoter and transformed into Arabidopsis. The transformants did not display the typical *atal* phenotype. When we found out that At3g03370 was annotated as a dehydration responsive protein this might be a better candidate responsible for the *atal* phenotype.

In *vajra,* the transposon tag insert was found adjacent to series of CAD (cinammyl-alcohol dehydrogenase) like genes. Our candidate genes were At1g09480 and At1g09490 as they were well annotated as CAD like genes and in the close proximity of the enhancer sequence. We also included At1g09486 and At1g09500 for our expression study. We found higher transcript level in all the genes compared to the WT, except At1g09480, where no transcript was detected. However At1g09490 transcript was significantly higher (data not shown) expressed than all other genes tested suggesting that altered expression of At1g09490 could have caused the *vajra* phenotype. A construct was made with the predicted coding region of the At1g09490 gene under control of the

CaMV35S promoter and transformed into Arabidopsis. However, the transformants did not display the typical *vajra* phenotype. The transformants displayed curly and small rosette and cauline leaves, with short, thin looking plant (data not shown). It could be that the other gene At1g09500 adjacent to At1g09490, which is also a CAD like gene might be responsible for the *vajra* phenotype or the co-expression of both these genes might be responsible for the *vajra* phenotype.

atal and _vajra_ confer increased drought tolerance and water use efficiency in Arabidopsis

The screen for drought resistance showed that the *atal* and *vajra* mutants survived longer periods (3 days longer) of drought stress than the WT plants (Fig. 4A) and recovered well to attain maturity and set seeds after rehydration. Since the size of *atal* and *vajra* plants were smaller compared to the WT, it was necessary to know the exact soil water content at which the plants can survive drought stress. Hence, a more controlled drought stress test was carried out, in which sets of replicate pots (each set had n≥9 pots) with 4 plants each were subjected to drought stress at 14 DAG by withholding water to the pots. The pots were weighed daily and the water transpired was replenished in the control set of pots (n≥4) maintained at 100% FC. The exact amount of water was calculated in the soil at the beginning of drought stress (14 DAG) and later in the drought period when the WT died (11DOD) and this minimum level of water threshold was tested on both *atal* and *vajra* mutant lines to see if they could survive at these water levels. In this test the WT did not survive beyond 12.7 % soil water content levels at 11DOD (Fig.4A). On this day (11DOD) *atal* and *vajra* plants showed 13.8 % and 11.32 % of water levels respectively in the soil and survived the drought stress. However, on the 12^{th} and 13^{th} DOD the pots with the *atal* plants had 11.08 % and 9.54 % soil water content respectively, whereas for *vajra* these values were 9.58 % and 7.12 % and both mutants survived upon rehydration (Fig. 4A).

The amount of water transpired per day, both under stress and non-stress conditions, was measured for WT, *atal* and *vajra*. And showed that the mean transpiration rate (MTR) of *atal* and *vajra* per day was much less compared to the WT both under stress and non-stress condition (Fig 4B). Further we measured the WUE of *atal* and *vajra* compared to the WT through a gravimetric method (see materials and methods) where WUE accounts for the ratio between the total gain in

the biomass during the experimental period and the cumulative water transpired (CWT). The *atal* and *vajra* mutant lines showed an almost two times higher WUE, under drought stress compared to the WT which showed 25% decrease in WUE under drought stress (Fig. 4D). The percentage reduction in the total dry biomass (shoots + roots) for *atal* and *vajra* was less reduced (28.7-30.8 % and 35.8-38.0 % respectively) under drought stress compared to the WT, which showed a 74.6% decrease in dry matter accumulation under drought stress (Fig. 4C). This result suggests that *atal* and *vajra* not only are able to survive below the minimum threshold of water levels but also are able to prolong survival under the drought stress conditions by losing less water through transpiration. Also they are able to perform better in terms of growth compared to the WT under drought stress situation, not having much reduction in biomass yields.

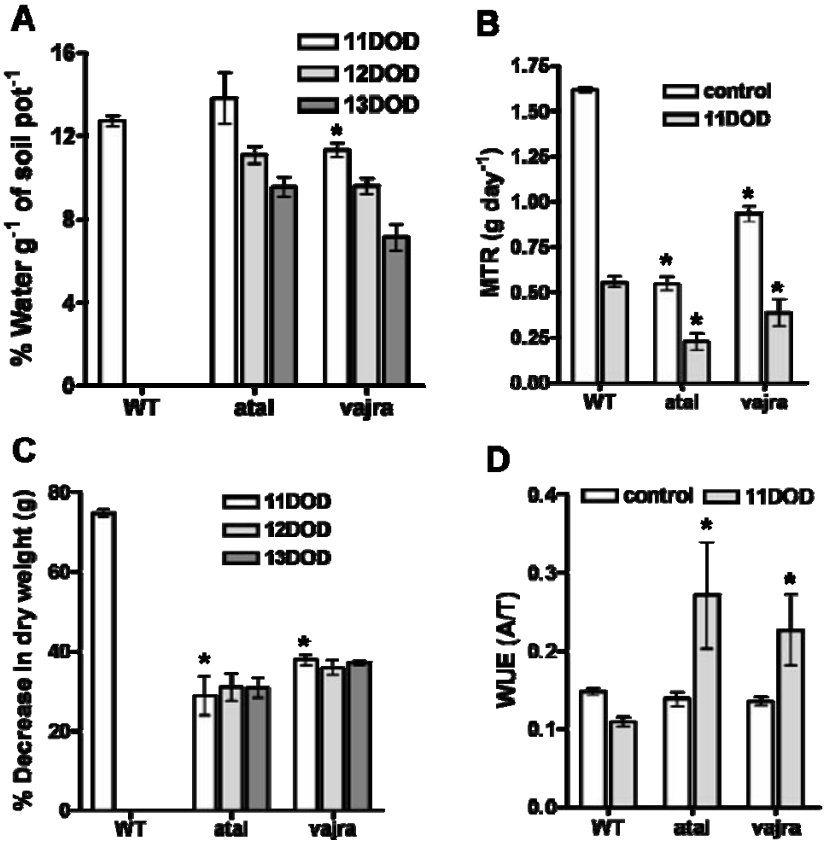

Fig. 4. *atal* and *vajra* improves WUE in Arabidopsis.

WT, *atal* and *vajra* plants were tested under well-watered and drought stress conditions, bars indicate SE ($n \geq 4$), P-values shown for *atal* and *vajra* compared to the WT. **(A)** The soil water content given in % of water per gram of soil per pot ($p<0.01$). **(B)** Mean transpiration rate (MTR) in *atal* and *vajra* compared to the WT, both under stress and non stress condition ($p<0.01$). **(C)** Percent decrease in total dry weight of *atal* and *vajra* compared to the WT. **(D)** *atal* and *vajra* shows higher water use efficiency (WUE) under drought stress condition compared to the WT ($p<0.05$). * indicates values significant at ($\alpha<0.05$) compared to the WT under the same conditions.

***atal* and *vajra* shows increase in root cortical cell layer**

We examined the roots of *atal* and *vajra* in comparison to the WT to see if there was any change in the pattern or structure of the root formation. The roots of 5-week old plants grown in sand with nutrition were carefully washed in water with minimum damage to the roots and examined. There was no visual difference found in the root structure between the WT and the mutants. However the mutants showed more lateral roots, which were very thin compared to the WT, giving the roots a more hairy appearance. We took the basal part of the root (2mm along the root base) for root sectioning to see if there were any internal changes in cell arrangement or pattern. We used thin sections of the roots, stained with phloroglucinol for lignin, and examined them under the microscope. No differences were observed in the thickness of the root at the root base, as the diameters of the WT, *atal* and *vajra* were the same (Fig. 5A and B). However the *atal* and *vajra* mutants showed more cortical cell layers and a smaller stele bearing the vascular tissues compared to the WT (Fig. 5B and C). In the WT lignin staining was observed mostly in the vascular tissues consisting of the xylem cells, and a faint staining was observed in the pith. *atal* showed a strong staining in the pith cells and scattered staining in the vascular tissue which mainly consists of the xylem cells (Fig. 5A). The cortical cells also showed faint patches of staining both in *atal* and *vajra* root sections, whereas it was absent in the WT (Fig. 5A). *vajra* showed stronger staining in the xylem tissues enclosed in the pericycle compared to the WT. It also showed strong staining in the pith cells compared to WT, but less compared to *atal* (Fig. 5A). These results indicate that *atal* and *vajra* have more storage place in the roots compared to the WT due to increase in the cortical cell layer.

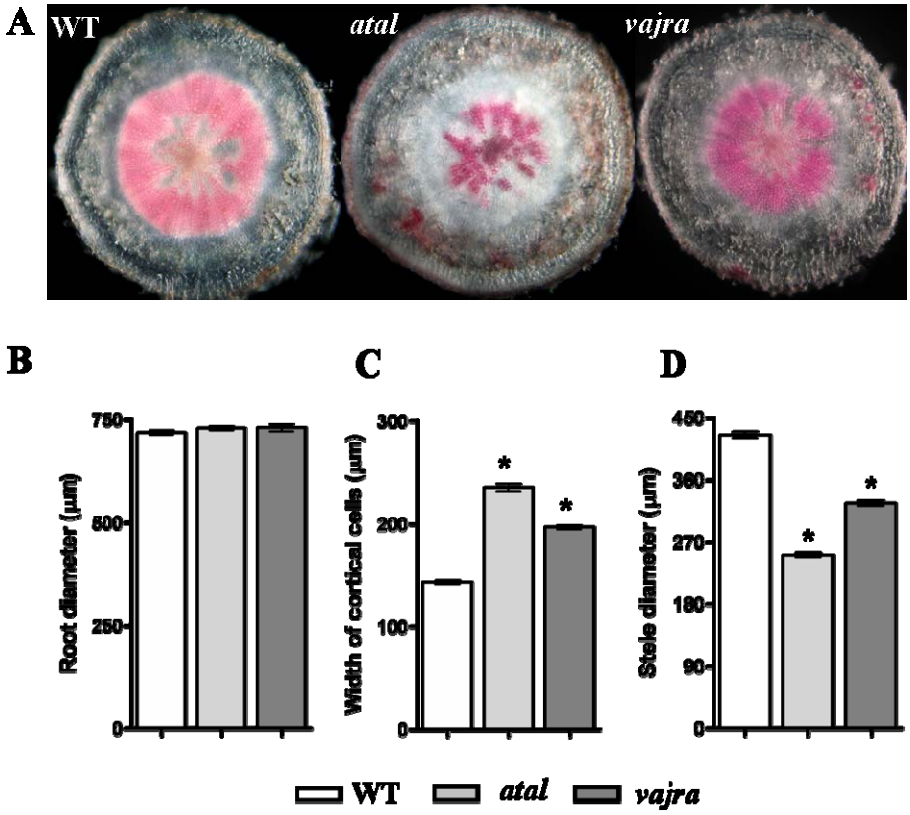

Fig. 5. *atal* and *vajra* bear more cortical cell layers in Arabidopsis.

(A) Root cross section of 5 week old plants (WT, *atal* and *vajra*). **(B)** Root diameter of WT, *atal* and *vajra* 2 mm from the base of the root. **(C)** Width of cortical cell layer in *atal* and *vajra* compared to the WT (p-value 1e-04). **(D)** Stele diameter in *atal* and *vajra* compared to the WT (p-value 1e-04). * indicates values significant at (α <0.01) compared to the WT.

***atal* and *vajra* confer increased salt tolerance in Arabidopsis**

A salt tolerance assay was employed (Cheong et al., 2003), treating the WT, *atal* and *vajra* mutants with 250 and 300 mM NaCl and then monitoring the plants for chlorosis and survival (Fig. 6A). The phenotype of the salt stressed plants was examined and tolerance estimated as the percentage undamaged non-chlorotic plants, which was higher at all NaCl concentrations for *atal* and *vajra* plants that could reach full maturity in contrast to the WT (Fig. 6A and Table 2). The percentage reduction in the shoot/leaf dry matter accumulation of *atal* and *vajra* under 250 mM NaCl stress was 35% and 30 % respectively, much lower compared to WT that displayed 64% reduction in growth (Fig. 6H). In 300mM NaCl stress the percentage reduction in the shoot/leaf dry matter accumulation of *atal* and *vajra* was 43% & 32% respectively compared to WT that displayed 69% reduction in growth (Fig. 6 H). Chemical compositional analysis revealed that *atal* and *vajra* accumulates lower amounts of Na^+ both under stress and non-stress condition compared to the WT (Fig. 6F & G) under salt stress. The Ca^{2+} levels are lowered under salt stress in WT,

whereas they are maintained in *atal* and even increases in *vajra* plant lines (Fig. 6C). The *atal* and *vajra* mutants also maintain a higher K^+ / Na^+ ratio both under non-stress and stress condition (Fig. 6D and E), which is a well known plant tolerance mechanism, by maintaining osmotic balance of the cells.

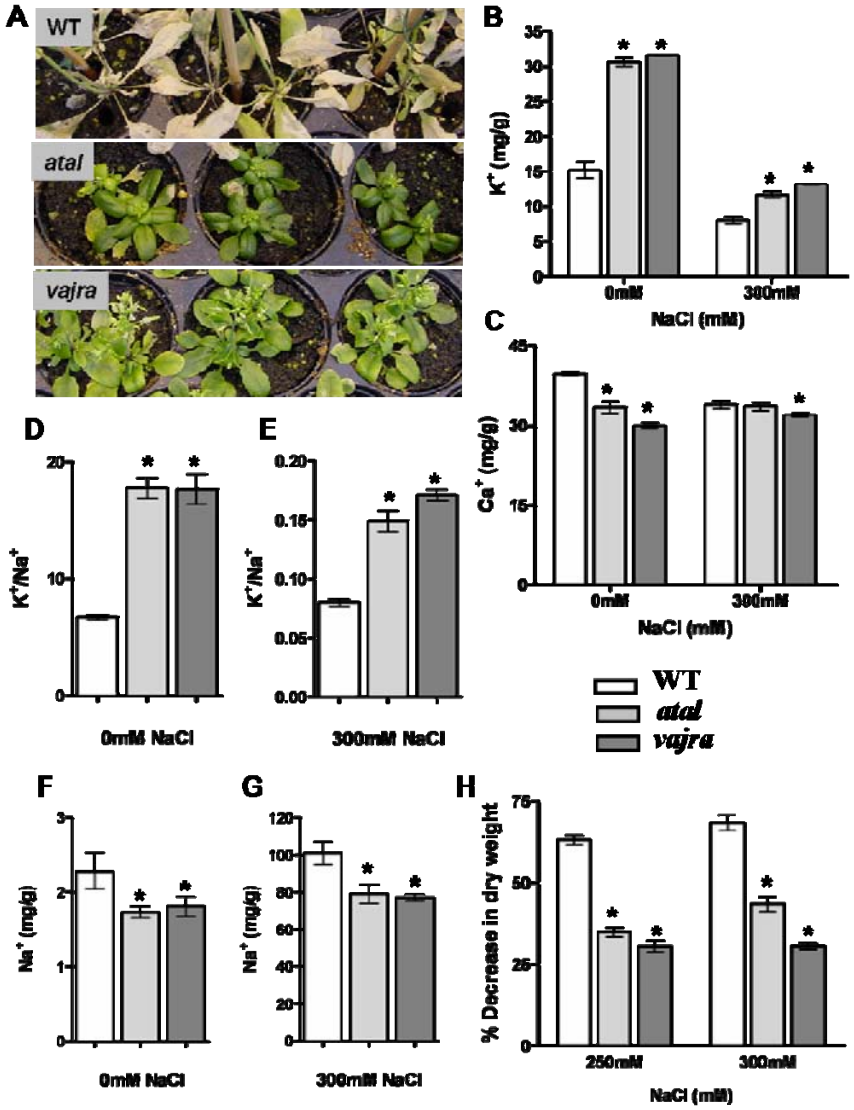

Fig. 6. Salt tolerance of *atal* and *vajra* vs. WT.

(A) WT, *atal* and *vajra* plants treated with 300mM NaCl for 21 days; (B-G) Concentrations of ions in the leaves and stems of the plants grown in soil and treated with and without high concentrations of NaCl (300mM). **(B)** K^+ levels of *atal* and *vajra* from treated and non-treated plants compared to the WT**; (C)** Ca^{2+} levels of *atal* and *vajra* from treated and non-treated plants compared to the WT**; (D)** K^+/Na^+ ratio of

atal and *vajra* under control non-treated conditions**; (E)** K^+/Na^+ ratio of *atal* and *vajra* with 300mM NaCl treatment**; (F)** Na^+ level**s** of *atal* and *vajra* under non-treated conditions**; (G)** Na^+ level**s** of *atal* and *vajra* under 300mM NaCl conditions**; (H)** Percentage decrease in the dry weight accumulation under 250mM and 300mM NaCl in *atal* and *vajra* compared to the WT. (bars indicate SE, n≥5; * indicates values significantly different from WT under the same conditions at ($\alpha < 0.01$)

Table 2. Tolerance of *atal* and *vajra* to NaCl stress

Genotype *	NaCl Treatment	Total plants	Non bleached	% Tolerant
WT	250mM	20	3	15
atal	250mM	25	23 *	92
vajra	250mM	25	25 *	100
WT	300mM	22	1	4
atal	300mM	25	21 *	84
vajra	300mM	26	25 *	96

*All the values comparing *atal* and *vajra* to the WT were tested for significance using chi-square test. The p-values for all the above comparisons is ($p<0.0001$).

Discussion

In this study we have tested the hypothesis that mutants overexpressing stress resistance pathways display compromised fitness in terms of vegetative and reproductive growth, and that pre-selecting such mutants can lead to identification of abiotic stress tolerant genes. We thereby only screened a subset of the activation tag population that displayed compromised fitness and identified several drought resistant mutants by high throughput screening for drought resistance. The frequency of 8 drought resistant mutants out of 51 or 16% from preselected mutants, and 1.3% (8/600 inserts) of inserts in the sub-population would make the screening of large genome saturating populations for drought stress feasible. These results also show that the previous estimate of ATag mutations is underestimated, as the sub-population used for this study (Population No. 3 in Table III, Marsch-Martinez et al., 2002) revealed only 4 mutants, while here we have uncovered twice that number of independent (semi)-dominant drought resistant mutants. Indeed a large proportion of the putative fitness compromised mutants also show visual phenotypic alterations, are mostly (semi)-dominant and independent. We estimate the ATag

observable phenotype mutant frequency to be around 10%, confirming other unpublished studies in the lab.

The mutants identified displayed different phenotypes related to reduced growth and reproductive fitness. We attempted to identify the tagged genes adjacent to the transposon in each mutant line by isolating DNA flanking the transposon. Due to the (semi)-dominant nature of the mutants, we expect the activating tag element to be responsible for the mutant phenotypes, by altering the expression of adjacent genes. However, the expression of these candidate genes needs to be checked and confirmed through expression analysis such as RT-PCR. Once the overexpression of these genes is confirmed, they can be tested by overexpression in a construct with the CaMV35S promoter and transformed into Arabidopsis for recapitulation of the mutant phenotype, confirming the action of the gene under investigation. Based on the position of the flanking genes to the transposon in the mutants we were able to select candidate genes (Fig. 4) for future overexpression studies.

In mutant *AT1* the transposon insertion was found at two positions in the genome, one on chromosome 5 and the other on chromosome 2. To identify the insertion responsible for the phenotype, the *AT1* mutant line should be back-crossed to WT and the phenotype of the progeny must be analyzed in relation to each insertion. At present we are left with four potential candidate genes closely flanking the ATag insert. These genes are At5g62200 and At5g62220 on chromosome 5, both positioned at ~2.7 kb on either side of the transposon enhancer, encoding an embryo specific protein and an exostosin family protein respectively. On the chromosome 2 insert, the two genes flanking on either side of the transposon enhancer are positioned at ~3.5-4.0 kb distant and are a putative calcium binding protein (At2g46600) and a pyridoxine 5'-phosphate oxidase-related protein (At2g46580).

The embryo specific protein (At5g62200) contains two domains of lipase/lipooxygenase (PLAT/LH2) and embryo-specific 3 and belongs to a small group of protein family involved in plant seed storage. The lipoxygenases are a class of iron-containing dioxygenases that catalyze the hydroperoxidation of lipids, containing a cis, cis-1,4- pentadiene structure. They are common in plants where they may be involved in a number of diverse aspects of plant physiology including growth and development, pest resistance, and senescence or responses to wounding. Embryo-

specific 3 domain proteins belongs to the family of plant seed-specific proteins identified in Arabidopsis (Nuccio and Thomas, 1999). Hence, At5g62200 might be involved in the accumulation of seed storage compounds. The other candidate, an exostosin (EXT) family protein (At5g62220) is a cellular membrane protein with catalytic activity and contains an exostosin-like domain. One of the EXT family genes encode a glycosyltransferase involved in heparan sulfate biosynthesis (Busse et al., 2007). Its role in plants is unclear, but might be involved in some cellular membrane activity.

In the second ATag insertion site, one candidate gene At2g46600 contains a calcium binding domain. Expression of such genes has been reported in several studies on abiotic stress tolerance, especially salt tolerance (Liu and Zhu, 1998; Ishitani et al., 2000; Horie et al., 2006; Pandey et al., 2007). The other candidate gene, encoding a pyridoxine 5'-phosphate oxidase-related protein (At2g46580), shows homology to Pyridoxine (pyridoxamine) 5′-phosphate oxidase (PPOX), an enzyme that is known as to be involved in the pyridoxine biosynthetic process. They catalyze the oxidative conversion of pyridoxamine 5′-phosphate (PMP) or pyridoxine 5′-phosphate (PNP) to pyridoxal 5′-phosphate (PLP). They are also known to be involved in rendering oxidative stress resistance in yeast (Yuying et al., 2007) and affect growth and stress responses in plants (Gonzalez et al., 2007).

In *AT2* the closest gene to the transposon enhancer is about 4 kb away (At5g57140), encoding a purple acid phosphatase 28 (ATPAP 28). The Purple acid phosphatases (PAPs) are metallo-phosphoesterases, that are characterized by the presence of seven conserved amino acid residues involved in coordinating the dimetal nuclear center in their reactive site (Zhu et al., 2005). In Arabidopsis there are 29 PAPs identified based on protein sequence similarities. They are believed to be involved in phosphate mobilization and the metabolism of reactive oxygen species (Li et al., 2002).

In *AT3* the closest gene to the transposon enhancer is about 3.8 kb away (At3g27240), encoding a putative cytochrome c1 like protein. Cytochrome c1 is formed in the cytosol and targeted to the mitochondrial intermembrane space. It is one of the constituents of complex III, which forms the third proton pump in the mitochondrial electron transport chain.

In *AT4* the transposon insertion was found at two positions in the genome. One was found in chromosome 5 and the other in chromosome 3. In order to have a single insertion mutant line, *AT4* should be back crossed with WT and analyzed for the effect of a single ATag insertion. The closest gene flanking the transposon enhancer is at ~774-bp distance encoding a leucine-rich repeat (LRR) transmembrane protein kinase (At5g01890). Leucine-rich repeats (LRR) consist of 2-45 motifs of 20-30 amino acids that generally fold into an arc or horseshoe shape (Enkhbayar et al., 2004). LRRs occur in proteins ranging from viruses to eukaryotes, and appear to provide a structural framework for the formation of protein-protein interactions (Kobe and Deisenhofer, 1994). Proteins containing LRRs include tyrosine kinase receptors, cell-adhesion molecules, virulence factors, and extracellular matrix-binding glycoproteins, and are involved in a variety of biological processes, including signal transduction, cell adhesion, DNA repair, recombination, transcription, RNA processing, disease resistance, apoptosis, and the immune response (Rothberg et al., 1990).

On chromosome 3 the two genes flanking on the either side of the transposon enhancer at ~3.0 kb are an F-box family protein (At3g10990) and a wound-responsive protein (At3g10985). F-box proteins are proteins containing at least one F-box motif, a protein structural motif of about 50 amino acids that mediates protein-protein interactions. The first identified F-box protein is one of three components of the SCF complex, which mediates ubiquitination of proteins targeted for degradation by the proteasome. F-box motifs commonly exist in proteins in concert with other protein-protein interaction motifs such as leucine-rich repeats and WD repeats (Kipreos and Pagano, 2000). F-box proteins have also been associated with cellular functions such as signal transduction and regulation of the cell cycle cell cycle (Craig and Tyers, 1999). In plants, many F-box proteins are represented in gene networks broadly regulated by microRNA-mediated gene silencing via RNA interference (Jones-Rhoades, 2006). The other gene encoding a wound responsive protein (At3g10985) is annotated as a senescence-associated gene whose expression is induced in response to treatment with Nep1, a fungal protein that causes necrosis. It is also a stress responsive gene and contains a wound-induced domain WI12. This family consists of several plant wound-induced protein sequences related to WI12 from *Mesembryanthemum crystallinum* (common ice plant). Wounding, methyl jasmonate, and pathogen infection is known

to induce local WI12 expression. WI12 expression is also thought to be developmentally controlled in the placenta and in developing seeds. WI12 preferentially accumulates in the cell wall and it has been suggested that it plays a role in the reinforcement of cell wall composition after wounding and during plant development (Yen et al., 2001).

In *AT5* and *AT6* the activation tag insert was found on chromosome 1 at positions 25,687,382 and 25,687,405 respectively, which was a difference of just 23 bps, with different orientations of ATag insertion. This also explains the similar looking phenotype of the *AT5* and *AT6* mutants, supporting that similar gene(s) might be overexpressed in the mutants. On one side of the ATag inserts the gene encodes a putative zinc finger (JAG) transcription factor family protein (At1g68480) on the other side the other gene encodes an exostosin family protein (At1g68470). The zinc finger protein is ~4 kb away from the transposon enhancer in *AT5*, whereas in *AT6* it is only ~2 kb away. The exostosin gene is further away (~5.4 kb) from the transposon enhancer in *AT5* and ~7.4 kb away in *AT6* mutant. Considering the distance from the transposon enhancer, we suppose that the zinc finger protein is a likely candidate for being overexpressed under the effect of the transposon enhancer in both the mutants.

The zinc finger (JAG) protein (At1g68480) is annotated as a transcription factor that is necessary for proper lateral organ shape and is sufficient to induce the proliferation of lateral organ tissue. Together with NUB, it is involved in stamen and carpel development and contains a zinc finger with classical (C2H2) type domain.

In *atal*, two genes flank the transposon enhancer at ~4.0 kb distance, one encodes a dehydration responsive protein (At3g03370) and the other a short-chain dehydrogenase reductase (SDR) family protein (At3g03350). The dehydration responsive gene (At3g03370) contains a DUF 248 domain encoding a protein of unknown function, with some members of this family being putative methyltransferases. The SDR gene contains two domains of glucose/ribitol dehydrogenase and short chain dehydrogenase/reductase. The short-chain dehydrogenases/reductases family (SDR) (Jörnvall et al., 1995) is a very large family of enzymes, most of which are known to be NAD- or NADP-dependent oxidoreductases. The dehydration responsive gene was annotated as an expressed protein at the first time we searched for the flanking genes. Hence we decided to make the overexpression construct with 35S-promotor and

the *SDR* gene and transformed into Arabidopsis, but the transformants did not display the *atal* phenotype. When the other gene was thereafter annotated as a dehydration responsive gene, we hypothesized that this might actually be the gene that confers the *atal* mutant phenotype. Further overexpression studies with the dehydration responsive gene in Arabidopsis would help to identify the gene responsible for the *atal* phenotype. Genevestigator expression data analysis (https://www.genevestigator.ethz.ch/; Zimmermann et al., 2004) shows that the dehydration responsive gene (At3g03370) is active during the late vegetative stage, internode elongation stage and during early reproductive stage (flowering, silique and seed formation). It's expression is found in pollen, hypocotyls and xylem. However no induction was found under any kind of abiotic stress or hormone activity.

In *vajra*, the transposon is surrounded by a cluster of genes annotated as cinnamyl alcohol dehydrogenase (CAD) like protein. The closest gene to the transposon enhancer is a pseudogene at ~2.4 kb distance, next to it a CAD-like gene (At1g09490) at a distance of ~2.8 kb from the enhancer. The other two CAD-like genes on the either side of the transposon enhancer are ~5.5 kb away. The RT-PCR results showed that the transcription of the pseudogene and the two CAD-like genes At1g09490 and At1g09500 was upregulated, unlike the other CAD-like gene At1g09480, whose transcription level was not detected. Hence, we made an overexpression construct with the At1g09490 gene and transformed it into Arabidopsis, but the transformants did not display the *vajra* phenotype. It could be that the other CAD-like gene (At1g09500) might be responsible for the *vajra* phenotype or the co-expression of both the CAD-like genes is required. Further, overexpression analysis with At1g09500 in Arabidopsis would help identify the gene(s) responsible for the *vajra* phenotype.

The cinnamyl-alcohol dehydrogenase (CAD) like gene contains a domain of NAD-dependent epimerase/dehydratase. This family of proteins utilizes NAD as a cofactor, normally using nucleotide-sugar substrates for a variety of chemical reactions. The gene is probably involved in lignin biosynthesis. Genevestigator expression data analysis shows that the At1g09500 gene is expressed during the inflorescence stage and later during seed maturation and , also expressed in the sepals. Its expression is most strongly induced by osmotic stress (24x) and pathogens (7-145x) as well as by ABA (10x) potassium (6x) and NaCl stress (4x). The expression

pattern of the other CAD-like gene (At1g09490) was found to be similar with At1g09500, with higher basal levels and expression induced during the rosette maturation stage and the entire inflorescence stage and silique formation. Its expression was specifically found in sepals and senescent leaf. It was induced similarly to the other, but with higher basal levels, the induction level was lower, being more that 2x under heat and osmotic stress.

Overexpression of the *atal* and *vajra* gene in Arabidopsis confers drought resistance and salt tolerance, two abiotic stress components of desiccation tolerance (Verslues et al., 2006). Also they give increased WUE under drought stress by transpiring less water. The *atal* and *vajra* plants were also able to tolerate and survive very low soil water content (9.54% and 7.12% respectively) compared to the WT (12.7%) (Fig. 4A), due to lower transpirational water loss. They also showed less decrease in biomass under drought stress compared to the WT (Fig. 6C)

atal and vajra also conferred increased salt tolerance in Arabidopsis. Many physiological studies have demonstrated that Na^+ toxicity is not only due to toxic effects of Na^+ in the cytosol, but also because K^+ homeostasis is disrupted possibly due to the ability of Na^+ competing for K^+ binding sites. In Arabidopsis, both *atal and vajra* showed less Na^+ accumulation in the cytoplasm and increased levels of K^+ both under non-stress and stress conditions maintaining a higher K^+/Na^+ ratio, a mechanism well known for maintaining cell homeostasis and thereby reducing Na^+ toxicity in the cell (Zhu, 2001). According to the Genevestigator analysis (Zimmermann et al., 2004), the expression of the *vajra* candidate gene At1g09500 is induced by K^+, NaCl and other osmotic stresses supporting its involvement in protection to osmotic stress created by Na^+ toxicity and drought. It is therefore possible that the At1g09500 gene is a good candidate in expressing the phenotype in the mutant *vajra*.

Our current understanding of *atal* and *vajra* providing drought and salt tolerance in Arabidopsis indicates that *atal* and *vajra* might be involved in providing abiotic stress tolerance by maintaining cell homeostasis. The inducibility of the candidate genes for the *vajra* mutant suggests a role of the genes in stress induction pathways and thus a biological function under this environmental challenge. However the action of *atal* and the candidate genes in stress tolerance is not very clear, and needs to be investigated further. How *atal* is able to accumulate less Na^+ compared to the WT and also maintain higher K^+/Na^+ ratio under salt stress is still to be

investigated. Further characterization of *vajra* and *atal* in relation to osmotic stress, by metabolic analysis of the levels of osmoprotectants like proline in the cells, which is known to increase ability of plants to tolerate high salinity and drought (Delauney et al., 1993), could help to shed some light on their physiological role in providing osmotic stress. Moreover further characterization of other genes identified in the *AT* drought resistant mutants will provide a better understanding of the mechanisms involved in drought stress resistance and provide insights for other abiotic stresses.

Chapter 6

General Discussion

Abiotic stresses like drought, salinity, cold, heat, flooding, heavy metal toxicity and oxidative stress causes losses worth hundreds of million dollars each year due to reduction in crop productivity and crop failure. Among these stress factors, drought and salinity affect more than 10 percent of arable land. Desertification and salinization are rapidly increasing on a global scale declining average yields for most major crop plants by more than 50 percent (Bray et al., 2000). Hence it is important to develop stress tolerant crops.

Drought, high salinity and low temperature are very complex stimuli that possess many different, yet related, attributes, each of which may provide the plant cell with quite different information. Understanding the mechanisms by which plants perceive environmental signals and transmit these signals to their cellular machinery to activate adaptive responses is of fundamental importance to biology. Knowledge about stress signal transduction is also vital for continued development of rational breeding and transgenic strategies to improve stress tolerance in crops.

Traditional breeding strategies that have attempted to utilize genetic variation arising from varietal germplasm, interspecific or intergeneric hybridization, induced mutations and somaclonal variation of cell and tissue cultures, have shown to be difficult (Flowers and Yeo, 1995). Traditional approaches are limited by the complexity of stress tolerance traits, low genetic variance of yield components under stress conditions and the lack of efficient selection techniques (Ribaut et al., 1997; Carla Frova, 1998). Furthermore, quantitative trait loci (QTLs) that are linked to tolerance at one stage in development can be different from those linked to tolerance at other stages (Foolad, 1999). Once identified, desirable QTLs can require extensive breeding to restore the undesirable traits that came along with the introgressed tolerance trait by linkage drag, especially when the stress tolerance was introduced from wild germplasm.

Present genetic engineering strategies rely on the transfer of one or several genes that are either involved in signaling and regulatory pathways, or that encode enzymes in pathways leading to the synthesis of functional and structural protectants, such as osmolytes and antioxidants, or that encode stress-tolerance-conferring proteins. The current efforts to improve plant stress tolerance by gene(s) transformation have resulted in important achievements (Vinocur and Altman, 2005). However, the nature of the genetically complex mechanisms of abiotic stress tolerance, and the potential detrimental side effects, make this approach also difficult. Various

engineering strategies to enhance plants adaptive response to such environmental stress have been proposed (Cushman and Bohnert, 2000), which indicate the complexity of the stress adaptation and the scope for improvement in area of biochemical and signaling pathways.

One of these strategies is the use of transcription factors (TFs) in engineering stress tolerant plants, which could be a rapid and convenient method. Significant improvement of stress tolerance was found upon overexpression of single TFs in engineered Arabidopsis plants (Jaglo-Ottosen et al., 1998; Thomashow, 1998; Kasuga et al., 1999; Gilmour et al., 2000). Some TFs with different stress tolerant phenotypes regulate the same set of stress-responsive genes, which is also reflected by the significant overlap of the gene-expression profiles induced in response to different stresses (Bohnert et al., 2001; Seki et al., 2001; Chen et al., 2002; Fowler and Thomashow, 2002). Members of the CBF/DREB1 family, such as CBF1, CBF2, and CBF3 (or DREB1B, DREB1C, and DREB1A, respectively) are themselves stress-inducible. DREB/CBF proteins are encoded by AP2/ERF multigene families and mediate the transcription of several genes such as *RD29A*, *RD17*, *COR6.6*, *COR15A*, *ERD10*, *KIN1*, *KIN2* and others in response to cold and water stress (Ingram and Bartels, 1996; Stockinger et al., 1997; Gilmour et al., 1998; Liu et al., 1998; Seki et al., 2001; Thomashow et al., 2001). However, the functions of many TFs can be redundant, as was shown for CBF1, CBF2 and CBF3 which when overexpressed or mutated showed identical profiles of induced genes indicating their similar biological function (Gilmour et al., 2004). This suggests that to characterize and test different mechanisms for stress tolerance, we have to look at more diverse TF genes than members of the same family that have been analyzed.

Although plant transformation with stress-responsive TFs permits overexpression of multiple downstream stress-associated genes, it may also activate additional genes that adversely affect the normal agronomic characteristics of a crop and which are not required for the tolerance mechanism/phenotype. One common negative effect of TF-modified plants is the growth retardation under non-stress conditions in transgenic plants that constitutively express TFs (Kasuga et al., 1999; Hsieh et al., 2002; Kang et al., 2002; Abe et al., 2003). In this thesis, we describe the use of *AP2/ERF* TFs genes (chapter 2 and 3), which when overexpressed provide enhanced drought and salt tolerance in the model plant Arabidopsis as well as enhanced salt tolerance in rice without growth retardation or yield penalties both under non-stressed and

stressed condition.

In Chapter 2, we describe the analysis of an Arabidopsis gene *HARDY* (*HRD*), an AP2/ERF-like TF, which when overexpressed in a gain-of-function mutant, shows an increase in number of leaf mesophyll layers, extended root branching and root thickness. The overexpression of *HRD* in Arabidopsis gives drought tolerance to plants, increases their WUE and maintains growth under drought stress due to less transpirational water loss, a more stable and enhanced cell wall integrity (Karaba et al., 2007) and a robust root system that can harvest scarce water from the soil. The root traits such as its biomass, length, density and depth have been proposed as the main drought avoidance traits that contribute to seed yield under terminal drought environments (Ludlow and Muchow, 1990). Another important trait under water deficit conditions is water use efficiency (WUE), the ratio of biomass produced to the water lost by transpiration (Condon et al., 2004). Variation in WUE can be brought about by changes in either biomass or transpirational water loss. The higher mesophyll efficiency of *HRD* leaves could lead to increased photosynthesis, which helps the plant to maintain its biomass under stress conditions.

HRD overexpression also confers enhanced salt tolerance both in Arabidopsis and rice by reduced Na^+ accumulation in the cytoplasm and by maintaining a higher K^+/Na^+ ratio, which mechanism is well known for maintaining cell homeostasis and thereby reducing Na^+ toxicity (Zhu, 2001). A high K^+/Na^+ ratio in the cytosol is essential for normal cellular functions of plants. Na^+ competes with K^+ uptake through Na^+-K^+ cotransporters, and may also block the K^+ –specific transporters of root cells under salinity (Zhu, 2003). This results both in toxic levels of Na^+ as well as in an insufficient K^+ concentration for enzymatic reactions and osmotic adjustment.

The overexpression of *HRD* in rice generates plants with significantly higher biomass, independent of salt stress. Higher biomass in *HRD* rice is attributed to its ability to photosynthesize better under both non stress and osmotic stress conditions due to enhanced mesophyll growth and an intact PSII system (Karaba et al., 2007). *HRD* overexpression increases root biomass under salt stress, indicating an ability to adapt by inducing roots to avoid or minimize the osmotic stress shock, which is created due to higher salt concentration in the soil water, which limits water absorbance. Most of the *HRD* lines monitored in rice showed increase in seed yield (21%, 26% and 46%) under non-stress conditions compared to the WT and under

150mM salt stress they showed 50% seed set in contrast to the WT, which failed to set seeds.

HRD overexpression in Arabidopsis also results in resistance to the fungal wilt pathogen Verticillium, which affects the roots of plants, probably by activating a general stress resistance mechanism. The *HRD* gene in Arabidopsis is expressed in inflorescence tissue including petals, inflorescence stems, in mature seeds and in pollen. Therefore, the gene is probably involved in the maturation of the inflorescence, which structure requires protection against desiccation. Thus, the Arabidopsis *HRD* gene, normally active in the inflorescence tissue, exhibits by ectopic expression a novel vegetative stage phenotype of enhancement of root and leaf structure, resulting in an adaptive mechanism for drought resistance and WUE (Condon et al., 2004).

In chapter 3 we studied another AP2/ERF-like TF Arabidopsis gene called *SHINE* (*SHN/WIN*) in relation to abiotic stress, which when overexpressed increases epicuticular wax and alters epidermal properties in Arabidopsis resulting in the *shn* glossy leaf surface phenotype (Aharoni et al., 2004). Plants loose water in the process of transpiration, either through the stomata, allowing movement of gases and water vapor, or the cuticle, a hydrophobic heterogeneous layer comprising of lipids, cutin and intra-cuticular wax. Although stomatal water loss accounts for most of the transpiration loss, the amount of water lost through the cuticle (cuticular transpiration) is significant when stomata close due to a stress response (Chaves and Oliveira, 2004). The variation for cuticular wax in rice has been suggested to be related to drought resistance (O'Toole and Cruz, 1980; Haque et al., 1992), indicating that increase in wax or change in its composition might be used to improve water use and drought resistance.

Plants overexpressing *SHN1/WIN1* in Arabidopsis were altered in the cuticle layer leaving the cuticle more permeable to water loss and in addition displayed a reduced stomatal density. However, overexpression of *SHN1* in Arabidopsis enhances drought resistance and increase water use efficiency (WUE) by reducing transpirational water loss probably due to the lower stomatal density. Enhanced drought tolerance was described in rice overexpressing Arabidopsis *SHN2* (Trijatmiko, 2005; Karaba, 2007), although the rice *SHN2* overexpressor plants did not exhibit any visible wax phenotype (Trijatmiko, 2005). However, overexpression of Arabidopsis *SHN1* in tomato showed an increase in total epicuticular wax and a decrease in cuticular permeability of the leaves and fruits. Furthermore the transformants displayed an increase in WUE compared to

the WT (Karaba, 2007). These results in Arabidopsis, rice and tomato helped dissecting the role of epicuticular wax in drought resistance of *SHN* overexpressing plants and indicated that the epicuticular wax amount is probably not essential for drought resistance. However, probably changes in specific components of the complex cuticular wax layer determine the permeability of the cuticle. Rice has an epicuticular wax network due to covalent modification of aldehydes which are the primary rice wax component, and this makes analysis by simple extraction in chloroform not feasible (Haas et al., 2001). As *SHN* primarily increases the alkane component of waxes (Aharoni et al., 2004), an analogous change in rice would alter the covalent wax network as alkanes are less reactive than the normally in rice present aldehydes. These studies are underway using different extraction procedures for rice wax (Jetter and Pereira, pers. commun.). The increase in WUE indicates that the plant can accumulate more carbon for growth using less water. The drought resistance and increase in WUE observed in Arabidopsis (chapter 3) and in rice (Karaba et al., 2007) did not result in a decrease, but rather in an increase in total biomass due to reduced stomatal density under noth non-stress and stress conditions compared to the WT.

Overexpression of *SHN1* in Arabidopsis and *SHN2* in rice enhanced salt tolerance also by maintaining higher K^+/Na^+ ratio and higher Ca^{2+} and K^+ levels under salt stress condition thereby maintaining cell homeostasis. Ca^{2+} plays an important role in providing salt tolerance to plants. Externally supplied Ca^{2+} reduces the toxic effects of NaCl, presumably by facilitating higher K^+/Na^+ selectivity (Cramer et al., 1987; Liu and Zhu, 1998). High salinity results in increased cytosolic Ca^{2+} that is transported from the apoplast as well as from the intracellular compartments (Knight et al., 1997). This transient increase in cytosolic Ca^{2+} initiates the stress signal transduction leading to salt adaptation. The rice transformants also displayed similar mechanisms as observed in Arabidopsis under salt stress and showed increased biomass and yield (20-30%) under salt stress condition. The increased membrane stability under salt stress condition in *SHN* overexpressors demonstrates their increased capacity to avoid membrane damage due to osmotic stress and helps stabilizing the underlying photosynthetic apparatus.

In chapter 2 and 3 we studied the effects of overexpressing two AP2/ERF-like TF's in mediating drought resistance and salt tolerance in plants without yield penalties under stress conditions compared to the WT. This exemplifies the importance of use of TF's in complex stress

related studies and in unraveling some of the mechanisms through which plants acquire stress tolerance. Most transcription factors don't work alone. Often for gene transcription to occur, a number of transcription factors must bind to DNA regulatory sequences. This collection of transcription factors in turn recruits intermediary proteins such as cofactors that allow efficient recruitment of the pre-initiation complex and RNA polymerase. Thus, for a single transcription factor to initiate transcription all of these other proteins must also be present and the transcription factor must be in a state where it can bind to them if necessary. Hence, TF-cofactors play an equally important function as the TFs themselves. In chapter 4, we characterized an Arabidopsis DNA-binding gene called *BOUNTIFUL* (*BFL*), which functions as a TF-cofactor, in relation to increase in biomass and stress tolerance when overexpressed. *BFL* overexpression in Arabidopsis displays a distinct and robust plant phenotype by producing larger and more rosette leaves and more lateral roots and siliques. The *BFL* overexpressing plants also display late flowering time compared to the WT; hence their phenotype resembles the late flowering phenotypes of several Arabidopsis mutants (Koornneef et al., 1991; El-Lithy et al., 2004). *BFL* overexpressors display a densely branched root structure due to elongation of the lateral roots; which may be a result of elevated auxin levels due to high *BFL* expression. The BFL lines also display enhanced salt tolerance and resistance to the wilt pathogen Verticillium in Arabidopsis. The resistance to Verticillium is probably due to the capacity of *BFL* overexpressing plants to produce more lateral roots and withstand the fungal growth, providing a continuous supply of water and nutrients to the plant, compared to wild-type where the roots get infected and are unable to support further plant growth. A better root system is related to higher seed yield and salt tolerance (Yasin Ashraf et al., 2005) possibly by avoiding or minimizing the osmotic stress shock which is created by the higher salt concentration in the soil water. *BFL* overexpression also provided salt tolerance in terms of plant growth by a reduced decrease in the total dry weight under different salt concentrations compared to the WT (Chapter 4). *BFL* overexpression affects the ion homeostasis in the cells by accumulating less Na^+ and maintaining higher Ca^{2+} and K^+ levels under salt stress condition compared to the WT. It also maintains a higher K^+/Na^+ ratio under salt stress. These factors play a crucial role in determining cell death due to Na^+ toxicity in the cell (Chinnusamy et al., 2005). Probably the salt overly sensitive (SOS) signaling pathway (Shi and Zhu, 2002) is activated under

BFL expression during salt stress, which helps maintaining ion homeostasis in the plant cells. Osmotic homeostasis can be achieved either by compartmentation of Na^+ into the vacuole or by biosynthesis and accumulation of compatible solutes (Zhu, 2002). *BFL* overexpressors exhibit a larger cell size, meaning larger cellular components including the vacuolar space, where it might be able to efflux Na^+ which prevents the occurrence of salt stress in the cell.

The salt stress tolerance, pathogen resistance and root/leaf structure phenotype suggest a coordinated mechanism of induced resistance conferred by overexpression of the *BFL* gene, with a potential value to crop plants. Strong overexpression of *BFL* gives a severe phenotype with stunted growth and very late flowering. Expressing *BFL* under the control of stress-inducible promoters may solve this problem. Microarray results showed induction of genes, which are involved in the plant response to salt and pathogens (abscisic acid, ethylene), which is in agreement with the salt tolerance and pathogen resistance of *BFL* overexpression in plants. Hence with ectopic BFL expression the plant can benefit by acquiring salt stress tolerance and higher biomass, giving general stress resistance to the plant.

Most signal transduction pathways in plant stress responses involve hundreds of interacting genes with redundant functions that are intractable through classical mutant analysis A significant limitation of classical loss-of function screens designed to dissect genetic pathways is that they rarely uncover genes that function redundantly, or which losses are compensated by alternative metabolic or regulatory circuits. The alternative pathways might have an additional role in early embryo or gametophyte development, which would lead to lethality of knockouts. To overcome the problem to genetically identify genes with functions useful for agriculture, a strategy to overexpress plant genes in a high-throughput way using activation tagging to select for stress tolerant lines was utilized (Chapter 5).

We used an efficient transposon-based activation tag library (Marsch-Martinez et al., 2002) that generates gain-of-function dominant phenotypes to positively select for phenotypes of interest. These 'Activation Tag' (**ATag**) transposon inserts contain a multiple enhancer of the CaMV35S promoter that can act on adjacent plant promoters (~10 kb) and enhance or activate their expression. Results show that about 3 % of the insertions cause dominant gain-of-function phenotype. In contrast the commonly used T-DNA-based activation tagging displays $1/10^{th}$ this

frequency of phenotypes (Weigel et al., 2000), probably due to multiple copy based gene silencing (Chalfun-Junior et al., 2003). In this study we have tested the hypothesis that mutants overexpressing stress resistance pathways display compromised fitness in terms of vegetative and reproductive growth, and that pre-selecting such mutants can lead to identification of abiotic stress tolerant genes. In a subset of 51 fitness-compromised lines of the activation tag population, we identified eight drought resistant mutants by high throughput screening for drought resistance. The frequency of 8 drought resistant mutants out of 51 or 16% from preselected mutants, and 1.3% (8/600 inserts) of inserts in the sub-population would make the screening of large genome saturating populations for drought stress feasible. This frequency is much higher for ATag mutations than predicted for this sub-population in earlier studies (Population No. 3 in Table III, Marsch-Martinez et al., 2002). We described in chapter 5 the putative tagged genes, which might be overexpressed in each mutant and which most likely is responsible for drought resistance. The identities of the genes as shown by their predicted annotation are: embryo specific protein, exostosin family protein, calcium binding protein, pyridoxine 5'-phosphate oxidase-related protein, purple acid phosphatase 28 (ATPAP 28), cytochrome c1 like protein, leucine-rich repeat (LRR) transmembrane protein kinase, F-box family protein, wound-responsive protein, putative zinc finger (JAG) transcription factor family protein, dehydration responsive protein, short-chain dehydrogenase reductase (SDR) family protein and cinnamyl alcohol dehydrogenase (CAD) like protein. All these genes are predicted to have a role in stress adaption like osmotic homeostasis, osmotic adjustment, stress damage control and repair, detoxification, growth and development control, membrane stability, oxidative stress, signal transduction, transcription, disease resistance, cell cycle regulation, seed maturation and lignin biosynthesis (Chapter 5). Two of the mutants named *atal* and *vajra* were further characterized and showed increased water use efficiency in Arabidopsis by decreasing transpirational water loss compared to the WT. They also give enhanced salt tolerance in Arabidopsis by accumulating less Na^+ in the cytoplasm and increased levels of K^+ both under non-stress and stress conditions maintaining a higher K^+/Na^+ ratio. Further characterization of all the genes identified in the drought resistant mutants will provide a better understanding of the mechanisms involved in drought stress resistance and provide insights for other abiotic stresses.

Conclusion and future prospects

The improvement of crop abiotic stress tolerance by classical breeding is hampered by difficulties because of the multigenic nature of this trait. Further complications arise from the large variability in stress sensitivity in different periods during the life cycle of a given plant. Among the various general types of plant response to salinity and drought stress, avoidance mechanisms mainly result from morphological and physiological changes at the whole-plant level. These are less amenable to practical manipulations. By contrast, tolerance mechanisms are caused by cellular and molecular biochemical modifications that lend themselves to biotechnological manipulation. All types of abiotic stress evoke cascades of physiological and molecular events and some of these can result in similar responses; for example, drought, high salinity and freezing can all be manifested at the cellular level as physiological dehydration (Verslues et al., 2006). A full elucidation of abiotic stress tolerance mechanisms, and an intelligent breeding strategy for stress tolerance, requires clear and fact-based answers to a number of questions. Which genes and proteins are upregulated or downregulated by the different types of abiotic stresses? What are the functions of these stress-responsive genes and proteins? Which genes can be used as genetic markers for the breeding and selection of stress-tolerant genotypes or successfully engineered in transgenic plants?

Plant genetic engineering strategies for abiotic stress tolerance (Wang et al., 2003) rely on the expression of genes that are involved in signaling and regulatory pathways (Seki et al., 2003; Shinozaki et al., 2003) or genes that encode proteins conferring stress tolerance (Wang et al., 2004) or enzymes present in pathways leading to the synthesis of functional and structural metabolites involved in stress tolerance (Apse and Blumwald, 2002; Rontein et al., 2002; Park et al., 2004). Current efforts to improve plant stress tolerance by genetic transformation have resulted in several important achievements (Vinocur and Altman, 2005); However, the genetically complex mechanism of abiotic stress tolerance makes the task difficult. For this reason, biotechnology should be fully integrated with classical physiology and plant breeding (Wang et al., 2003; Flowers, 2004). Nonetheless, marker-assisted selection of specific secondary traits that are indirectly related to yield (Ribaut et al., 1996; Ribaut et al., 1997), osmotic adjustment (Zhang et al., 1999), membrane stability (Frova et al., 1999a) or physiological tolerance indices (Frova et

al., 1999b) might prove increasingly useful as the resolution of the genetic maps of the major crops improve. This strategy could be used in combination with 'pyramiding' strategies or consecutive selection for, and accumulation of, physiological yield-component traits (Flowers and Yeo, 1995).

This research demonstrates the use of TFs identified from Arabidopsis (dicot) in engineering drought and salt tolerant plants, which when transferred to an agronomically important crop like rice (monocot), confers traits of economic value to the crop by improving abiotic stress resistance in them. Our study also shows that these genes in rice do not adversely affect the crop growth and yield with or without stress, which is very positive, since some earlier overexpression studies on TF genes exhibit negative effects on plants growth and development. However, these genes could be used in combination with other genes to induce different pathways. The use of drought inducible promoters to drive the expression of these genes might be useful to get an optimal level of expression at the right time and to avoid the negative effects of the use of a constitutive promoter on growth and yield. Also, in this study we successfully tested our hypothesis of preselecting mutant lines which showed poor growth/fitness in a transposon based activation tag population in identifying drought resistant mutants with much higher (16%) frequency than predicted earlier. This would make the screening of large genome-saturating mutant populations for drought stress feasible.

Perspectives

In carrying out studies on tolerance to abiotic stress, a number of factors should be taken into consideration. Firstly, we should keep in mind that a given tolerance-related mechanism should always be assessed with respect to its cross-talk with other stress-related genes/mechanisms. Secondly, most current molecular studies use short-term stress treatments, rather than observing the effects of stress over longer periods — conditions that more closely mimic the life span of most crops. As physiological and molecular responses during short and long exposures to stress could differ, conclusions regarding actual tolerance, which must involve agronomic factors such as biomass, yield data and survival, cannot always be drawn. Thirdly, cycles of stress and recovery from stress (e.g. rehydration) are the prevalent processes occurring

under natural conditions during different seasons, and under agricultural practices such as irrigation and salt leaching. Thus, the degree of recovery from stress, which also has its molecular basis, is as relevant as the response to stress.

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Appendix: I

Table 1. Life growth parameters of *bfl_D* and WT- Ws plants[a].

Genotype[a]	RL[b]	Flowers[c]	PLS[d]	SLS[e]	Siliques[f]	Seed weight [g] (g)
bfl-D homozygote	15.53 ± 0.63 *	68 DAS *	11.47 ± 0.50 *	4.27 ± 1.11	181.13 ± 20.88	66.04 ± 10.71 *
bfl-D heterozygote	14.00 ± 0.82	47 DAS *	9.00 ± 0.65 *	2.89 ± 1.43	283.44 ± 26.95	138.67 ± 13.82
WT	12.36 ± 0.65	26 DAS	5.93 ± 0.52	5.00 ± 1.14	219.00 ± 21.61	133.26 ± 11.08

* indicates values significant at (α<0.01) compared to the WT.

[a] Sample number for all measurements ranged from 15 to 17.

[b] Number of rosette leaves (RL) at the time of emergence of the first flower bud.

[c] Number of days after germination (DAG) at which 100% of the flowers were opened.

[d] Number of primary lateral shoots (PLS).

[e] Number of secondary lateral shoots (SLS).

[f] Number of siliques at maturity.

[g] Seed weight in grams.

Table 2. Root length (RL) and number of primary lateral roots (PLR) in *bfl-D* and WT plants 14 DAG.

Genotype	RL (cm)[a]	Total length of PLR			No. of PLR[a]	No. of PLR/cm[a]
		>2cm	0.1-2cm	<0.1cm		
bfl-D homozygote	6.14 ± 0.48	40.3%	32.5%	27.2%	24.64 ± 3.18	4.11 ± 0.61
bfl-D heterozygote	6.67 ± 0.97	100%	-	-	29.67± 4.53	4.56 ± 0.52
WT	9.32 ± 0.35	2.1%	72.0%	25.9%	37.12 ± 3.21	3.83 ± 0.23

[a] Average and standard error values are reported.

Appendix II

A. Proteins that showed similarity to BFL protein in other crops

Oryza sativa AHL1 (OsAHL1), AK068379;

Oryza sativa AHL2 (OsAHL2), AK105419;

Zea mays AHL1 (ZmAHL1), AY106980;

Zea mays AHL2 (ZmAHL2), AY105124;

Pisum sativum AHL1 (PsAHL1), T06584).

B. Proteins that showed similarity to BFL protein in Arabidopsis

AHL1 (At4g12080), NP_192945

AHL2 (At4g22770), NP_194008;

AHL3 (At4g25320), NP_194262;

AHL4 (At5g51590), NP_199972;

AHL5 (At1g63470), NP_176536;

AHL6 (At5g62260), NP_201032;

AHL7 (At4g00200), NP_191931;

AHL8 (At5g46640), NP_199476;

AHL9 (At2g45850), NP_182109;

AHL10 (At2g33620), NP_565769;

AHL11 (At3g61310), NP_191690;

AHL12 (At1g63480), NP_176537;

AHL13 (At4g17950), NP_567546;

AHL14 (At3g04590), NP_187109;

AHL15 (At3g55560), NP_191115;

AHL16 (At2g42940), NP_181822;

AHL17 (At5g49700), NP_199781;

AHL18 (At3g60870), NP_191646;

AHL19 (At3g04570), NP_566232;

AHL20 (At4g14465), NP_567432;

AHL21 (At2g35270), NP_181070;

AHL22 (At2g45430), NP_182067;

AHL23 (At4g17800), NP_193515;

AHL24 (At4g22810), NP_194012;

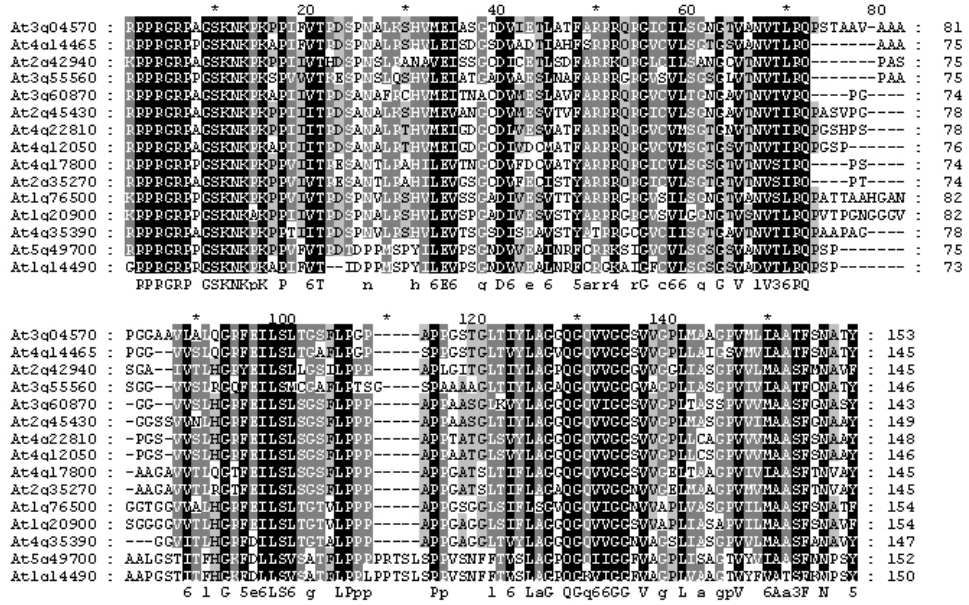
AHL25 (At4g35390), NP_195265;

AHL26 (At4g12050), NP_192942;

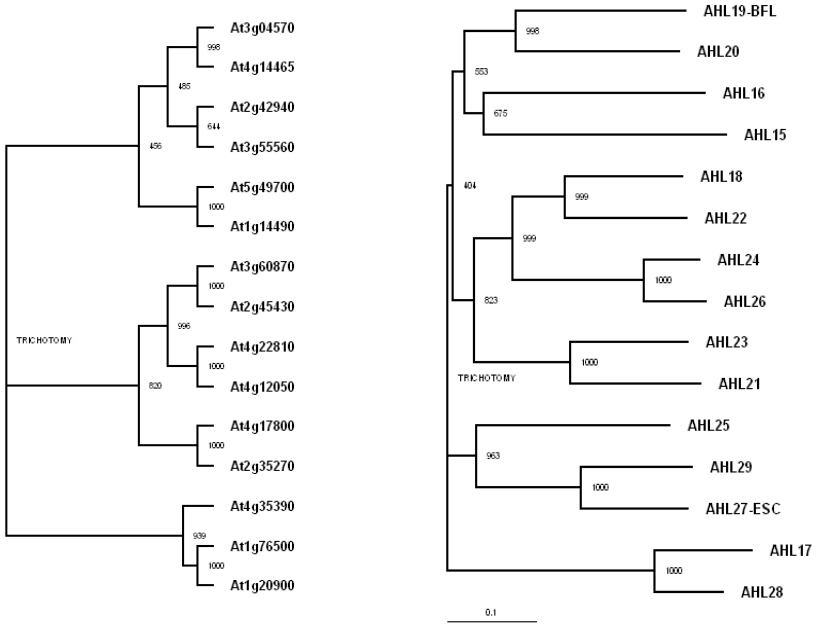
AHL27 (At1g20900), NP_173514;

AHL28 (At1g14490), NP_172901;

AHL29 (At1g76500), NP_177776



C. Phylogenetic analysis of the BFL protein and other closely related paralogs



Summary

Abiotic stress is the principal cause of crop failure world wide, among which drought and salinity affect more than 10 percent of arable land, reducing average yields for most major crop plants by more than 50 percent. Understanding plant tolerance to drought and salinity and improving crops capacity to withstand such stress is therefore of fundamental importance.

The use of transcription factors (TFs) in engineering stress tolerant plants is a rapid and convenient method compared to traditional breeding strategies. However, plants overexpressing stress-responsive TFs, although exhibiting stress tolerance, often cause growth retardation which adversely affects the normal agronomic characteristics of the crop. In this thesis, we identified two AP2/ERF TF genes from the model plant Arabidopsis which induced enhanced drought and salt tolerance when overexpressed in Arabidopsis. When these genes were expressed in rice they induced enhanced salt tolerance without growth retardation or yield penalties both under non-stressed and stressed conditions.

The *HARDY* (*HRD*) TF gene was discovered via a gain-of-function Arabidopsis mutant which shows an increase in leaf mesophyll layers and extended root branching and thickness. The overexpression of *HRD* in Arabidopsis conferred drought tolerance to plants, and maintained growth under drought stress due to less transpirational water loss, more stable and enhanced cell wall integrity and a robust root system harvesting scarce water from the soil (Chapter 2). Root traits such as biomass, length, density and depth have been proposed as the main drought avoidance traits to contribute to seed yield in terminal drought environments. Another important crop trait under water deficit conditions is water use efficiency (WUE), the ratio of biomass produced to the water lost by transpiration. *HRD* overexpression in Arabidopsis conferred higher WUE under drought, which supports the results in rice. The higher mesophyll efficiency of the *HRD* overexpressors lead to increased photosynthesis which helps the plant to maintain its biomass under stress conditions. *HRD* overexpression also conferred enhanced salt tolerance both in Arabidopsis and rice by less Na^+ accumulation in the cytoplasm and maintaining a higher K^+/Na^+ ratio, a mechanism well known for maintaining cell homeostasis and thereby reducing Na^+ toxicity in the cell. The overexpression of *HRD* in rice resulted in plants with significantly

higher biomass and seed yield both in non-stressed as well as salt stress conditions, showing 50% seed set under 150mM NaCl stress as compared to failure of seed set in the WT. *HRD* overexpression increased root biomass under salt stress which is viewed as an adaptive response in combating stress. *HRD* overexpression in Arabidopsis also conferred resistance to the fungal wilt pathogen Verticillium, which affects the roots of plants, probably by activating a general stress resistance mechanism.

Another AP2/ERF-like TF Arabidopsis gene called *SHINE* (*SHN*) was studied in relation to abiotic stress. Overexpression of *SHN* increased epicuticular wax and altered epidermal properties in Arabidopsis. Under water stress plants lose water in the process of transpiration mostly through the cuticle since drought induces reduced stomatal conductance and stomatal closure. Therefore, decreasing transpiration by changing plant cuticular properties (eg. increase in epicuticular wax content) can be used to improve water use and drought resistance in plants. Plants overexpressing *SHN1* in Arabidopsis were altered in the cuticle layer leaving the cuticle more permeable to water loss and displayed a reduced stomatal density. Overexpression of the *SHN1* gene in Arabidopsis enhanced drought resistance and increased water use efficiency (WUE) by reducing transpirational water probably due to the lower stomatal density (Chapter 3) indicating that epicuticular wax amount is probably not essential for drought resistance. Overexpression of *SHN1* in Arabidopsis and *SHN2* in rice induced enhanced salt tolerance by maintaining higher K^+/Na^+ ratio and higher Ca^{2+} and K^+ levels under salt stress conditions thereby maintaining cell homeostasis. The rice transformants also displayed similar mechanisms as observed in Arabidopsis under salt stress and showed increased biomass and seed yield (20-30%) under salt stress conditions compared to WT.

Another Arabidopsis gene studied was called *BOUNTIFUL* (*BFL*), a DNA-binding transcriptional cofactor. *BFL* overexpression in Arabidopsis displayed a robust plant phenotype with a distinct plant architecture of larger and more rosette leaves, lateral roots and siliques (Chapter 4). The *BFL* overexpression mutant also displayed a delay in flowering time compared to the WT; hence its phenotype could be related to the late flowering phenotypes observed in several Arabidopsis mutants. *BFL* overexpression however also enhanced cell expansion which leads to larger leaf size contributing to the biomass accumulation. *BFL* overexpression resulted in

a densely branched root structure due to elongation of the lateral roots. This enhanced root network probably contributes to its phenotype of salt tolerance and resistance to the pathogen Verticillium. BFL overexpressors showed ion homeostasis in the cell under salt stress by accumulating less Na^+ and maintaining higher Ca^{2+}, K^+ and K^+/Na^+ ratio compared to the WT. Plant growth (as measured by plant dry weight) was less severely affected by saline conditions in the *BFL* overexpressors than in the WT. The salt stress tolerance, pathogen resistance and root/leaf structure phenotype signifies a coordinated mechanism of induced resistance conferred by overexpression of the *BFL* gene, with potential adaptive value to crop plants.

The primary tool for dissecting genetic pathways has been the screen for loss-of-function mutations that disrupt such a pathway. However, a limitation of loss-of-function screens is that they rarely identify genes that act redundantly. To overcome these problems to genetically identify genes with functions useful for agricultural traits, a strategy to overexpress plant genes using transposon-based activation tagging in a high-throughput way was utilized (Chapter 5). These activation tag transposon inserts contain a multiple enhancer of the CaMV35S promoter that can act on adjacent plant promoters and enhance or activate their expression. In Chapter 5 we have tested the hypothesis that mutants overexpressing stress resistance pathways display compromised fitness in terms of vegetative and reproductive growth, and that pre-selecting such mutants can lead to identification of abiotic stress tolerant genes. We thus selected a subset of 51 fitness compromised mutant lines out of 1200 lines from the activation tag population and screened them for drought resistance. We identified eight drought resistant mutants, and describe the putative tagged genes in each mutant with a potential role in stress adaptive responses (Chapter 5). Two of the mutants named *atal* and *vajra* were further characterized and show increased WUE in Arabidopsis by decreasing transpirational water loss compared to the WT. They also give enhanced salt tolerance in Arabidopsis by accumulating less Na^+ in the cytoplasm and increased levels of K^+ both under non-stress and stress conditions maintaining a higher K^+/Na^+ ratio. Our results demonstrate that preselecting mutant lines which showed poor growth/fitness in a transposon based activation tag population enables the identification of drought resistant mutants with much higher (15.68%) frequency than predicted earlier. This would make the screening of large genome-saturating populations for drought stress feasible.

The genetically complex mechanism of abiotic stress tolerance makes improvement of crops for this trait difficult. Current efforts to improve plant stress tolerance by genetic transformation have resulted in several important achievements. For this reason, biotechnology should be fully integrated with classical physiology and breeding. The research presented in this thesis demonstrates the use of Transcription Factors that were found to confer drought and salt tolerance in Arabidopsis (dicot) which when transferred to an agronomically important crop like rice (monocot), exhibit increased biomass and seed yield as well as improved stress tolerance. These results offer possibilities to improve a wide variety of crops for a combination of abiotic stress tolerance traits providing stability to crop production with increased economic value in a changing environment.

Samenvatting

Abiotische stress is de voornaamste oorzaak van gewasverliezen in de wereld. Droogte en hoge zoutgehaltes zijn een probleem op meer dan 10% van de landbouwgronden waarbij de gemiddelde opbrengsten van de belangrijkste gewassen met meer dan 50% worden gereduceerd. Het is daarom van fundamenteel belang om de wijze waarop planten droogte en hoge zoutconcentraties kunnen weerstaan beter te leren begrijpen en met deze kennis de weerstand van gewassen tegen deze vormen van stress te vergroten. Het gebruik van Trancriptie Factoren (TF) om de weerstand van planten tegen stress te vergroten is een snelle en relatief eenvoudige strategie in vergelijking met traditionele veredelingsstrategieën. Overexpressie van een TF, die betrokken is bij de stress reactie van de plant leidt echter, naast verbeterde weerstand tegen stress, vaak ook tot verminderde groei, wat een nadelig effect heeft op de agronomische eigenschappen van een gewas. In dit proefschrift worden twee AP2/ERF TF genen van de modelplant *Arabidopsis thaliana* beschreven, die een verhoogde weerstand tegen droogte en hoge zoutgehaltes gaven, wanneer ze tot overexpressie werden gebracht. Overexpressie van deze genen zorgde ook in rijst voor een hogere tolerantie voor zoutstress zonder de negatieve bijeffecten van vertraagde groei of verminderde opbrengst zowel onder normale als onder stress omstandigheden.

Het *HARDY* (*HRD*) TF gen werd ontdekt met behulp van een “gain of function” Arabidopsis mutant, die een toename van mesofyl in het blad vertoonde, samen met een verhoogd aantal vertakkingen van de wortels, en dikkere wortels. Overexpressie van *HRD* in Arabidopsis had een verhoogde tolerantie voor droogte tot gevolg zonder dat dit ten koste ging van de groei tijdens droogtestress. Dit wordt mogelijk gemaakt door verminderd waterverlies als gevolg van verdamping, een stabielere en verbeterde celwandintegriteit, en een robuust wortelstelsel dat in staat is het weinige water te onttrekken uit de droge grond (hoofdstuk 2). Worteleigenschappen zoals biomassa, lengte, dichtheid en diepte worden beschouwd als de voornaamste droogte-vermijdende eigenschappen, die bijdragen aan zaadopbrengst in zeer droge omgevingen. Een andere belangrijke gewaseigenschap in omstandigheden met watertekort is “Water Use Efficiency” (WUE), de verhouding tussen de geproduceerde biomassa en verlies van water door verdamping. Overexpressie van *HRD* in Arabidopsis gaat gepaard met een hogere WUE tijdens droogtestress. Dit resultaat ondersteunde wat bij rijst werd gevonden. De verbeterde mesofyl

efficiëntie van de *HRD* overexpressors zorgde voor een verhoogde fotosynthese, wat de plant in staat stelt haar biomassa te behouden onder stress condities. *HRD* overexpressie zorgt ook voor verbeterde tolerantie voor zoutstress in zowel Arabidopsis als in rijst als gevolg van verminderde Na^+ ophoping in het cytoplasma en behoud van een hogere K^+/ Na^+ ratio. Dit is een bekend mechanisme om de cel homeostase te handhaven en daarmee de toxiciteit van Na^+ te verminderen. Overexpressie van *HRD* in rijst resulteerde in planten met een significant hogere biomassa en zaadopbrengst zowel onder normale condities als tijdens condities met hoge zoutgehaltes, waarbij in de overexpressors onder condities van 150mM NaCl nog 50% van de zaadzetting behouden bleef, terwijl in de wild type (WT) planten helemaal geen zaad meer werd geproduceerd. *HRD* overexpressie verhoogde de biomassa van de wortels bij verhoogde zoutgehaltes, wat beschouwd wordt als een aanpassingsreactie om stress te weerstaan. Arabidopsis planten werden als gevolg van *HRD* overexpressie ook resistent tegen het pathogeen Verticillium, dat met name de wortels van de planten aantast door mogelijk een algemeen stress resistentiemechanisme te activeren.

Een tweede AP2/ERF TF Arabidopsis gen *SHINE* (*SHN*) is bestudeerd in relatie tot abiotische stress. Overexpressie van *SHN* verhoogt de epicuticulaire waslaag en verandert de epidermale eigenschappen van Arabidopsis planten. Tijdens watergebrek verliezen planten voornamelijk water via verdamping door de opperhuid; droogte induceert daarom een verminderde stomatale geleidbaarheid en sluiting van de stomata. Verminderde verdamping door middel van het veranderen van de opperhuid eigenschappen van de plant (door toename van de epicuticulaire waslaag) kan daarom gebruikt worden als mechanisme om watergebruik en daarmee droogteresistentie van de plant te verbeteren. Arabidopsis planten met overexpressie van *SHN1* vertoonden een verbeterde resistentie tegen droogte, en een verhoogde WUE als gevolg van een verminderd verlies van water door verdamping, hoogstwaarschijnlijk door een verlaagde stomatale dichtheid (hoofdstuk 3). Dit suggereert dat de hoeveelheid epicuticulaire was niet essentieel is voor droogteresistentie van deze overexpressor. Overexpressie van *SHN1* in Arabidopsis en van *SHN2* in rijst resulteerde in verhoogde tolerantie tegen hoge zoutgehaltes. Dit was het gevolg van handhaving van een hoger K^+/Na^+ ratio, en hogere Ca^{2+} and K^+ gehaltes tijdens de zoutstressperiode, waardoor de homeostase van de cel behouden bleef. De rijst

transformanten vertoonden vergelijkbare mechanismen met de Arabidopsis mutanten tijdens zoutstress, en ook een verhoogde biomassa en zaadopbrengst (20-30%) in vergelijking met de WT planten bij zoutstress. Een derde Arabidopsis gen dat is bestudeerd heet *BOUNTIFUL (BFL)*, een DNA-bindende transcriptionele cofactor. Overexpressie van *BFL* in Arabidopsis resulteerde in een plant met een robuust fenotype: meer en grotere rozet bladeren, meer laterale wortels en meer hauwtjes (hoofdstuk 4). De bloei van de *BFL* overexpressie mutant was ook verlaat in vergelijking met WT, en daarom kon dit fenotype ook gerelateerd worden aan de late bloei fenotypes van verschillende andere Arabidopsis mutanten. Overexpressie van *BFL* resulteerde echter ook in toegenomen celstrekking, waardoor de bladeren groter worden en wat bijdraagt aan de toename van de biomassa. Verder vertoonden de wortels een sterk vertakte structuur als gevolg van hun langere laterale wortels. Dit verbeterde wortelnetwerk draagt hoogstwaarschijnlijk bij aan de verbeterde zouttolerantie, en verhoogde resistentie tegen Verticillium. De *BFL* overexpressors behielden hun ion homeostase in de cel tijdens zoutstress door een verlaagde ophoping van Na^+, hogere Ca^{2+} en K^+ gehaltes, en een hogere K^+/Na^+ ratio in vergelijking met het WT. De negatieve effecten van zoutstress op plant groei (gemeten als drooggewicht van de plant) waren minder in de *BFL* overexpressors dan in het WT. De verhoogde tolerantie voor zoutstress, de pathogeenresistentie en het robuuste fenotype wijzen op een goed gecoördineerd mechanisme van verhoogde weerstand als gevolg van overexpressie van *BFL* met potentie voor verbetering van gewassen.

Een belangrijke benadering om de samenstelling van genetische routes op te helderen is de analyse van “verlies van functie” mutanten waarin een dergelijke pathway is onderbroken door uitschakeling van een essentieel onderdeel van de route. Een belangrijke beperking van deze benadering is echter dat genen, wier functie kan worden overgenomen door andere genen niet kunnen worden opgespoord. Om toch dit type genen met voor de landbouw interessante eigenschappen te kunnen bestuderen werd gebruik gemaakt van een methode waarbij plantgenen tot overexpressie werden gebracht door middel van een op transposon-gebaseerde gemarkeerde activatie in een “high throughput” opzet (hoofdstuk 5). De activatie-gemarkeerde transposon inserties bevatten een meervoudige ”Enhancer” van de CaMV35S promotor die in staat is om promotoren in de directe nabijheid van de insertie te activeren of hun activiteit te verhogen. In

Hoofstuk 5 testen we de hypothese dat mutanten die stress resistentie pathways tot overexpressie brengen daarvoor een fitness prijs moeten betalen inhun vegetatieve en reproductieve groei. Een selectie van de mutanten op basis van verminderde groei zou dan kunnen leiden tot identificatie van abiotische stress tolerantiegenen. Op deze wijze hebben we uit een set van 1200 gemarkeerde activatie lijnen een subset van 51 mutantlijnen geselecteerd met verminderde fitness. Deze 51 lijnen zijn getest op droogteresistentie. In totaal zijn acht droogte-resistente mutanten geïdentificeerd, en de mogelijk geactiveerde genen in elke mutant en de rol die deze genen zouden kunnen spelen in de aanpassing aan de droogtestress condities zijn beschreven in hoofdstuk 5. Twee van deze mutanten, genaamd *atal* en *vajra* zijn uitgebreider gekarakteriseerd en vertonen een verhoogde WUE in Arabidopsis door een verminderd waterverlies door verdamping in vergelijking met het WT. Tevens zijn deze mutanten beter bestand tegen zoutstress in Arabidopsis doordat ze minder Na^+ ophopen in het cytoplasma en K^+ gehaltes zijn toegenomen waardoor een hogere K^+/Na^+ ratio kan worden gehandhaafd zowel onder normale als onder zoutstress omstandigheden. Deze resultaten laten zien dat voorselectie van mutantlijnen met een verminderde groei/fitness in een transposon-gebaseerde activatie-gemarkeerde populatie de identificatie van mutanten die droogteresistent zijn mogelijk maakt, waarbij de frequentie van mutanten veel hoger is (16%) dan eerder werd verwacht. Dit maakt het mogelijk om zeer grote, genoom-verzadigde populaties te analyseren op resistentie tegen droogtestress.

Het genetisch complexe mechanisme dat ten grondslag ligt aan abiotische stress tolerantie bemoeilijkt de verbetering van gewassen voor deze belangrijke eigenschappen. Door inspanningen om de stress tolerantie van planten te verbeteren via genetische transformatie zijn al enkele zeer belangrijke doelen bereikt. Reden des te meer om biotechnologische benaderingen volledig te integreren met de klassieke fysiologie en veredeling. Het onderzoek in dit proefschrift beschrijft de identificatie van Transcriptie Factoren die droogte en zoutstress tolerantie in Arabidopsis (dicotyl) en rijst bewerkstelligen, met een verhoogde biomassa en zaadopbrengst in een belangrijk gewas als rijst (monocotyl). Deze resultaten bieden mogelijkheden voor de verbetering van een groot aantal gewassen waarin biotische stresstolerantie eigenschappen kunnen worden gecombineerd om te komen tot een stabiele gewasproductie met verhoogde economische waarde in een veranderende omgeving.

Acknowledgements

I came to the Netherlands on the 15th of August 2001 to do my masters in plant breeding at Wageningen University and Research Centre (WUR). Later I started my PhD project in January 2004 and I am very glad today that I could finish my project in time with the satisfactory outcome of my thesis. This was achieved with collaborative efforts by many people and I would like to acknowledge my sincere thanks to them. Firstly I would like to thank God for giving me the opportunity, strength and courage to fulfill my dream. I would like to thank my supervisor Prof. Andy Pereira, for showing his confidence in me. Andy you constantly challenged me to get the best out of me and this wasn't always a pleasant experience for me. But eventually we both came to know each other better and learnt to work together. You are a person with immense intelligence and knowledge and surely I benefitted a lot from it. I enjoyed lots of freedom you gave me to design and carryout the research work which made me a very confident and self reliant researcher. It also made it less difficult for me to cope up with your departure from PRI in 2006, when you went to United States of America, to join the Virginia tech. Institute as a professor. I was almost getting immune to peoples departure by that time as I had seen each and every member of our group departing one by one (almost each year) including you. Now I am the only last member left in your ex-group at PRI. Even from the distance you remained my supervisor and helped me to put this whole thesis together beautifully and in such an efficient manner and I thank you for that cooperation. I would like to thank my promoter Prof. Maarten Koornneef (Dept. of Genetics, WUR) for his valuable guidance throughout my PhD period. Sir, I appreciate the time you took from your busy schedule (although you work only one day per week in Wageningen) to come and visit me at PRI for discussions over my research and helped me to focus more on academic aspects in order to complete this thesis in time. I would like to thank Aafke (secretary for Genetics Dept.) for helping me to settle the bills from my trips and reminding me of deadlines for filling certain important forms and most importantly she helped me to stay in touch with my colleagues at dept. of genetics and always listened to my problems and showed lot of care and support, this meant a lot to me and I thank you very much for that. Also I would like to thank our secretaries at PRI, Mariame, & Annie for their help with administrative things.

It has been six years and nine months that I stayed in Wageningen and during this time I made many friends and met many interesting people from around the world. Wageningen has almost become my second home now. Hence, I would like to thank my friends and colleagues for there lovely friendship and support and making my time in Wageningen the most memorable in my life. I would like to thank Nayelli & Stefan, Raffa, Odette, Junming, Fabio, Lt. Mimi, Asaph, Teun, Rada, Rudi, Rudd, Harro, Gert, Doret, Annelies, Caroline, Marjon, Wim, Eric, Edwin, Aarati, Wang, Marco, Mark and Xingying for being such a fantastic colleagues and friends at PRI and with most of you I enjoyed especially the evenings we spent dining at the restaurants. Teun, we have shared our special friendship now for a very long time and I wish you good luck for graduating soon after me, finally I won the bet ! I graduated before you did hahaha… Since plant breeding dept. moved to PRI, I made some new friends and recovered some old friends for which I am very glad. Anoma, Estelle, Nicolas, Marten, Jaap thank you all for making the labs at PRI more vibrant and enjoyable to work. Benoit, Thierry, Stefano, Ningwen, Anitha, Sameer, Nadeem, Aneemesh, Rajshekharji, thank you for your friendship. I would also like to thank Francios, Fengfeng and Radjan, my students for there contribution to my work. I would like to thank my ex-officemate Hossein for being very helpful and friendly with me and I absolutely enjoyed the interesting talks we had over our great Iranian and Indian culture. When Hossein left Netherlands, I was joined by another colleague from Slovenia, Katya Cankar. Katya, I think we got along very well and shared some funny moments as well as serious ones together and I thank you for your friendship. I hope by now you might have agreed that CEO is indeed an international abbreviation for Chief Executive Officer and not an Indian habit of talking in abbreviation language. I would like to thank some of my friends from PRI with whom I shared a special friendship. Bram, I would never forget the conversations we used to have over practically anything on this earth, especially over history, politics, culture you name it, although I must admit our favorite topic was history. Hey Radi!!! This is the way most of the time I greeted you. I loved the way we bonded and shared some of our feelings towards life, work and people around us. It was a pleasure to know you. I would like to thank Prof. Evert Jacobson for always checking on me and encouraging me all the time especially with finishing my thesis. I will remember you as the funniest, caring and helpful person. I would like to acknowledge Mr. Roel van de Weg, my

counselor from Rotary International from Wageningen, for his support and kindness he showed during my first two years in Wageningen and later as I continued with my PhD you stayed in touch and supported me morally in my endeavor. I would like to thank NWO-WOTRO for funding my PhD project.

In the last one and half year of my PhD, Dr. Gerard van der Linden took over my project after Andy left. I would like to thank him for his valuable help in arranging finance related matters for my project and I enjoyed being the part of your group. I also appreciate the concern, support and care that you showed towards me especially when I was not doing very well with my health during the period of my thesis writing. I would also like to thank Jan Knuiman, who was always there for me to help me with any kind of problems related to computer. You were more like a good friend to me than just a colleague. I will profoundly remember the company and friendship of Reza, Ali Reza I & II. I would like to thank Marian, Irma, and colleagues from Greenomics Tamara, Marleen, and Paul for there help in the lab.

Staying in the Netherlands was not possible without a healthy social life. I would like to acknowledge Hugo, Henry, Nico, Florence, Peter-I & II, Maurice, Janneke and others from the KSV-international students organization, for sharing the Dutch student culture with me and working together to built a common platform for international students to interact with each other and share there culture. I would like to thank the Indian "Desi" community in Wageningen for sharing the Indian atmosphere especially during dinners and parties where we danced like a crazy crowd on our popular bhangra music (Oh balle balle…). I would specially like to thank Meenakshi for her love and affection towards me like my elder sister. I also share a special bond and friendship with Veda (also known as my twin). Although we look alike we are two very different personalities, yet most of our views towards life and our passion for our great country are so similar. I thank you for being my friend. I would like to remember the "Indian curry group "members for sharing the spicy evenings in the weekends. I specially like to remember Srinivas, Ram, Nagesh, Ana, Neeraja, Krushi & Raman, Raghu, Jui, Sara and Nader for there special friendship and love. I am sure we will stay friends forever.

My passion for Indian classical dance and singing continued during my stay in Wageningen and this was only possible due to the perfect companionship I got from Usha and

Rinia. We belong to three different generations although we come along so well together like friends. I enjoyed dancing and teaching "Kathak" in Wageningen. I would like to thank Ludwig for giving me an opportunity to join in his band for singing bollywood numbers. I absolutely enjoyed the time we spent together practicing and performing. Apart from this I would like to thank both Mr. and Mrs. Baldew and Usha and Ludwig for making me feel at home away from my home and accepting me as a part of their family. Thank you once again for showering this love and compassion on me. Another family Mr. and Mrs. Rammallu was always by my side supporting me and checking on me with the progress of my work and life in Wageningen. The kindness and compassion that you showed towards me is speechless and I thank you for that.

Most importantly I would like to thank my family members for constantly believing in me and supporting me endlessly. You are a great family one could ever ask for and I am so thankful to almighty to bless me with such a wonderful family. I am sure I made everyone in my family proud especially my father and a great scientist. Mummy you are my true hero and my inspiration. You are a women with great strength and a heart full of love and forgiveness. You have a great sense of humor and you are a very positive person and I am still learning a lot from you. I would like to thank my grand parents for there blessings. Shilpa didi, you are more than a sister to me, my best friend. You cannot hear a single negative thing about me from anyone. This proves your confidence and trust in me. I thank you for loving me so much. I would also like to thank Ranvir Jijaji for supporting and encouraging me from a distance. The kids of our family Rani, Jairaj and Aastha, you have kept me motivated as you have always looked up to your Aunt. You have also given me an opportunity to closely feel my own childhood. They say that Wageningen is a place where people find the love of there life and indeed its true. I found Raghu, my best friend and my soul mate in Wageningen. He is an "all in one" family package for me in Wageningen. A person with great compatibility and understanding, who loves me overwhelmingly and took care take of me and guided me as my mentor through difficult times. I am thankful to god for bringing you into my life and to my family and cherish each moment I spent with you.

Shital Dixit

Wageningen, 14th March 2008

List of Publications

*Karaba, A., ***Dixit, S.,** Greco, R., Aharoni, A., Trijatmiko, K.R., Marsch-Martinez, N., Krishnan, A., Nataraja, K.N., Udayakumar, M., and Pereira, A. (2007). Improvement of water use efficiency in rice by expression of HARDY, an Arabidopsis drought and salt tolerance gene. **Proc. Natl. Acad. Sci.** USA 104**,** 15270-15275.
*(shared first authorship).

Aharoni, A., **Dixit, S**., Jetter, R., Thoenes, E., van Arkel, G., and Pereira, A. (2004). The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in Arabidopsis. **Plant Cell** 16**,** 2463-2480.

*Marsch-Martinez, N., *Greco, R., Becker, JD., **Dixit**, **S**., Bergervoet JH, Karaba A, de Folter S, Pereira A. (2006). *BOLITA*, an Arabidopsis AP2/ERF like transcription factor that affects cell expansion and proliferation/differentiation pathways. **Plant Mol. Biol**. 62, 825-843.
*(shared first authorship).

To be Submitted

Dixit, S., Greco,R., Marsch-Martinez,N., Krishnan,A., Pereira, A. *BOUNTIFUL*, an Arabidopsis AT-hook DNA-binding protein, confers a robust plant phenotype, disease resistance and salt tolerance.

Dixit, S., and Pereira, A. *SHINE*, an AP2 transcription factor, confers salt tolerance in Arabidopsis and rice.

Marsch-Martinez, N., Greco, R., **Dixit, S**., Kuijt, S.J.H., de Folter, S., Meijer, A.H., Ouwerkerk, P.B.F., Pereira, A. The PICOS/WIP2 transcription factor functions as a direct activator of the homeobox gene BREVIPEDICELLUS/KNAT1

Curriculum Vitae

Shital Dixit was born on 22nd March 1979 in a district called Bhavanagar, in Gujarat state, India. She completed her higher secondary school in 1996, with Biology, Chemistry, Physics and Mathematics as her majors and later joined for Bachelors in Agriculture and Animal Husbandry at the Anand Agriculture University, Anand, Gujarat State, India. She was awarded the merit scholarship for her four years bachelors and she graduated in 2000, with two gold medals in the subject of Economics and Extension education. In 2001, she was awarded the prestigious "Ambassadorial Scholarship", by Rotary International to pursue her Masters studies in Crop Sciences with specialization in Plant breeding, at Wageningen University. On completion of her MSc. in 2003, she joined the PhD programme at Plant Research International, Wageningen University and Research Center in Plant molecular genetics in 2004. The PhD programme was funded by the Netherlands Organization for Scientific Research, NWO-WOTRO. In her PhD research she worked on "Identification of plant genes for abiotic stress resistance" and this thesis is the final product of her PhD research with some significant outcomes.

Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Shital Dixit
Date: 14 March 2008
Group: Plant Breeding & Genetics, Wageningen University and Research Centre

1) Start-up phase	date
► **First presentation of your project**	
Identification of genes for abiotic stress tolerance-cluster presentation	Nov 8, 2004
► **Writing or rewriting a project proposal**	
Identification of Plant genes for abiotic stress resistance.	Feb 2004
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
Vrij University, Amsterdam	Jun 3, 2004
Radboud University Nijmegen	Jun 2, 2005
Wageningen University	Sep 19, 2006
Wageningen University	Sep 13, 2007
► **EPS theme symposia**	
EPS Theme 1 ' Developmental Biology', Wageningen	Apr 26, 2005
EPS Theme 4 'Genome Plasticity', Wageningen	Dec 9, 2005
EPS Theme 3 ' Metabolism and Adaptation", Utretch	Nov 24, 2005
► **NWO Lunteren days and other National Platforms**	
ALW/EPW Lunteren meeting	Apr 5-6, 2004
NWO-ALW meeting Experimental Plant Sciences, Lunteren	Apr 4-5, 2005
NWO-ALW meeting Experimental Plant Sciences, Lunteren	Apr 3-4, 2006
NWO-ALW meeting Experimental Plant Sciences, Lunteren	Apr 2-3, 2007
► **Seminars (series), workshops and symposia**	
Geneyous Symposium: Utrecht	Jan 20, 2004
Dr. Joerg Bohlmann, University of British Columbia, Vancouver, Canada,at PRI,Wageningen.	Feb 9, 2004
Title: 'Making scents with terpenoids: Plant defense and floral fragrances'	
Arabidopsis'	Jun 28, 2004
Seminar Dr. Freek Bakker and Dr. Ewald Groenewald,PRI, Wageningen	Oct 29, 2004
complex'	
Flying Seminar Prof.dr. Joseph R. Ecker,'Discovery of Functional Elements in the Arabidopsis Genome',Wageningen.	Sep 26, 2005
Collaborative meeting 'Plant Biotechnology' - Wageningen Univ. & Univ. of Bonn, Bonn (Germany)	Oct 5, 2005
Flying Seminar prof.dr. Philip Benfey- 'A systems biology approach to understanding root development'	Oct 25, 2005
VIB Microarray User Group Meeting, VIB Institution, Ghent (Belgium)	Nov 16-18, 2005
Intern. Frontis workshop Gene-Plant-Crop Relations: Scale and complexity in plant systems research	Apr 23-26, 2006
European Flying Seminar: Professor Jim Carrington,'Diversification of Small RNA Pathways in Plants'	March 26, 2007
► **Seminar plus**	
► **International symposia and congresses**	
2nd EPSO Conference 'Interactions in Biology: Cells, Plants and Communities', Ischia (Italy)	Oct 10-14, 2004
16th International Arabidopsis Conference, Madison, Wisconsin (USA)	Jun 14-19, 2005
18th International Conference On Arabidopsis Research, Beijing (China)	Jun 19-24, 2007
► **Presentations**	
Poster: 2nd EPS Conference, Ischia (Italy)	Oct 10-14, 2004
Poster: PhD day Nijmegen	Jun 02, 2005
Posterl: 16th International Arabidopsis Conference, Madison, Wisconsin (USA)	Jun14-19, 2005
Oral: Plant Biotechnology Wageningen - Bonn, Bonn (Germany)	Oct 05, 2005
Poster:Int. Frontis workshop Gene-Plant-Crop Relations: Scale and complexity in plant systems research	April 23-26, 2006
Oral Presentation in Annual plant science meeting-Lunteren:	Apr 03, 2006
Title:Conserved function of the SHINE clade of AP2/ERF transcription factors	
Poster: EPS PhD students day : "Hardy, An Arabidopsis gene, confers salt and drought tolerance	Sep 13, 2007
in Arabidopsis"	
► **IAB interview**	Sep 18, 2006
► **Excursions**	
Subtotal Scientific Exposure	*17.9 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
Systems Biology Course: Principles of ~omics data analysis	Nov 07-10, 2005
EPS Summer School (Utrecht): Environmental Signaling: Arabidopsis as a model	Aug 22-24, 2005
EPS Summer School (Wageningen): Signaling in Plant Development and Defence: towards Systems Biology	Jun 19-21, 2006
EPS hands on course "Gateway to Gateway technology"	Nov 20-24, 2006
► **Journal club**	
Participation in literature studygroup at PRI BU	2004-2008
Biweekly cluster/group meeting, plant breeding.	2004-2007
► **Individual research training**	
Subtotal In-Depth Studies	*7.2 credits**

4) Personal development	date
► **Skill training courses**	
Techniques for writing and presenting a scientific paper (by Michael Grossman, Illinois,USA)	Jun 29- Jul 02, 2004
► **Organisation of PhD students day, course or conference**	
PhD student day 2006, Wageningen University	Sep 19, 2006
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*2.7 credits**

TOTAL NUMBER OF CREDIT POINTS*	**35.3**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*

Cover layout and design by the author

Cover: A severe drought situation in the field and a surviving drought tolerant Arabidopsis plant. This conveys our goal to identify abiotic stress resistant genes in order to engineer crops for resistance.

PROPOSITIONS

1. Transcription factors identified from the dicot model Arabidopsis can be used to improve abiotic stress resistance in an important monocot crop like rice without any negative agronomic effects. (This thesis)

2. The overexpression of transcription co-factors can enhance a plant's performance in multiple ways. (This thesis)

3. Current molecular studies on drought often use short-term dehydration stress treatments to test survival, while in actual field situations such occurrences are rare. Therefore selection for drought tolerance cannot rely on such short term treatments.

4. In order to engineer a truly stress resistant plant, biotechnology must work together with classical physiology and plant breeding.

5. The term "genetically modified" itself is a controversial term and is a part of the problem of acceptance of GMO crops, as it prejudices the debate over its benefits, use, safety, hazards, etc. to begin with. This term can be replaced by a more positive term like "genetically improved".

6. A right attitude determines a person's circumstance, behavior and its ability to succeed in almost any of life's endeavors.

Propositions belonging to the PhD thesis:

"Identification of Plant Genes for Abiotic Stress Resistance"

by Shital Dixit

Wageningen, 14th March, 2008