COMPARATIVE ANALYSIS OF DROUGHT RESISTANCE GENES IN ARABIDOPSIS AND RICE

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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 woensdag 7 september 2005
des namiddags te vier uur in de Aula

Trijatmiko, K.R. 2005
Comparative analysis of drought resistance genes in Arabidopsis and rice
Ph.D. thesis Wageningen University, Wageningen, The Netherlands- with summaries in English, Dutch and Indonesian.
Plant Research International, P.O. Box 16, 6700 AA Wageningen,
The Netherlands.

ISBN: 90-8504-260-7

Contents

This thesis is dedicated to my beloved wife, Nana.

Chapter 1

GENERAL INTRODUCTION

Rice and Drought

Abiotic stresses like drought and salinity are one of the most limiting factors for stable crop production world-wide but especially for developing countries where the largest growth in populations will put an enormous demand on stable food supplies. Environmental stress factors have been estimated to cause depreciation in crop yield up to 70% when compared to the yield under favorable conditions (Boyer, 1982). Stability of crops to changes in environmental factors is, therefore, one of the most valued traits for breeding. As world population grows to an estimated 8.9 billion in 2030, agriculture must respond to the increasing demand for food and compete for scarce water with other users. Many of the over 800 million people in the world who still go hungry live in water scarce regions (FAO report 2002, Crops and Drops). Of the 1,500 million hectares of global cropland, only 250 million hectares (17%) are irrigated (FAO report 2002, Crops and Drops).

Rice is the world's single most important food crop and a primary food for more than a third of the world's population (David, 1991). More than 90% of the world's rice is grown and consumed in Asia where 60% of the earth's people live (Khush, 1997). The present annual rice production is 589 million tons (FAOSTAT data, 2004), and it must be increased (more than 40%) to 850 millions tons by the year 2025 to feed the increased population. There are no additional lands available for rice cultivation. In fact, the area planted to rice is going down in several countries due to pressures of urbanization (Khush, 1997). While it has been a very difficult task to break the present yield ceiling, in many rice areas the present yield potential has not been fully exploited because of many biotic and abiotic stresses (Herdt, 1991).

Rice is grown under diverse growing conditions. Four major ecosystems are generally recognized (Khush, 1984) as follows: (1) irrigated, (2) rainfed lowland, (3) upland, and (4) floodprone. Approximately, 55% of the world rice area planted to rice, is irrigated and is the most productive rice growing system, perhaps contributes 75% of the world rice production

(Khush, 1997). Rainfed lowland rice is grown in bunded fields where water depth does not exceed 50 cm for more than 10 consecutively days and the fields are inundated for at least part of the season (IRRI, 1982). Although rainfed lowland area occupies 25% (approximately 37 m ha) of the world's rice crop area, it contributes only 17% of the total production (Wopereis et al., 1996). About 16 millions hectares (or 12%) of world rice land is classified as upland. Upland rice is grown under rainfed, naturally well drained soils in bunded or unbunded fields without surface water accumulation. Upland area contributes 4.5% of the world's rice production (Khush, 1997). Floodprone rice is grown in low lying lands in river deltas of South and Southeast Asia. Standing water depth may vary from 50 cm to more than 3 m. About 9 millions hectares (or 8%) are planted to floodprone rice, and contributes around 3.5% of the world's rice production (Khush, 1997).

Rice uses two to three times more water than other food crops such as wheat or maize. In total, rice production in the world uses about 1,578 km^3 of water, which is 30% of the fresh water used worldwide. The scarcity of water internationally (IWMI 2000; Gleick, 1993) threatens the sustainability of the irrigated rice ecosystem.

It was known that a few countries were naturally short of water because they were arid. But there had not been a true awakening to the global threat of water stress caused by the rapidly increasing world population and the accompanying rapid increases in water use for social and economic development (Cosgrove and Rijsberman, 2000). Because of the increase in water withdrawals, the pressure on water resources will grow significantly in more than 60% of the world, including large areas of Africa, Asia, and Latin America (Alcamo et al., 1999).

In some parts of world, such as South Asia, most of the recent expansion in irrigated areas has been in wells through private investments to exploit groundwater (United Nations, 2003). There are worrying signs in some areas that groundwater resources are being over-exploited, with groundwater levels falling, which could develop into a crisis. To meet the increasing demand of rice in the future, therefore, we can not continue to depend solely on irrigated area. We need to exploit more the recent yield potential of rice crop in rainfed areas.

Drought is a major constraint to rice production in rainfed environment (Zeigler and Puckridge, 1995). Unreliable monsoon and uneven distribution of rainfall cause year-to-year fluctuations in crop yields (Hossain, 1995). Improving drought resistance to increase productivity and stability has become a challenge to many plant breeders.

Experimentally growing high-yield lowland rice varieties under aerobic conditions has shown great potential to save water, but with a severe yield penalty. In the early 1970s, De Datta et al. (1973) tested lowland variety IR20 in aerobic soil under furrow irrigation at IRRI. Water savings were 55% compared with flooded conditions, but the yield fell from about 8 ton/ha to 3.4 ton/ha. Therefore, achieving high yields under deficit irrigated or rainfed conditions requires diversified drought tolerant varieties that use water efficiently with high yield potential, good grain quality, and specifically adapt to aerobic conditions.

Gene identification in model plants

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.

Another model is the monocot crop rice, whose genome has been completely sequenced at the end of 2004 by the International Rice Genome Sequencing Project (IRGSP) a consortium of publicly funded laboratories. Rice has a compact genome size (three times Arabidopsis) and is easy to transform enabling 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 Arabidopis 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

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; Kojima et al., 2002; Takahashi et al., 2001). Recently, map-based cloning of *D*3 gene controlling tillering number in rice (Ishikawa et al., 2005) revealed that it encodes a F-box leucine-rich repeat (LRR) protein orthologous to Arabidopsis MAX2/ORE9 (Stirnberg 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.

Conservation in function of abiotic-stress-responsive genes

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., 1998), some of which are also induced by low temperature stress (Tomashow, 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). There is an overlap between the responsiveness of many of these genes, most dehydration-inducible genes also respond to cold stress and vice versa, suggesting parallel mechanisms of induction. The analysis of promoters of stress responsive genes revealed a cis-acting dehydration-responsive element (DRE) (Yamaguchi-Shinozaki and Shinozaki, 1994) and a similar low-temperature C-repeat (CRT) element (Baker et al., 1994), providing an explanation for the similar stress responses. 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. Thus tolerance affected to one stress response might provide cross-tolerance to another.

In various species a large number of different genes respond to drought-stress. These genes can be divided into two groups: genes involved in cell protection during drought stress and genes involved in the regulation of other genes involved in the drought responses (Shinozaki and Yamaguchi-Shinozaki, 2000). The first group includes proteins involved in for example osmotic adjustment, degradation, repair, detoxification and structural adaptations. The second group contains regulatory proteins, such as protein kinases and transcription factors. Genetic engineering at the level of the transcription factors is a particularly promising strategy for stress tolerance improvement. The potential of this strategy lies in the fact that most if not all stress-related transcription factors are likely to regulate a wide range of target genes of which the gene products contribute to stress tolerance. Therefore, the approach to engineer stress tolerance by modification of transcription factor levels may have clear advantages over other strategies that focus on enhancing levels of individual proteins with a protective function. A few years ago it was shown that overexpression of the AP2/EREBP factors CBF1, DREB1A and CBF4 resulted in drought/salt/cold tolerance in Arabidopsis (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999, Haake et al., 2002).

The orthologous genes of *CBF*/*DREB* have been found in most crop plants examined so far, including canola (*Brassica napus*), soybean (*Glycine max*), broccoli (*Brassica oleracea*), tomato (*Lycopersicon esculentum)*, alfalfa (*Medicago sativa*), tobacco (*Nicotiana tabacum*), cherry (*Prunus avium*), strawberry (*Fragaria* spp.), wheat (*Triticum aestivum*), rye (*Secale cereale*), maize (*Zea mays*), rice (*Oryza sativa*), and barley (*Hordeum vulgare*) (Jaglo et al., 2001; Choi et al., 2002; Gao et al., 2002; Owens et al., 2002; Shen et al., 2003a; Shen et

al., 2003b; Vagujfalvi et al., 2003; Xue, 2003; Dubouzet et al., 2003). Many of the putative orthologs have been functionally tested, indicating conservation of the stress tolerance pathway in many plants. Constitutive overexpression of the Arabidopsis *CBF*/*DREB* genes in canola results in increased freezing tolerance (Jaglo et al., 2001) and drought tolerance. Similarly, constitutive overexpression of *DREB* orthologs from rice (*OsDREB1*) in transgenic Arabidopsis resulted in salt, cold, and drought tolerance (Dubouzet et al., 2003). Likewise, the overexpression of a *ZmCBF* gene in maize also resulted in increased cold tolerance (Chaiappetta, 2002). In these and other studies, it was discovered that ectopic overexpression of the *CBF* genes in plants produced, in addition to increased stress tolerance, dark-green, dwarfed plants with higher levels of soluble sugars and proline (Liu and Zhu, 1998; Gilmour et al., 2000). To overcome these problems, stress-inducible promoters that have low background expression under normal growth condition have been used in conjunction with the *CBF* genes to achieve increased stress tolerance without the retarded growth (Kasuga et al., 1999; Lee et al., 2003).

These are excellent examples that modification of transcription factor levels can successfully produce stress tolerance. It is, therefore, realistic to hold an optimistic view that these and other stress-related transcription factors would also be able to confer drought tolerance in diverse crop species ranging from dicots to monocots. Presently, the major bottleneck for testing such hypothesis is knowledge of transcription factors involved in the drought response of specific crop species is still very limited.

Drought resistance mechanisms

The mechanisms of drought resistance can be grouped into three categories (Mitra, 2001), viz. drought escape, drought avoidance and drought tolerance. 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 avoidance is the ability of plants to maintain relatively high tissue water potential despite a

shortage of soil-moisture, whereas drought tolerance is the ability to withstand water-deficit with low tissue water potential. Mechanisms for improving water uptake, storing in plant cell and reducing water loss confer drought avoidance. The responses of plants to tissue water-deficit determine their level of drought tolerance. Drought avoidance is performed by 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 survive by doing a balancing act between maintenance of turgor and reduction of water loss. 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.

However, most of these adaptations to drought have disadvantages. A genotype of short 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. 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. Consequently, 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, awn, 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. 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 fructan, 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.

QTL mapping for grain yield under drought

Drought has long been recognized as the primary constraint to rainfed rice production (Mackill et al, 1996). The practical progress in breeding for drought resistance has not been significant (Reddy et al., 1999). Drought, therefore, requires an analytical approach of dissecting and studying the contribution of different trait components using a quantitative trait loci (QTLs) model. This approach is particularly suited to crops like rice for which dense genetic linkage maps are already available (Causse et al., 1994; Temnykh et al., 2001; Harushima et al., 1998; McCouch et al., 2002). The availability of the complete genomic sequence of rice (Feng et al., 2002; Sasaki et al., 2002; The Rice Chromosome 10 Sequencing Consortium, 2003; http://rgp.dna.affrc.go.jp/cgi-bin/statusdb/irgsp-status.cgi) and the integration of a BAC-based physical map with genetic markers (Chen et al., 2002) allows us to predict the putative candidate genes in the chromosomal regions identified by QTL analysis.

Many QTLs have been reported in rice for traits that are putatively associated with performance under drought, such as root system morphology (Champoux et al., 1995), osmotic adjustment (Robin et al., 2003), leaf membrane stability (Tripathy et al., 2000), and visual symptoms of leaf stress such as rolling and drying (Courtois et al., 2000). But it is still unclear if these secondary traits significantly contribute to grain yield under drought stress.

In recent years more attention has started to be given to mapping of QTLs for grain yield and its components under managed stress environments (Zhang et al., 1999; Lafitte et al., 2002; Venuprasad et al., 2002; Babu et al., 2003; Lanceras et al., 2004; Lafitte et al., 2004). Up to now, there have been three different populations used in these QTL mapping analyses.

Lafitte et al. (2002) reported mapping of grain yield and its components in a lowland experiment and in six upland experiments using a population of doubled haploids derived from a cross between IR64xAzucena. All QTLs that were identified in two or more aerobic

experiments were also detected in the lowland experiment. In that study, the semi-dwarfing gene *sd1* dominated differences in yield in aerobic fields, just as it had in the lowland study. This gene is known to affect many aspects of plant morphology and physiology through pleiotropic effects: height, tillering, panicle length, responsiveness to fertilizer, biomass and harvest index (Xia et al., 1991; Courtois et al., 1995), as well as root system development (Yadav et al., 1997).

Identification of stress resistance genes by overexpression

Genes that confer stress resistance can be identified from model plants like Arabidopsis and applied in other crop plants to obtain resistance to multiple stress factors. This is supported by the identification of two EREBP/AP2 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 (HTP) way and selection for stress resistant lines would, therefore, provide the most direct way of identifying genes that can confer stress resistance.

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. 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. 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 (~5kb) and enhance or activate their expression. Results show that about 1% of the insertions cause an obvious 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 required for biotechnological use or for marker-assisted breeding towards a durable agroproduction system, providing stable production by helping plants fight environmental challenges.

Novel Mechanisms

The mechanisms of drought resistance can be grouped into three categories: drought escape, drought avoidance and drought tolerance. Crop plants use more than one mechanism at a time to resist drought. However, most genetic analyses handle single genes that might provide one or part of some of these mechanisms. To be able to create drought and other stress resistant cultivars, a combination of mechanisms would be able to provide resistance that is more sustainable, i.e. with minimal growth reduction during favorable conditions, but induced resistance during stresses like drought. There are, therefore, other complementary mechanisms that are worth considering.

The genotypic variation for drought resistance in rice has been attributed to epicuticular wax (EW) content and mostly to long chain alkanes (O'Toole and Cruz, 1983; Haque et al., 1992). Recently, it was suggested that a cuticular skin composed of highly structured waxy domains and not a high wax load is responsible for water transport (Kerstiens, 1996). Furthermore, a wide chain length distribution of aliphatic wax constituents, contributing to an amorphous structure, was suggested as the main determinant of barrier properties. However, rice has been documented to have a very low level of epicuticular waxes (O'Toole et al., 1979). Thus increasing levels of these epicuticular waxes would be expected to provide an effective protective layer against environmental stresses like drought. A number of genes involved in EW have been identified from Arabidopsis, like the *CER1* gene involved in formation of alkanes (Aarts et al., 1995) and the *CER6* (*CUT1*) gene (Fiebig et al., 2000; Millar et al., 1999) involved in elongation of the carbon backbone in Very Long Chain Fatty Acid synthesis. In addition, a regulator of the EW biosynthesis pathway SHINE

has been isolated recently (Aharoni et al., 2004) that could be used to regulate and increase the EW in heterologous plants.

Other Arabidopsis genes conferring dehydration or osmotic tolerance in transgenic/mutant plants

Gene name	Gene action	Phenotypic expression	Reference
AtHAL3a	Phosphoprotein phosphatase	Regulate salinity and osmotic tolerance and plant growth	Espinosa-Ruiz et al., 1999
AtGolS2	Galactinol and raffinose accumulation	Reduced transpiration	Taji et al., 2002
LWR1, LWR2	Solute accumulation (proline)	Growth, osmotic adjustment, water status	Verslues and Bray, 2004
ALDH3	Aldehyde dehydrogenase	Tolerance to dehydration, NaCl, heavy metals (Cu^{2+} and Cd^{2+}), MV, H_2O_2	Sunkar et al., 2003
AtRabG3e	Intracellular vesicle trafficking	Salt and osmotic stress tolerance	Mazel et al., 2004
AZF1, AZF2, AZF3, and STZ	Cys2/His2-Type Zinc-Finger Proteins	Reduced growth and drought resistance	Sakamoto, 2004
CBL1	Ca sensing protein	Salt and drought tolerance – reduced transpiration, cold sensitivity	Cheong et al., 2003, Albrecht et al., 2003
Gli1	Mutant lack glycerol catabolism	Accumulated glycerol confers dehydration resistance	Eastmond, 2004
AtNCED3	Increased ABA synthesis	Reduced transpiration and drought resistance	Iuchi et al., 2001
AtMRP4	Stomatal guard cell plasma membrane ABCC-type ABC transporter	Disruption of *AtMRP4* leads to increased drought susceptibility due to loss of stomatal control	Klein et al., 2004
AVP1	Vacuolar proton-pumping pyrophosphatase	Drought and salt tolerance	Gaxiola et al., 2001
PIP2;2	Plasma membrane aquaporin	Disruption of *PIP2;2* leads to reduced hydraulic conductivity of root cortex cells	Javot et al., 2003

Scope of the thesis

The research presented in this thesis describes a comparative study on drought-related genes between Arabidopsis and rice. Resistance to drought stress is a quantitative trait and molecular markers can be a powerful tool to understand the complex genetic control of drought resistance. *Chapter 2* of this thesis describes a genetic dissection of components of drought resistance in rice. The results uncover a region conferring drought avoidance conserved across different genetic backgrounds.

Analysis of gene expression using Arabidopsis whole genome microarrays on three drought resistant genotypes and a drought-treated wild type (*Chapter 3*) revealed the detection of several drought resistance associated genes. Identification of putative orthologs of these genes in rice might be useful to understand drought resistance mechanisms in rice.

Chapter 4 of this thesis reports on the functional analysis of the Arabidopsis SHINE AP2/ERF transcription factor when overexpressed in rice, causing an alteration in cuticle permeability, stomatal density and enhancement of drought resistance. This observation demonstrates conservation of function of the *SHINE* genes between dicots and monocots.

Chapter 5 describes the isolation and characterization of a rice homolog *OsSHINE1* of the Arabidopsis gene encoding the SHINE AP2/ERF transcription factor. The results in Arabidopsis suggest that *OsSHINE1* has a similar function to its Arabidopsis homologs. It is most significantly enhancing drought resistance when overexpressed in Arabidopsis.

Several drought and other stress resistance genes have been identified in Arabidopsis by a high-throughput overexpression method using transposon-based activation tagging. *Chapter 6* of this thesis reports the development of activation tag constructs using the *Ac-Ds* and *En-I* maize transposon systems and transformation of the constructs into rice. The results suggest that the *Ac-Ds* system transposes actively and that transposition of the *En-I* system occurs at a low efficiency in rice.

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Chapter 2

Parameters determining grain yield of rice under pre-flowering drought stress

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ABSTRACT

The identification of molecular markers linked to genes controlling drought resistance factors in rice is a necessary step to improve breeding efficiency for this complex trait. Quantitative trait loci (QTL) for grain yield and other traits were identified using a recombinant inbred population derived from a cross between two rice cultivars, IR64 and Cabacu. Synchronizing the variable flowering times between lines was conducted by staggering planting date. Drought stress treatment was given at the sensitive reproductive growth stage. The research was conducted at the Banguntapan Field Station, Yogyakarta, Indonesia. A candidate QTL mapping approach was taken where microsatellite and SNP markers near known drought loci were used to test for linkage in the population. Out of 12 selective markers, one marker located close to the *sd1* locus on chromosome 1 was associated with grain yield, percent seed set, grains per panicle, and leaf rolling. Comparison with other populations showed this region conferring drought avoidance conserved across different genetic backgrounds

INTRODUCTION

Rice is particularly susceptible to water deficit compared to other crop species, and this sensitivity is especially severe around flowering (Lafitte et al., 2004). The relative importance of this problem is shown below from an analysis of yield loss parameters of rainfed lowland rice (Figure 1, adapted from Widawsky and O'Toole, 1990).

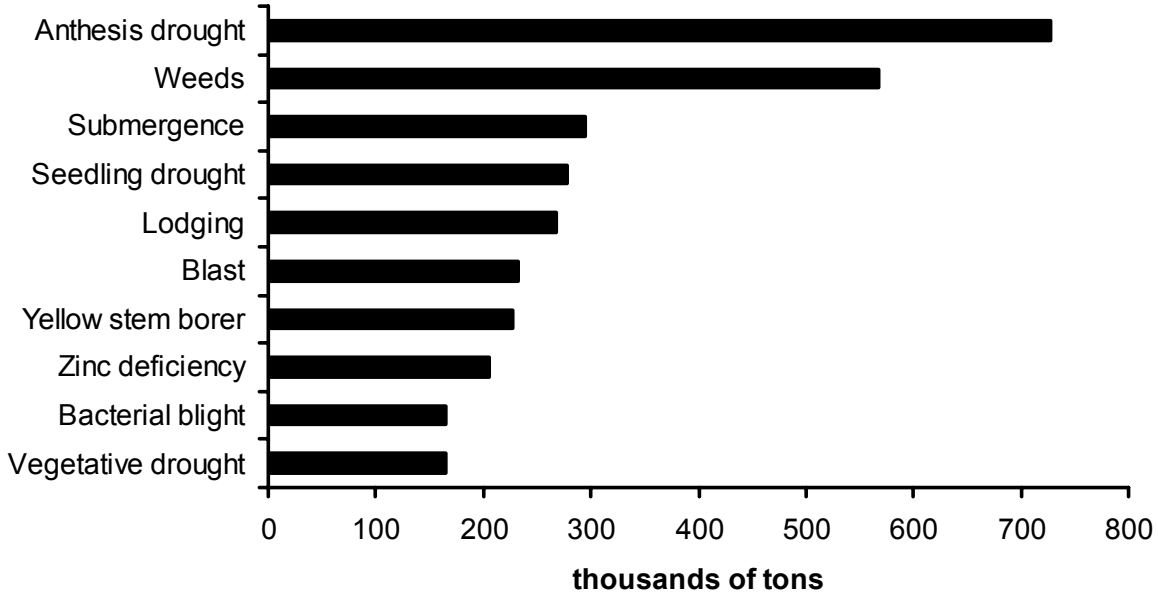

Figure 1. Top 10 causes of yield losses in rainfed lowland rice, eastern India (adapted from Widawsky and O'Toole, 1990)

The practical progress in breeding for drought resistance in rice has not been significant (Reddy et al., 1999). Drought, therefore, requires an analytical approach of dissecting and studying the contribution of different trait components using a quantitative trait loci (QTLs) model. This approach is particularly suited to crops like rice for which dense genetic linkage maps are already available (Causse et al., 1994; Temnykh et al., 2001; Harushima et al., 1998; McCouch et al., 2002). The availability of the complete genomic sequence of rice (Feng et al., 2002; Sasaki et al., 2002; The Rice Chromosome 10 Sequencing Consortium, 2003; http://rgp.dna.affrc.go.jp/cgi-bin/statusdb/irgsp-status.cgi) and the integration of a BAC-based physical map with genetic markers (Chen et al., 2002) allows us to predict the putative candidate genes in the chromosomal regions identified by QTL analysis.

Many QTLs have been reported in rice for traits that are putatively associated with performance under drought, such as root system morphology (Champoux et al., 1995), osmotic adjustment (Robin et al., 2003), leaf membrane stability (Tripathy et al., 2000), and visual symptoms of leaf stress such as rolling and drying (Courtois et al., 2000). But it is still unclear if these secondary traits significantly contribute to grain yield under drought stress.

In recent years more attention has started to be given to mapping QTLs for grain yield and its components under managed stress environments (Zhang et al., 1999; Lafitte et al., 2002; Venuprasad et al., 2002; Babu et al., 2003; Lanceras et al., 2004; Lafitte et al., 2004).

Fukai et al. (1999) reported that flowering time is a major determinant of grain yield under late season drought conditions in the rainfed lowland ecosystem. They also emphasized the ability of rice plants to maintain high leaf water potential as a trait relevant to stabilize yield in rainfed rice planting areas.

In this study, we tested the association between some candidate target loci and grain yield under pre-flowering drought stress using a recombinant inbred population derived from a cross between IR64 (a lowland *indica* cultivar) and Cabacu (an upland tropical *japonica* cultivar). We tried to minimize the effect of flowering time by choosing parental lines differing very little in flowering time and minimize the remaining effect of flowering time by synchronizing the flowering date of the RI population in the experiment. Our results showed that a region close to the *sd1* locus, that is associated with leaf rolling, has a significant effect on grain yield under drought stress. Comparison with three different populations showed that this region conferring drought avoidance has been conserved across different genetic backgrounds.

MATERIALS AND METHODS

Plant material

This study used the progeny of a cross between the cultivars IR64 and Cabacu. IR64 is a lowland-adapted semi-dwarf *indica* cultivar from the International Rice Research Institute, Philippines. It is noted for its high yield and a low level of drought resistance. It has shallow,

thin roots. Cabacu is a tropical *japonica* cultivar from Brazil. It has a comparatively thick, deep root system, and low but stable yields under drought stress in upland conditions. A total of 280 F7 lines were produced by single seed descent.

Screening for pre-flowering drought resistance

Lines were evaluated for pre-flowering drought resistance at the Banguntapan Field Station, Yogyakarta Indonesia in the 2004 dry season. The number of days to flowering was obtained for each line from previous F6 records. The seeding date for each line was then allocated to one of four planting dates, spaced at 4-days intervals. The latest maturing lines were assigned to the early date, and the earliest maturing lines were seeded last. The objective was to synchronize the flowering dates of all lines as close as possible. The experiment was laid out in a randomized complete block design with two replications. All plots were surface irrigated to field capacity once every four days, except when water stress was imposed by withholding irrigation from 65 days after seeding. Fifteen days after withholding irrigation, leaf rolling scores (lrs) were made at midday on a 0-9 scale standardized for rice (IRRI, 1996). After scoring, the stress was relieved and, thereafter, all plots were regularly irrigated until maturity. Ten plants from each plot were randomly chosen for the evaluation of 8 traits: (1) days to heading (dth), (2) plant height (ph), (3) panicles per plant (ppl), (4) spikelets per panicle (spp), (5) grains per panicle (gpp), (6) percent seed set (pss), (7) 100-grain weight (gw), and (8) grain yield per plant (gy).

Screening for root pulling force

The lines were evaluated for root pulling force (rpf) at Banguntapan Field Station, Yogyakarta Indonesia in the 2005 wet season. A randomized complete block design with four replications was used. Plot size was 4 rows x 11 hills (25 cm between rows and 25 cm between hills) with one plant per hill. All plots were surface irrigated to field capacity once every four days. Root pulling force was determined at the flowering stage (O'Toole and

Soemartono, 1981). Two hills per plot in each replication were randomly selected for determining the rpf.

DNA marker analysis

Genomic DNA was isolated from leaves of 3 weeks old seedlings according to Pereira and Aarts (1998). Two hundred and fifty-six microsatellite primers were tested for polymorphism using DNA from two parents. PCR reactions were performed in a PTC 100 thermocycler (MJ Research Inc., Watertown, Mass.) as described by Chen et al. (1997) with the exception that 10 µl of reaction mixture was used instead of 25 µl, and 10 ng of DNA, 4 pmol of each primer and 0.5 unit of *Taq* DNA Polymerase were added per reaction. The temperature profile was: 5 min at 94^0C, 35 cycles of 1 min at 94^0C, 1 min at 55^0C, 2 min at 72^0C, and 5 min at 72^0C. PCR products were separated on 6% polyacrylamide denaturing gels and marker bands were revealed using the silver-staining protocol as described by Panaud et al. (1996). Ninety four RI lines were used for selective genotyping using 11 selective microsatellite markers and 1 SNP marker (Table 1). Depending on data of fragments size differences between the two parents, PCR products were separated on standard or Metaphor agarose gels.

Statistical Analysis

Simple statistics of each trait and correlation coefficient among the traits were computed using phenotypic means. Single marker analysis was conducted using Kruskall-Wallis test at a 0.005 significance level.

Table 1. Selective markers used for selective genotyping on 94 RI lines

Markers	Chr	Primer sequence	Target QTL/gene	Reference
RM104[a]	1	www.gramene.org	*sd1*[b]	Septiningsih et al., 2003
RM220	1	www.gramene.org	gyf	Septiningsih et al., 2003
RM85	3	www.gramene.org	*Hd6*	Takahashi et al., 2001
RM218	3	www.gramene.org	gys	Lanceras et al., 2004
RM119	4	www.gramene.org	gys	Lanceras et al., 2004
RM127	4	www.gramene.org	gys	Lanceras et al., 2004
RM267	6	www.gramene.org	*Hd1*,*Hd3a*	Yano et al., 2000 Kojima et al., 2002
RM222	10	www.gramene.org	gys	Lanceras et al., 2004
RM21	11	www.gramene.org	gys, rpf	Zhang et al., 1999
RM19	12	www.gramene.org	rpi	Trijatmiko et al., unpublished
RM247	12	www.gramene.org	rpi	Trijatmiko et al., unpublished
SNP12	12	GATCACAGGTGCATGCTCAA (IR64 F) GTGAGGTCTGGGTGCTCACA (IR64 R) CAGGTGCATGCTCAAATATTC (Cab F) CTAGGACAAAAGAACGCAAAT (Cab R)	gys yri	Babu et al., 2003 Zhang et al., 1999

[b]The closest convenient markers to the target QTLs or genes

[b]Abbreviations: *sd1* semidwarf 1, *gyf* grain yield under favorable condition, *gys* grain yield under drought stress, *Hd6* heading date 6, *Hd1* heading date 1, *Hd3a* heading date 3a, *rpf* root pulling force, *rpi* root penetration index, *yri* yield resistance index.

RESULTS

Pre-flowering drought resistance parameters and their correlations

The single seed descent (SSD) derived F7 recombinant inbred (RI) population was tested in replicated field tests for different yield determining parameters under drought stress at flowering. The mean and range of values for days to heading (dth), plant height (ph), panicles per plant (ppl), spikelets per panicle (spp), grains per panicle (gpp), percent seed set (pss), 100-grain weight (gw), grain yield per plant (gy), root pulling force (rpf), and leaf

rolling score (lrs) in the RI population are summarized in Table 2. Under the given drought stress, grain yield of IR64 was similar to Cabacu with means of 17.5 gram and 16.0 gram per plant, respectively. However, in submerged conditions the grain yield of IR64 is much higher (41.8 gram per plant, Trijatmiko et al., unpublished data). In the RI population, gy ranged from 0.2 – 24.3 gram per plant and showed higher negative transgressive variation (Figure 2). Transgressive variation in both directions was observed for dth, ph, ppl, spp, gpp, pss, gw, lrs and rpf.

Table 2. Phenotype performance of parents and 280 recombinant inbred lines

Traits	IR64	Cabacu	RI lines	
	Mean	Mean	Mean	Range
Days to heading (dth)	99	108	97	81- 122
Plant height (ph)	65.4	97.3	74.7	34.4 -132.5
Panicles per plant (ppl)	19	11	13	4 – 35
Spikelets per panicle (spp)	83	103	81	22 - 194
Grains per panicle (gpp)	67	88	52	1 – 147
Percent seed set (pss)	85.4	87.5	60.1	0 – 93.4
100-grain weight (gw)	2.14	3.21	2.31	1.35 – 3.54
Grain yield per plant (gy)	17.5	16.0	8.8	0.2 – 24.3
Root pulling force (rpf)	13.2	22.0	17.4	6.5 – 32.4
Leaf rolling score (lrs)	2.2	0.6	2.5	0 - 9

The correlation coefficients among all traits were calculated and shown in Table 3. Grain yield had significant high positive correlations with gpp ($r = 0.591$, $P < 0.001$), pss ($r = 0.571$, $P < 0.001$), gw ($r = 0.271$, $P < 0.001$), and spp ($r = 0.264$, $P < 0.264$). There was no significant correlation between gy and ph. Root pulling force had significant positive correlations with ph ($r = 0.317$, $P < 0.001$) and gpp ($r = 0.205$, $P < 0.01$). Leaf rolling score had a significant positive correlation with dth ($r = 0.239$, $P < 0.001$) and a significant negative correlation with gw ($r = -0.186$, $P < 0.01$). There was no significant correlation between leaf rolling score and root pulling force.

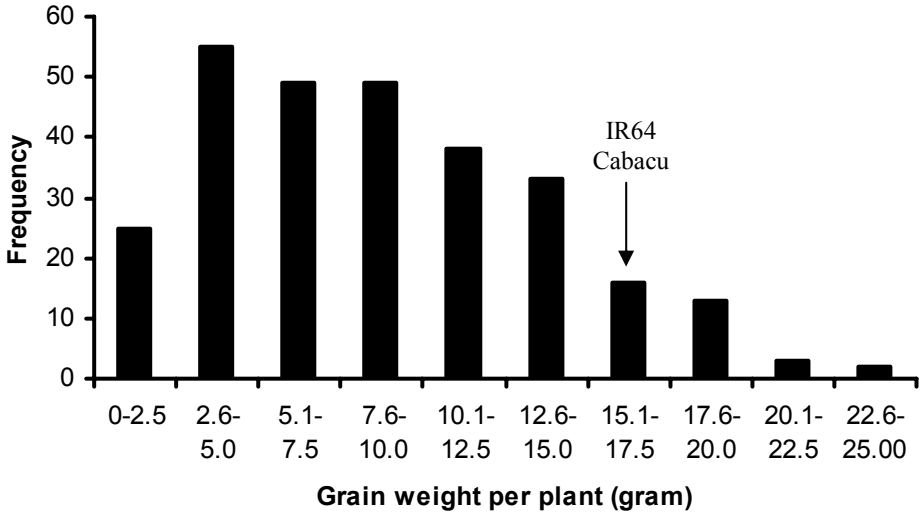

Figure 2. Frequency distribution of grain yield under pre-flowering drought stress in the RI population

Table 3. Phenotypic correlations for grain yield and other traits[a]

Trait	ppl[b]	spp	pss	gpp	gw	dth	ph	rpf	lrs
gy	0.205*	0.264**	0.571**	0.591**	0.271**	-0.153	-	0.123	-0.135
ppl		-	-0.221**	-0.203*	-0.156	0.252**	-0.188*	-	-
spp			-	0.582**	-	-	0.436**	0.140	-
pss				0.756**	0.261**	-0.307**	0.133	-	-0.140
gpp					0.230**	-0.225**	0.328**	0.205*	-0.133
gw						-0.189*	0.145	0.152	-0.186*
dth							-	-	0.239**
ph								0.317**	-
rpf									-

*Significant at $P < 0.01$

**Significant at $P < 0.001$

[a]All correlations shown are significant at $P < 0.05$

[b]Abbreviations: *ppl* panicles per plant, *spp* spikelets per panicle, *pss* percent seed set, *gpp* grains per panicle, *gw* 100-grain weight, *dth* days to heading, *ph* plant height, *rpf* root pulling force, *lrs* leaf rolling score, *gy* grain yield

Microsatellite marker polymorphisms and segregation in the mapping population

In order to map the drought traits a set of 256 available microsatellite primer pairs (Temnykh et al. 2001) were used in the parental polymorphism survey. Out of the 256 microsatellite loci, 61.33% (157 markers) showed polymorphism between the upland and lowland parents. These polymorphic markers were used to make a choice for selective screening of candidate target loci in the population.

Without selection, the expected genotypic ratio in the F7 generation should be 1:1 for the homozygous IR64 to homozygous Cabacu genotype classes and the level of heterozygosity should be around 1.6%. Out of 12 marker loci, 25% (3 markers) located on chromosomes 3, 10 and 12 were skewed towards IR64 (Table 4). There was no distortion in the region close to the *sd1* locus (marker RM104). As expected, the level of heterozygosity on most of 12 markers was around 1.6%.

Table 4. Segregation ratio on 12 marker loci tested in 94 RI lines derived from IR64xCabacu

Markers (Chr)	Segregation ratio			P-value for departure from 1:1 ratio	Heterozygosity level (%)
	A1A1 (IR64)	A2A2 (Cabacu)	A1A2 (H)		
RM104 (#1)	50	41	0	0.345	0
RM220 (#1)	46	37	0	0.196	0
RM85 (#3)	39	41	1	0.823	1.23
RM218 (#3)**	58	30	1	0.003	1.12
RM119 (#4)	46	48	0	0.837	0
RM127 (#4)	41	52	0	0.254	0
RM267 (#6)	42	48	0	0.527	0
RM222 (#10)**	63	31	0	0.001	0
RM21 (#11)	48	42	1	0.527	1.10
RM247 (#12)	40	37	1	0.732	1.28
RM19 (#12)**	51	28	2	0.010	2.47
SNP12 (#12)	45	49	0	0.680	0

QTL analysis

Among the 12 selective markers used for the RI population to target candidate loci, there was one marker (RM104) associated with grain yield under drought stress as identified by single marker analysis (Table 5). Marker RM104 located close to the *sd1* locus on chromosome 1 (genetic distance is around 16 cM, Septiningsih et al. 2003; physical distance is around 1.8 Mb, www.gramene.org) showed very significant association with grain yield under drought stress ($P < 0.000$) (Table 5). This locus explained 29.7% of grain yield variation, with the IR64 parental line contributing the favorable allele. Marker RM104 was also associated with grains per panicle ($R^2 = 15.9\%$), plant height ($R^2 = 15.9\%$), percent seed set ($R^2 = 10.5\%$). Interestingly, this locus was also associated with leaf rolling score ($R^2 = 12.6\%$), with IR64 contributing the favorable allele.

Table 5. QTLs for grain yield under drought stress detected in an IR64/Cabacu RI population

QTL	Marker	Chr	Increased effect	P value	R^2 (%)
Grain yield					
gys 1	RM104	1	IR64	0.000[a]	29.7[b]
Grains per panicle					
gpp 1	RM104	1	IR64	0.000	15.9
Plant height					
ph 1	RM104	1	Cabacu	0.001	15.9
Leaf rolling score					
lrs 1	RM104	1	Cabacu	0.002	12.6
Percent seed set					
pss 1	RM104	1	IR64	0.004	10.5

[a]QTL detected at $P < 0.005$ using Kruskall-Wallis test

[b]Adjusted R^2 calculated using generalized linear model

DISCUSSION

Variation and correlation

Grain yield under drought stress is a function of yield potential and drought resistance mechanism of crop plants. These two factors should be considered in any attempt to study the genetic mechanisms controlling grain yield under drought stress. Flowering time is a major determinant of grain yield and its components under drought stress, especially for late season drought (Fukai et al. 1999). We, therefore, used parents differing very little in flowering time and tried to eliminate or at least minimize the remaining effect of flowering time by synchronizing the flowering date of the RI population in the experiment. We examined the grain yield along with its components and two putative drought-related traits, i.e. root pulling force and leaf rolling score. All phenotypes showed transgressive variation in both directions, indicating that the two parental lines contribute favorable alleles for all the traits.

Aerobic rice is a cultivation method that conserves water use and would, therefore, be useful in water scarce regions and periods. This condition is also susceptible to drought at specific stages and the stability of crop production depends on resistance of the rice crop at various stages during the life cycle. In this study, we provided aerobic conditions by giving surface irrigation to field capacity once every four days with a period of mild drought stress during pre-flowering stage. In this condition, the grain yield of the submergence-adapted parent (IR64) decreased significantly. More than 80% of the RI population showed grain yields less than either parental line, and in some cases very low absolute values (Figure 2). This could be due to segregation of unfavorable alleles from both parents or also maybe an indication of hybrid breakdown (sterility and weakness in F2 or later generations) commonly seen in crosses between *indica* and *japonica* varieties (Stebbins 1958; Oka 1988). More studies are needed to distinguish the basis of lower grain yield in the populations.

In this analysis, the grain yield under drought stress was predominantly determined by its components, i.e. grains per panicle (r = 0.591), percent seed set (r = 0.571), 100-grain weight (r = 0.271), and spikelets per panicle (r = 0.264). These results support the concept that an important criterion to obtain stable and high yield of rainfed lowland rice under drought conditions is, to have high potential yield under well-watered conditions (Fukai et al. 1999). On the other hand, the most significant traits related to grain yield (grains per panicle and percent seed set) are the capacity of plants to maintain the fertility of spikelets during

drought stress, and this might be related to their ability to maintain high leaf water potential through avoidance mechanisms. Despite being low, the grain yield under drought stress also correlated with the leaf rolling score (r = -0.135). This parameter is an indirect indicator of drought avoidance mechanisms of plants. It may support the proposal that the leaf rolling score is useful to estimate drought resistance if it is done during flowering time.

Another trait related to a drought avoidance mechanism is root pulling force that was also correlated to grain yield under stress (r = 0.123). This trait also showed a significant positive correlation with grains per panicle (r = 0.205). Considering high correlation between the grains per panicle and the grain yield under stress (r = 0.591), root pulling force may be a useful indirect selection criteria for grain yield under stress.

Marker segregation

From previous reported mapping results for grain yield under drought in other rice populations, we could identify a number of candidate QTL loci. Therefore, we used a candidate QTL approach to check if similar loci were involved in our RI population. In this study, we used the microsatellite marker RM104 located close to the semi-dwarf *sd-1* gene locus (physical distance around 1.8 Mb). There is also a non-shattering gene locus (*sh-2*) in this region. Analysis of departure from the expected 1:1 ratio showed that there was no distortion on this marker, indicating that our single seed descent method was not biased for selection at this major region. Out of 12 markers, 25% (3 markers) located on chromosomes 3, 10 and 12 were skewed toward IR64. Skewed segregation for several chromosomal regions is a normal phenomenon encountered in a mapping population.

QTL for grain yield under drought stress

We showed here a QTL for grain yield under drought stress on locus RM104. This locus explained 29.7% of the grain yield variation, with the lowland parental line contributing the favorable allele. Marker RM104 was also associated with grains per panicle (R^2 = 15.9%), plant height (R^2 = 15.9%), percent seed set (R^2 = 10.5%). Interestingly, this locus was also associated with leaf rolling score (R^2 = 12.6%). Despite that the performance of IR64 for grains per panicle, percent seed set, and leaf rolling score were inferior compared to Cabacu, in the RI population IR64 contributed favorable alleles for these traits. This phenomenon is common for interspecific populations in rice (Xiao et al. 1997, Moncada et al. 2001, Thomson

et al. 2003, Septiningsih et al. 2003) and tomato (de Vicente and Tanksley 1993, Eshed and Zamir 1995). It supports the idea that a phenotype of a plant is not always a good predictor of its genetic potential (de Vicente and Tanksley 1993; Xiao et al. 1997).

Fukai et al. (1999) emphasized the importance of the ability of rice plants to maintain high leaf and panicle water potential to avoid high spikelet sterility during pre-anthesis drought stress. The leaf rolling score is an indirect indicator of the ability of rice plants to maintain high leaf water potential. One explanation of the same position of QTLs for leaf rolling, percent seed set, and grains per panicle is that through an unknown avoidance mechanism the resistant rice plants can maintain high panicle water potential that keeps the percent seed set and the grains per panicle high.

We compared the QTL for leaf rolling that we identified to the other QTLs detected in different populations. We found that QTLs for leaf rolling, leaf dying, and relative water content were mapped in the region close to RM104 in a DH population derived from IR64 x Azucena (Courtois et al. 2000). Price et al. (2002) mapped QTLs for leaf rolling and leaf drying on the similar position using a RI population derived from Bala x Azucena. Robin et al. (2003) mapped a QTL for osmotic adjustment in the similar position using a BC3F3 population derived from IR622266 x IR60080. In all cases, the semi-dwarf *indica* parental lines contributed favorable alleles. A comparative map for these drought resistance components between the four populations in the region close to RM104 is summarized in Figure 3.

Like IR64, Bala carries the semi-dwarf allele at the *sd1* locus (Price et al. 2002). The pedigree of IR622266, indicates it may carry the *sd1* allele derived from Dee Geo Woo Gen, just like IR64 and Bala (www.iris.irri.org). It might be that this major gene, controlling the semi-dwarf stature, gives a positive effect in reducing biomass and, therefore, in decreasing transpiration. Biomass has been shown to influence leaf drying under water stress, the genotypes with larger biomass tending to have higher scores (Mitchell et al. 1998). On the other hand, it cannot be ruled out that osmotic adjustment might also explain leaf rolling in this region. It was documented that IR64 shows more osmotic adjustment than upland lines (Lilley and Ludlow, 1996).

There seems to be a relatively consistent pattern of QTLs identified across different genetic backgrounds for leaf rolling. This conserved region might be a good candidate for derivation of near-isogenic lines and further dissection.

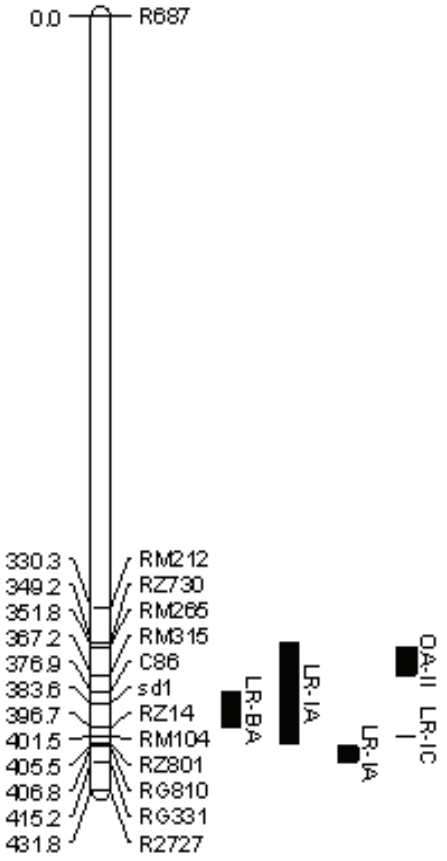

Figure 3. Comparative map for leaf rolling and osmotic adjustment between four different populations. The map was built using The Gramene TIGR Pseudomolecule Assembly of IRGSP Sequence 2005 (www.gramene.org) as anchoring map. The numbers in the left side describe the approximate position in the genome with scale 1 = 100 kb. Abbreviations: *LR-BA* leaf rolling in Bala x Azucena (Price et al., 2002), *LR-IA* leaf rolling in IR64 x Azucena (Courtois et al. 2000), *LR-IC* leaf rolling in IR64 x Cabacu (this study), *OA-II* osmotic adjustment in IR62266 x IR60080 (Robin et al., 2003).

ACKNOWLEDGEMENTS

We thank O. Dolstra, Z. Yan, D. Wouters, H. van der Schoot, W. van 't Westende, D. Esselink, E.M. Septiningsih and M. Yunus for helpful discussions, Masumah, Dadang, Iman, Harnati, S.R. Wardayanti for technical assistance in the greenhouse, and Agung for technical assistance in the field. We gratefully acknowledge the use of the spring balance instrument for root pulling force measurement from Soemartono.

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Chapter 3

Drought stress associated genes revealed by expression analysis of drought induction and drought resistant genotypes in Arabidopsis

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ABSTRACT

To dissect the genetic mechanisms of drought perception and tolerance we conducted whole genome microarray experiments in Arabidopsis. Genes differentially expressed under drought conditions in Arabidopsis were compared to the transcriptomes of three drought resistant genotypes. The three resistant genotypes were obtained by overexpression of the AP2/ERF transcription factors DREB1A, SHINE1 and HARDY. Microarray experiments of the three drought resistant genotypes compared to wild-type revealed the genes differentially expressed in each genotype that defined the specific gene associated 'regulon'. Comparative analysis of the drought transcriptome and the resistant gene regulons revealed genes that are important for the regulation of drought physiological responses, signaling and resistance. In addition to confirm the results of other reports on stress transcriptome analysis, our results on the whole genome microarrays provide additional genes in the drought stress transcriptome using a guilt-by-association analysis.

INTRODUCTION

Environmental stress is a major factor limiting stable crop production world-wide and has been estimated to cause depreciation in crop yield up to 70% (Boyer, 1982). The environmental factors that impose stress on plants can be broadly defined as: osmotic stress in the form of dehydration (drought) and salinity; temperature stress including chilling, freezing and high (heat) temperature; biotic stresses conferred by pathogens or pests (including wounding) as well as extremes in illumination or atmospheric conditions. In controlled laboratory studies a single stress factor can be imposed and the plant reactions precisely monitored at the level of transcripts, proteins, morphology and growth parameters. In nature most environmental stresses do not occur alone, for example osmotic stress due to drought in summer is accompanied by high temperature stress. Cold stress can also cause osmotic stress as it reduces water absorption and transport. The study of the interrelationships between different stresses is, therefore, relevant for applications in improving stress resistance under field conditions.

Plants respond quickly to stress stimuli through signal transduction processes and changes in gene expression. Osmotic stress caused by water deficit or high salt can change the expression of a set of genes (Zhu et al., 1997) that are also affected by low temperature stress (Thomashow, 1998). Many genes have been identified in response to different stresses by expression analysis and have been classified as *rd* (responsive to dehydration), *erd* (early responsive to dehydration), *cor* (cold-regulated), *lti* (low-temperature induced) and *kin* (cold-inducible). However, there is an overlap in the response of many of these genes, most dehydration-inducible genes also respond to cold stress and vice versa. Thus many of the identified genes have multiple names depending on their discovery. The analysis of stress responsive genes and promoters has enabled the identification of a cis-acting dehydration-responsive element (DRE) (Yamaguchi-Shinozaki and Shinozaki, 1994) and a similar low-temperature C-repeat (CRT) element (Baker et al., 1994).

Osmotic and cold stress influence ABA levels and many osmotic-stress and cold-stress responsive genes can also be induced by ABA (Zhu et al., 1997). The analysis of stress responsive gene expression studied in ABA-deficit (*aba*) and ABA-insensitive (*abi*) mutants has revealed the presence of ABA-dependent and ABA-independent signal transduction pathways in response to different stresses (Shinozaki and Yamaguchi-Shinozaki, 2000). At

least four independent pathways function under drought, two ABA-independent and two ABA-dependent. Two additional ABA-independent pathways are involved in low-temperature responsive gene expression. 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 are proposed to not necessarily act in a parallel manner but interact and converge to activate stress genes (Ishitani et al., 1997).

Plants respond to dehydration and low temperature by accumulating various proteins and smaller molecules including sugars, proline and glycine betaine. Analysis of the drought-stress inducible genes additionally reveal the plethora of responses that include the induction of transcription factors (MYC, MYB), phospholipase C, protein kinases (MAPK, CDPK), proteinases, water channel proteins, chaperons, detoxification enzymes (GST, sEH), protection factors of macromolecules (LEA proteins) and osmoprotectant synthases (for proline, betaine, sugar). Cold acclimation involves complex physiological changes resulting in reduction of growth, water content, transient increase in ABA, changes in membrane composition and also accumulation of osmolytes like proline, betaine and soluble sugars. The overlap in the induced proteins and other molecules in the different stress responses emphasize the interrelationship of signaling pathways and also reveal their complexity confronted by analyses of the product of the stress response.

It has been demonstrated that expression of a single gene can confer stress tolerance in model plants. Apart from the possibility of application in crop plants, these genes and the downstream pathways offer a model to understand the underlying mechanisms in stress tolerance. Overexpression of transcription factors (TFs) conferring stress tolerance provides the tools to study the downstream genes involved. It was shown that overexpression of the AP2/ERF factors CBF1, DREB1A and CBF4 resulted in drought/salt/cold tolerance in Arabidopsis (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Haake et al., 2002). The orthologous genes of *CBF*/*DREB* have been found in most crop plants examined and systematically characterized in Brassica and rice (Jaglo et al., 2001; Dubouzet et al., 2003). Many of the putative orthologs have been functionally tested, indicating conservation of the stress tolerance pathway in many plants. Constitutive overexpression of the Arabidopsis *CBF*/*DREB* genes in canola results in increased freezing tolerance (Jaglo et al., 2001) and drought tolerance.

A number of other AP2/ERF TFs display stress tolerance when overexpressed. Recently, an AP2 TF SHINE has been shown to confer drought resistance in Arabidopsis (Aharoni et al., 2004) using a different mechanism as that of the *DREB*/*CBF* genes. Another AP2/ERF gene named *HARDY* has been identified recently (Dixit et al., unpublished), that confers drought and pathogen resistance, providing another gene to study the downstream mechanisms.

Gene expression analysis using microarrays enables a genome wide comparison of genes induced or repressed under specific conditions like specific stress responses. A number of studies have been done with various microarray systems and revealed sets of regulated genes (Shinozaki et al., 2003). Also a few genotypes conferring stress tolerance have been used and have revealed downstream expressed genes (Maruyama et al., 2004; Takahashi et al., 2004; Vogel et al., 2005). However due to the various differences in microarray systems and stress testing conditions a comprehensive description and compilation of stress induced genes has not been made. Drought stress induction has been undertaken in many ways, e.g. early induction or at specific stages. We employ here a set of drought resistant genotypes obtained by overexpression of three AP2/ERF genes: *DREB1A*, *SHINE1* and *HARDY* in Arabidopsis. In addition, we study the induction of drought induced genes in a gradual drought stress experiment with wild-type plants, using conditions that display drought resistance in the AP2/ERF overexpression lines.

MATERIALS AND METHODS

RNA isolation and amplification

Total RNA was isolated using TRIZOL reagent (Life Technologies, Inc.) using two-four rosette leaves of Arabidopsis according to the manufacturer's instruction and purified using RNeasy Minelute Kit (Qiagen, Carlsbad, CA, USA).

RNA amplification was performed using Amino Allyl MessageAmp Kit (Ambion, Austin, TX, USA). Briefly, first strand cDNA synthesis was carried out by reverse transcription using 3 μg Total RNA and 1 μl T7 Oligo(dT) primer. Full length dsDNA was synthesized with the reaction containing 1× second strand buffer, 4 μl dNTP mix, 2 μl DNA

polymerase, 1 µl RNase H, followed by incubation for 2 h at 16 °C. The cDNA was purified by applying to an equilibrated filter cartridge. The purified cDNA was concentrated and the volume adjusted to 14 µl. The anti-sense RNA amplification by T7 in vitro transcription was achieved in a solution containing 12 µl of ATP, CTP, GTP Mix (25 mM), 3 µl of aaUTP Solution (50mM), 3 µl of UTP Solution (50mM), 4 µl of 10 × reaction buffer, 4 µl of T7 enzyme mix at 37 °C for 8 h. Then the amplified aRNA was purified by applying to an equilibrated filter cartridge.

Preparation and hybridization of fluorescent-labeled aRNA

For hybridization we used spotted long oligonucleotide Operon array from David Galbraith's Laboratory comprising 29,000 genes of Arabidopsis (http://www.operon.com/arrays/omad.php). The aRNA samples were coupled to mono-reactive NHS esters of Cy3 or Cy5 (Amersham Biosciences) for 2 hrs. After dye quenching and purification, the targets were mixed to 80 µl hybridization solution containing 2xSSC, 6%Liquid Blocking Reagent (Amersham Biosciences), and 0.08% SDS. After heating to 95 °C for 2 min and then cooled on ice, the mixture was applied to the gap between the slides and coverslip (LifterSlip, Erie Scientific). Slides were incubated in a hybridization chamber for 9 h at 56 °C in an oven. Slides were washed at RT for 5 min in solutions of 2×SSC with 0.5% SDS, 0.5×SSC, and 0.05×SSC sequentially and then spin dried for 10 min at 1000 rpm.

Array scanning and data processing

Hybridized slides were scanned with the ScanArray Express HT scanner (Perkin Elmer), which generates Tiff images of both the Cy3 and Cy5 channels. ScanArray PMT voltage was set from 42 to 50 V depending upon the first sign of a saturated signal. The scanned images were analyzed with the software program ScanArray Express (Perkin Elmer). Adaptive circle method was used for quantification and for data normalization the LOWESS method was used.

For further analysis, the data was extracted into Excel spreadsheets and the gene expression values of channel 1 or channel 2 (Cy3 or Cy5) that displayed more than 2x background for the median intensity were selected. In addition, we filtered for significantly

regulated genes that displayed ratios of the medians that were more than 1.5 or less than 0.66 (Log_2 values +/- 0.58). These gene expression lists provided us with a long list of potentially regulated genes that were further used for other comparisons between experimental treatments. In the analysis of specific treatments (e.g. drought) the genes with expression values of at least one replication more than 2 fold (Log_2 values +/-1) were taken to compare to other treatments.

Plant materials and experimental setup

In our studies on screening for drought resistant Arabidopsis genotypes, wild type ecotype Wassilewskija (WS) displayed death after nine days of withholding water, whereas the rd29A::*DREB1A* overexpressor, 35S::*SHN1* overexpressor, and *HRD* overexpression mutant *hrd-D* still survived till 12 days of withholding water (Aharoni et al., 2004; Dixit et al., unpublished). For uniform testing of plant material we used 8 days of water withholding for wild type WS and harvested a leaf sample for RNA isolation, and at the same time took leaf samples from wild type WS control, rd29A::*DREB1A*, 35S::*SHN1* and *hrd-D* that were regularly watered. For each comparison (wild type control vs wild type drought or wild type control vs overexpressor) we used two biological replications, respectively. The wild type control aRNAs were labeled with Cy3 and the wild type drought or the overexpressor aRNAs were labeled with Cy5 in both biological replications. A number of replications were also done with dye swap experiments as technical control.

RESULTS

Combined Microarray experiment analysis

The objective of the microarray experiments was to identify genes induced/repressed in drought stress and compare them to genes regulated in drought stress resistant genotypes. For the comparative analysis we used plant leaf material harvested under the same greenhouse conditions, and the same wild-type leaf samples as control for all the samples so that comparisons could be made for all treatments. In total there were 4 treatments (Drought, DREB1A, SHINE, HARDY) with two biological replications each. After normalization

within experiments, the wild-type intensity values were compared and were very similar, suggesting that the experiments could be combined without normalization between experiments. Consistency between experiments was taken as a criterion for selection of the gene expression values in addition to the criteria described in Methods above.

For the combined analysis we first selected a list of 2459 potentially regulated genes that were induced/repressed more than 1.5 fold in at least one treatment and used this for further selection and analysis. To compile a list of genes for a specific treatment, genes with more than 2 fold induction/repression in a replication and more than 1.5 times in the second replication, were taken to generate a complete list of treatment (e.g. drought) associated genes. Only one gene was significantly overexpressed in all treatments: Lipid transfer protein LTP2 (At2g38530). Five genes were repressed in all treatments, including a BHLH TF (At5g39860), the expansin *EXPL5* gene (At3g29030) and 3 other genes of unknown function.

A summary of the most significant results of the differentially expressed genes is shown in Table 1, describing the distribution of induced and repressed genes in the most common functional categories observed among the genes.

The coregulation of drought stress associated genes is shown in Table 2 that displays the genes induced by drought stress that display different patterns of expression in the three stress resistant genotypes. The details of each treatment are described in the following sections of the results.

Response to gradual drought stress in Arabidopsis plants

In order to discover the genes induced by putting plants under gradual water stress, a condition simulating plants under field drought conditions, we set up an experiment to put Arabidopsis plants for various days of water deficit. In the experiment, wild-type WS ecotype plants were grown for 2 weeks and then water was withheld. At 9 days after no water the majority of the pot replicates showed wilting and at 10 days after drought the wilted plants were irreversibly dead as they could not be restored by re-watering. We chose day 8 grown plants at which stage the plants were under water deficit but not yet wilted and considered this as an early stage of gradual water stress.

Table 1. Summary of differentially expressed genes of different categories, induced or repressed in the genotypes or conditions

(>2 fold in both replications, in parenthesis are percentage among total differentially expressed genes, in bold are those categories significantly different)

Expression	**Category**	**Drought**	**DREB1A**	**SHINE**	**HARDY**
Differential		507	508	187	156
Induced	Total	188	173	81	78
	Abiotic	31 (6.1)	29 (5.7)	10 (5.3)	16 (10.2)
	TF	19 (3.7)	13 (2.6)	6 (3.2)	9 (5.7)
	Signaling	13 (2.5)	9 (1.8)	8 (4.3)	8 (5.1)
	Lipid	9 (1.8)	6 (1.2)	**14 (7.5)**	4 (2.5)
	Cell wall	4 (0.8)	7 (1.4)	**8 (4.3)**	5 (3.2)
	Protease	8 (1.6)	4 (0.8)	2 (1)	2 (1.3)
	Inhibitors	3 (0.6)	9 (1.8)	3 (1.6)	4 (2.5)
	Biotic	4 (0.8)	5 (1)	3 (1.6)	3 (1.9)
	Transport	9 (1.8)	8 (1.6)	1 (0.5)	0 (0)
	Enzymes	34 (6.7)	15 (2.9)	7 (3.7)	8 (5.1)
	Ribosomal	0 (0)	1 (0.2)	0 (0)	1 (0.6)
	Housekeeping	0 (0)	3 (0.6)	1 (0.5)	1 (0.6)
	Expressed	36 (7.1)	45 (8.8)	10 (5.3)	10 (6.4)
Repressed	Total	319	335	106	78
	Abiotic	8 (1.6)	10 (2)	**16 (8.6)**	1 (0.6)
	TF	18 (3.5)	24 (4.7)	2 (1)	3 (1.9)
	Signaling	21 (4.1)	**60 (11.8)**	5 (2.7)	8 (5.1)
	Lipid	9 (1.8)	12 (2.3)	7 (3.7)	9 (5.7)
	Cell wall	24 (4.7)	18 (3.5)	13 (6.9)	2 (1.3)
	Protease	3 (0.6)	5 (1)	2 (1)	2 (1.3)
	Inhibitors	3 (0.6)	4 (0.8)	1 (0.5)	3 (1.9)
	Biotic	3 (0.6)	16 (3.1)	4 (2.1)	3 (1.9)
	Transport	5 (1)	16 (3.1)	5 (2.7)	4 (2.5)
	Enzymes	30 (5.9)	64 (12.6)	27 (14.4)	23 (14.7)
	Ribosomal	**112 (22.1)**	0 (0)	0 (0)	0 (0)
	Housekeeping	15 (2.9)	14 (2.7)	1 (0.5)	0 (0)
	Expressed	35 (6.9)	59 (11.6)	11 (5.8)	8 (5.1)

Expression in leaves of the 8 day drought treated plants was compared to the same age plants that had not been given drought stress. From the combined analysis we chose genes that were induced/repressed more than 2 fold in at least one replication and more than 1.5 times at least in the second, to generate a consistent list of drought stress induced genes. This revealed 250 induced and 400 repressed genes, and on considering more than 2 fold induction or repression in both experiments there are 188 induced and 319 repressed genes. Further, these genes were compared for their patterns of expression in other experiments to reveal

genes expressed in drought resistant plants and those specific to drought stress. A representative set of drought induced genes not changed in drought resistant plants is shown in Table 3A and the drought repressed genes that are co-regulated in the resistant genotypes are shown in Table 3B. All significant results of co-regulated genes are summarized in Table 2.

The induced/repressed genes fall into certain general classes that were examined for relevance in drought mechanisms. Most remarkable is a class of 112 repressed genes encoding ribosomal proteins as well as chloroplast and histone proteins that signify the major effect of drought stress on housekeeping functions of the plant. In addition a large number of TFs and a set of expansins are repressed. Most significantly the two-component response regulator ARR6 was repressed in drought but induced in the stress tolerant genotypes. Among the genes specifically induced in drought but not significantly seen in the stress tolerant genotypes (Table 3), are a group of TFs including zinc finger and homeobox genes, a number of genes involved in phosphorylation signaling, various stress induced genes and some genes involved in metabolic processes. The well known stress induced genes are from the known class of *rd*, *erd*, *cor*, *lti*, *kin*, *lea* (late embryogenesis proteins), heat shock proteins and ABA induced proteins, many of them are induced in the stress tolerant overexpressor genotypes (shown in Table 2). The most significantly induced pathways involved in abiotic stress protection are for genes in osmolyte production; galactinol synthase, P5CS (proline synthesis), and ascorbate metabolism genes (dehydroascorbate reductase, ascorbate peroxidase). In addition, there are 36 induced and 35 repressed genes of unknown function, annotated as expressed proteins that can be now annotated as new drought related genes.

Microarray analysis of DREB1A overexpression in Arabidopsis

The stress tolerant gene *DREB1A*/*CBF3* displays drought, cold and salt tolerance when overexpressed in plants. Though microarray analyses of the genes induced or repressed by *DREB1A* overexpression have been published (Maruyama et al., 2004), they are based on different platforms using full length cDNA or Affymetrix and not for a complete Arabidopsis gene set. Here, we used a whole genome oligo array to analyze the genome wide expression of genes induced and repressed in plants expressing *DREB1A* under control of the rd29A promoter that has a medium level and stress induced activity compared to earlier studies of high over-expression under the CaMV 35S promoter. We used RNA isolated from leaf tissue

of DREB1A plants compared to wildtype, at 3 weeks after germination grown under ample water conditions. Two biological replicates were examined and revealed 219 induced and 422 repressed genes considering at least one replication more than 2 fold and second more than 1.5 fold. At more stringent criteria of both replications more than two fold change there were 173 induced and 335 repressed genes. A representative set of those DREB1A induced/repressed genes more than two-fold are shown in Table 4, along with a comparison to the other drought resistant genotypes and compared to the drought transcriptome in Table 2.

The pattern of genes up/down regulated in the DREB1A line was more similar to the genes regulated by drought. Among the DREB1A overexpressed genes 55 were also induced in drought, 41 in HARDY and 10 in SHINE lines. Among the DREB1A repressed genes 112 were also repressed in drought, 52 in HARDY and 22 in SHINE. To compile a list of genes significantly induced/repressed in only DREB1A plants, genes with expression values more than 2 fold in both replications were taken, showing 173 induced and 335 repressed genes. Among the DREB1A specific genes a large class is of unknown function, annotated as expressed proteins; making up to $1/4^{th}$ that of induced genes and $1/6^{th}$ of the repressed genes. The *DREB1A* gene is the highest induced gene in *DREB1A* overexpressor, but this gene is repressed in the SHINE and HARDY genotypes. Another AP2/ERF DREB subfamily member is also induced in the *DREB1A* overexpressor, including two other TFs. As expected this genotype revealed a large number of genes (29 induced and 10 repressed) that are known to be involved in stress responses. In addition, a major class of 9 proteinase inhibitors (included those classified as LTP proteins) and 3 cytochrome P450 genes were induced. Among the repressed genes there are 24 TFs and 4 defensin genes and interestingly the BRI1 brassinosteroid receptor and the GA oxidase dwarfing gene, both important in reducing plant height.

Expression analysis of the drought resistant SHINE1 gene overexpressor

Overexpression of the *SHINE1* gene in Arabidopsis induces an increased lipid composition and confers drought resistance (Aharoni et al., 2004). This is seen in the microarray results which reveal 141 induced and 158 repressed genes, considering genes with a change more than 2 fold in a replication and more than 1.5 fold in the second. At the more stringent criteria of both replications higher than two fold change, there are 81 induced and

106 repressed genes. Table 5 shows a part of the SHINE regulon, with genes induced and repressed specifically in SHINE1.

A major class of induced genes is 14 genes involved in lipid metabolism and 8 involved in cell wall synthesis, significantly higher represented than in the other treatments. Among the lipid biosynthesis genes a known class involved in wax biosynthesis was induced; including *CER2*, *CER1* (in one replication), 5 members of the beta-ketoacyl-CoA synthase family involved in elongation of very long chain fatty acids. Among the TFs, expression of the *SHINE1* gene is the highest as expected in the plant overexpressing the gene, but in addition 3 MYB genes and 3 other TFs. A significant number of genes involved in signaling were induced that included 4 kinases and a set of genes involved in Calcium binding and signaling. Among the genes involved in cell wall biosynthesis a xyloglucan endotransglycosylase and a transferase were induced and another pair of the same genes significantly repressed.

Overexpression of the *SHINE3* gene seemed to be also highly induced, but this was found out to be due to the homology between the specific *SHINE3* oligonucleotide probe and the *SHINE1* mRNA (Operon oligo sequence, http://www.operon.com/arrays/omad.php). Two other *SHINE3* oligos were present on the microarray without homology to *SHINE1* and they do not show induction.

Most interestingly a class of 5 GDSL-motif lipase/hydrolases and two additional undefined lipases were repressed that made up the entire set of repressed lipid metabolism associated genes. A set of genes involved in ascorbate synthesis were induced, including a cluster of dehydroascorbate reductases (At1g19550 and At1g19570), ascorbate oxidase and monohydroascorbate reductase (slightly below 2 fold). However, only two stress regulated genes; *RD22* and a LEA gene were induced specifically in SHINE (and drought), showing a different pattern from DREB1A.

Analysis of the drought resistant HARDY gene overexpressor by microarrays

The *HARDY* (*HRD*) gene displays drought and pathogen resistance when overexpressed in Arabidopsis (Dixit et al., unpublished). To understand the mechanism of combined abiotic and biotic stress responses, microarray experiments were done with the *HRD* overexpression mutant *hrd-D* that was obtained by activation tagging. The microarray experiments reveal 142 induced and 151 repressed genes using criteria of a replication more than 2 fold and second

more than 1.5 fold changed. At the stringent criteria of both replications more than two fold change there were 78 induced and 78 repressed genes. Table 6 shows a selection of the HARDY regulon with induced/repressed genes more than two-fold compared to wild-type.

The pattern of expression of HARDY is more similar to that of DREB1A than to the other treatments (Table 2). The genes co-induced with HARDY are 39 with DREB1A, 5 with SHINE and 6 with drought. These include stress induced genes, proteinase inhibitors, TFs and osmolyte pathway genes. Among the repressed genes, 36 are with DREB1A, 31 with SHINE and 7 with drought. The genes specifically induced in the HARDY regulon are a set of 4 TFs, Ca2+ signaling proteins as well as trehalose and galactinol metabolism genes. The biotic stress related genes are represented by the induced defensin PDF2 genes that have been shown to be induced by pathogen responses in a jasmonate pathway, and repression of the pathogenesis related PR-5 and Bet v1 genes.

DISCUSSION

The phenotypic analysis of Arabidopsis plants over-expressing the three AP2/ERF transcription factors (TFs) DREB1A, SHINE1 and HARDY showed that they could withstand drought for more than 9 days at which stage the wild-type plants underwent irreversible wilting and finally death. We, therefore, used wild-type plants just beginning to wilt, at 8 days of no water, and also the stress resistant genotypes at the same stage. Genome wide microarray expression analysis of the differentially expressed genes in these treatments provides us with a view of genes associated with drought responses and resistance.

We examined the genes induced and repressed under drought stress and compared them to the expression in the three drought resistant genotypes (Table 2). The transcriptome of the drought resistant genes can be termed the ‘regulon’ or set of genes regulated by the transcription factor (Vogel et al., 2005). The pattern of drought induced genes was most similar to the regulon of DREB1A, next to SHINE and least to HARDY. Among the 250 genes induced by drought, 77 are also induced by DREB1A, 45 by SHINE and 24 by HARDY. The overlapping set of genes induced in drought and in the stress resistant gene regulon suggests the association with drought response, signaling or resistance.

The class of genes known to be induced by abiotic stress, like *RD, ERD, COR, KIN, LTI, LEA* and ABA-induced are induced preferentially in the drought and DREBIA

experiments and a subset induced in HARDY. Thus half of the 18 genes induced by drought, DREB1A and HARDY are known stress induced genes. There are very few stress induced genes, e.g. *RD22* that are not induced in DREBIA but in another stress resistant line (SHINE). This supports the independence of the SHINE drought resistance mechanism as *RD22* contains MYC and MYB recognition sites in the promoter region and function in the drought- and ABA-induced pathway (Iwasaki et al., 1995; Abe et al., 1997). SHINE also displays a significantly higher proportion of genes involved in lipid metabolism and cell wall synthesis supporting the biological role proposed in plant protective layers (Aharoni et al., 2004).

The TFs ERF4/AP2, a WD40 family gene and a CCAAT-binding TF are induced in drought, DREB1A and HARDY. An annotated RD gene, *RD26* is induced in DREB1A and drought, and is a NAM (no apical meristem) TF shown to be involved in drought resistance (Fujita et al., 2004), in addition one NAM gene is induced and another repressed in drought and DREB1A genotypes. The TFs of the homeodomain class are prominent, ATHB1, ATHB6 are induced by drought alone, while ATHB7 and ATHB12 are induced both by drought and DREB1A. All these TFs are candidates to confer drought stress tolerance by overexpression analysis. In addition to activation of gene expression by TFs, repression is also involved (Sakamoto et al., 2004) and observed in our list of novel TFs that are in the repressed regulon and drought transcriptome.

The mechanism for conferring drought resistance in the three AP2/ERF genes is not identical as evident from the non-correspondence between their regulons. If one considers that the common set of induced genes would be the minimal for conferring drought tolerance, this would be very few, though more than what we show here as levels lower than 1.5 or 2 fold are not considered in the analysis, though there are many genes at low levels of induction that are common to multiple treatments. More accurate experimentation like real-time PCR analysis of these genes would certify their role in the resistant gene regulons. On the other hand it is evident that different classes of genes are expressed in each of the resistant gene regulons suggesting a partial difference in mechanism. At the same time genes involved in some physiological processes involved in drought tolerance are induced in most of the TF regulons. Thus genes involved in the metabolism of proline, trehalose, raffinose are induced, and also a number of cytochrome P450 genes involved in various metabolic processes and stress tolerance (Shinozaki et al., 2003; Narusaka et al., 2004).

Earlier studies on the cold tolerant gene regulons, DREB1A using about 8000 genes and CBF2 using the whole genome gene chip provided a set of genes involved in cold stress tolerance (Maruyama et al., 2004, Vogel et al., 2005). We compared our drought transcriptome and the drought resistant gene regulons to these results (Table 2) and compile a set of stress tolerance genes. In addition to the common stress induced genes a number of additional transcription factors of the earlier described families are induced. Thus new TFs of the MYB, AP2, bHLH/MYC, C2H2 Zn finger, and NAM class are described here in the drought stress transcriptome. In addition, we also showed that the TF members of the homeobox (HD-zip), YABBY and WD40-repeat families are involved in drought stress. The drought transcriptome also comprises a number of additional signaling related genes like kinases and phosphatases. A large number of new genes are observed in the DREB1A regulon as we described (Table 2), these new genes are due to a more extensive genome wide coverage in our experiments.

The dissection of the drought transcriptome using different drought resistant genes provides us with information on the different biochemical and physiological processes that contribute to drought. Most interestingly, in many of the resistant gene regulons only a few genes of particular biochemical pathways are consistently induced. Thus though the biochemical process is relevant to drought/stress resistance, complete pathways are not induced, only probably the key enzymes.

Our use of the whole genome microarray results provides us with many genes associated in the drought transcriptome than have been earlier described. Also our analysis of the 3 drought resistant genotypes and their regulons provide us with new genes and expression patterns. In comparison to other published work on drought and other stress, a guilt-by-association approach of analysis provides us lists of genes in a regulon that are correlated in expression to known genes.

The drought stress associated genes identified here provide us candidate genes for other studies. These are useful to compare to other environmental stress responses and stress resistant genotypes. The identified Arabidopsis genes can be used to identify orthologs or genes with similar sequences and possibly function in other plants like rice (Cooper et al., 2003) whose genome sequence is now available. The validation of the genes identified here for their role in drought resistance in other crop species will be an effort in the future.

ACKNOWLEDGEMENTS

We thank C. Broekgaarden, D. Wouters, M. Szemes, A. van Houwelingen, O. Vorst, O. Alda and R. Joosen for helpful discussions.

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Table 2: Drought induced genes in interaction to stress resistant gene regulons

		DROUGHT		DREB1A		HARDY		SHINE		CBF2	cDRE
Name	**Gene Description**	127	128	193	195	129	130	126	131		
At2g34720	CCAAT-binding transcription factor (CBF-B/NF-YA)	1.77	1.67	1.48	2.19	1.18	0.66	-0.2	0.01		
At3g15210	ethylene-responsive element-binding factor 4 (ERF4)	1.26	1.52	1.38	0.72	1.81	1.56	0.66	0.5		
At3g23000	CBL-interacting protein kinase 7 (CIPK7)	2.41	2.04	1.73	2.42	1.01	0.73	-0.2	-0.4		
At1g13930	drought-induced protein SDi-6	0.8	1.18	1.63	1.56	1.37	0.64	-0.5	-0.8		
At5g52310	desiccation-responsive protein 29A (RD29A) COR78	2.42	2.87	3.48	5.48	2.32	2.1	-0.2	-0.2		
At1g20440	dehydrin COR47/RD17	1.4	1.49	4.19	5.7	1.74	1.57	0.52	0.06		
At1g20450	dehydrin ERD10 (LTI45)	0.99	1.39	3.52	4.17	1.56	1.47	-0.2	-0.6		
At2g23120	expressed protein (COR8.5)	1.44	0.87	2.48	3.87	1.15	0.69	0.4	0.09		
At5g50720	ABA-responsive protein (HVA22e)	1.53	2.3	4.01	6.5	2.58	2.18	-0.3	-0.5		
At1g78070	WD-40 repeat family protein	1.06	1.19	2.95	3.46	1.78	1.12	-0.2	-0.3		
At4g04020	plastid-lipid associated protein PAP / fibrillin	1.1	1.28	2.6	2.23	1.52	0.59	-0.1	-0.3		
At3g57020	strictosidine synthase family	1.09	1.26	1.43	1.54	0.76	1.18	-0.2	-0.4		
At2g42540	cold-regulated protein cor15a: Lea protein	1.4	2.09	4.31	5.83	2.62	2.24	-2.8	-2.8		
At5g15970	stress-responsive protein (KIN2) / COR6.6	2.06	2.2	1.08	3.82	2.25	2.58	-1.6	-1		
At2g37760	aldo/keto reductase, similar to chalcone reductase	1.92	1.57	1.11	0.59	0.21	-1.29	0.78	1.03		
At2g39980	transferase family protein	1.23	1.19	4.67	0.61	0.15	-0.6	1.24	1.51		
At1g54000	GDSL-like Lipase/Acylhydrolase, myrosinase-associated	1.14	0.63	0	0	1.17	2.56	1.13	0.74		
At5g59320	lipid transfer protein 3 (LTP3) protease inhibitor	4.27	5	-2.7	0	1.59	0.73	1.68	3.19		
At5g04340	zinc finger (C2H2 type) family protein	2.73	0.81	3.2	1.18	0.06	1.09	0.37	0.32		
At1g01470	Late embryogenesis abundant protein Lea14-A	2.44	2.08	3.02	4.71	0.94	0.27	0.42	0.1		
At4g24960	ABA-responsive protein (HVA22d)	1.46	1.31	6.87	4.84	0.66	0.95	-0.7	-0.7		
At4g27410	no apical meristem (NAM) family protein (RD26)	3.36	2.64	1.74	1.35	0.94	-0.66	0.32	0.04		
At2g15970	cold acclimation protein WCOR413-like protein	0.89	1.22	2.82	1.53	-0.07	0.41	-0.5	-0.3		
At1g05170	galactosyltransferase family protein	1.32	1.33	4.01	3.2	1.01	0.54	-0.2	-0.3		
At1g58360	amino acid permease I (AAP1)	1.2	1.44	2.28	2.27	-0.98	-0.4	-0.3	-0.7		
At4g17550	glycerol 3-phosphate permease	1.14	1.43	2.63	2.52	0.52	0.38	-0.3	-0.2		
At3g12580	heat shock protein 70 / HSP70	1.1	1.5	3.55	2.35	-0.57	-0.02	0.18	0.07		
At3g28270	At14a related protein	3.27	3.55	3.16	2.7	-0.44	-0.63	-1.7	-0.3		
At5g17460	expressed protein	2.52	3.41	7.26	8.15	1.56	0.56	0.03	-0.6		
At3g61890	HD-ZIP transcription factor ATHB-12	3.86	4.1	1.1	1.64	0.34	0.63	-0.3	-1		
At2g46680	HD-ZIP protein ATHB-7	3.27	4.24	2.09	1.46	0.67	-0.87	0.4	-0.1		
At5g67300	myb family transcription factor	1.88	0.81	2.26	2.18	0.16	0.19	0.46	0.17		
At1g01720	no apical meristem (NAM) family protein	1.13	1.19	0.69	1.04	0.73	0.24	-0.2	-0		
At2g21620	universal stress protein (USP)/ RD2	0.77	1.1	1.52	0.9	0.48	0.26	0.27	-0.3		
At3g09390	metallothionein protein, putative (MT2A)	1.92	1.51	2.1	1.91	0.67	-0.66	0.5	0.38		
At1g53580	hydroxyacylglutathione hydrolase / glyoxalase II	1.72	1.42	1.67	2.06	0.57	0.1	-0.6	-0.3		
At4g16760	acyl-CoA oxidase (ACX1)	1.61	1.08	1.08	0.87	0.02	-0.07	0.37	-0.1		
At4g21580	oxidoreductase, zinc-binding dehydrogenase	1.22	1.24	1.31	1.04	0.37	-0.09	0.16	-0.3		
At1g17840	ABC transporter family protein	1.37	1.77	0.66	1.55	0.01	0.76	0.83	0.9		
At2g41190	amino acid/polyamine transporter, family II	3.75	4.52	1.75	1.66	0.37	0.56	0.35	0.21		
At1g64780	ammonium transporter 1, member 2 (AMT1.2)	1	0.82	0.75	0.76	-0.37	-0.78	-1.7	-1.2		
At1g19570	dehydroascorbate reductase,	2.09	1.22	1.6	1.82	1.1	0.28	-1.6	-1.7		
At1g19550	dehydroascorbate reductase	1.76	0.9	1.49	1.71	0.78	0.11	-1.4	-1.5		
At4g27560	UDP-glucoronosyl and UDP-glucosyl transferase	1.87	1.11	3.47	3.89	-0.17	0.18	-2.2	-2.3		
At4g39730	Polycystin-1, Lipoxygenase, Alpha-Toxin/Lipoxygenase	1.38	1.4	1.41	1.89	0.23	-0.6	-0.9	-1.3		
At1g02820	late embryogenesis abundant 3 protein / LEA3	1.95	1.71	2.25	3.29	0.27	-0.78	-1.2	-0.9		
At4g27570	UDP-glycosyltransferase	1.66	1.28	3.73	4.33	0.47	0.59	-1.1	-2.1		
At4g36010	pathogenesis-related thaumatin protein, PR5K	1.08	0.69	3.2	4.8	0.75	-0.28	-0.9	-1.2		

Microarray data are given for eight individual experiments (labeled 126-195) in the Log_2 ratios of the treatment to wildtype, genes in dark shade indicate induced and light shade repressed. Data in columns CBF2 and cDRE are published results from constitutive CaMV35S overexpression of CBF2 and DREB1A.

Table 2 (continued)

		DROUGHT		DREB!A		HARDY		SHINE		CBF2	cDRE
Name	**Gene Description**	127	128	193	195	129	130	126	131		
At5g10380	zinc finger (C3HC4-type RING finger)	2.01	0.63	1.27	0.13	1.1	-0.46	1.14	1.08		
At5g25610	dehydration-responsive protein (RD22)	2.19	2.36	-0.5	-0.2	0.3	-0.31	2.02	2.01		
At3g57260	glycosyl hydrolase family 17 protein	2.92	1.19	2.51	-1.9	0.34	-1.77	0.79	1.22		
At5g10760	aspartyl protease family protein	2.52	0.9	2.78	-3.6	1.13	-3.72	1.22	2.54		
At4g11890	protein kinase family protein	2.11	1.73	-0.6	-2.6	0.07	-1.49	1.96	1.64		
At3g57520	Raffinose synthase, seed imbibition protein Sip1	1.9	1.72	0	2.44	-0.87	0.11	0.66	1.37		
At5g55450	lipid transfer protein (LTP), protease inhibitor	1.87	1.41	1.8	-0.7	0.19	-2.42	1.25	2.47		
At2g14610	pathogenesis-related protein 1 (PR-1)	1.78	1.09	4.47	-3.9	2.34	-5.8	1.16	8.4		
At1g07720	beta-ketoacyl-CoA synthase family protein	2.06	2.45	-0.5	-0.8	-0.44	-0.51	1.55	1.44		
At1g04220	beta-ketoacyl-CoA synthase	1.78	3.45	1.58	-0.6	0.06	-0.35	1.55	1.81		
At1g68530	very-long-chain fatty acid condensing enzyme (CUT1)	2	2.08	-0.2	-0.1	0.34	-0.01	1.07	1.11		
At4g24510	eceriferum protein (CER2), Transferase family	1.56	2.29	0.15	1.46	0.48	1.41	1.41	1.2		
At1g01600	cytochrome P450,	1.05	1.49	2.67	0.23	0.14	-0.01	2.43	2.78		
At4g00360	cytochrome P450, putative	1.25	1.67	-0.3	-0.2	0.17	0.25	1.21	1.18		
At1g08450	calreticulin 3 (CRT3)	1.32	0.92	0.44	-2.3	0.3	-1.22	1.42	1.27		
At1g75040	pathogenesis-related protein 5 (PR-5)	1.94	1.05	0.65	-3.7	-0.71	-3.71	0.81	1.38		
At5g40780	lysine and histidine specific transporter,	1.45	0.91	-0.8	-1.1	0.47	-0.75	1.33	1.69		
At4g02380	late embryogenesis abundant 3 family protein / LEA3	0.84	1.34	-1.2	-1.2	-0.02	-1.16	1.41	1.29		
At5g22630	prephenate dehydratase family protein	1.9	1.56	0.37	0	2.54	3.59	-0.2	-0.6		
At5g41400	zinc finger (C3HC4-type RING finger) family protein	1.5	1.36	1.78	0.31	1.45	1.13	0	0.2		
At5g01600	ferritin 1 (FER1)	1.31	2.58	1.6	-0.3	3.42	1.04	-2.7	-0.3		
At4g23600	tyrosine transaminase like protein (CORI3/JR2)	1.29	1.15	-1.5	2.68	-0.11	0.36	-0.5	-0.7		
At1g11210	expressed protein (COR35)	2.22	2.07	6.95	0	0.06	0.41	-0.4	-0.9		
At5g52300	desiccation-responsive protein 29B (RD29B)/LTI65	4.82	4.72	3.87	0	-0.29	0.65	-0	-0.5		
At1g62570	flavin-containing monooxygenase family protein	2.21	1.38	4.87	0	0.15	0.93	-0.1	-0.2		
At2g39030	GCN5-related N-acetyltransferase (GNAT) family	1.79	0.91	2.9	0	0.81	-0.93	0.83	0		
At1g76180	dehydrin (ERD14)	0.83	1.09	0.83	0.83	0.88	0.93	0.64	0.33		
At5g66400	dehydrin (RAB18)	2.64	2.35	0.46	1.29	0.66	0.47	0.82	-0.7		
At4g22820	zinc finger (AN1-like) family protein	2.37	2.34	0.89	0.98	-0.48	-0.52	0.59	0.54		
At1g56600	galactinol synthase isoform GolS-1	3.54	3.26	4.93	-2.1	-0.63	-0.57	-2.1	-1.6		
At4g39800	myo-inositol-1-phosphate synthase 1 /MI-1-P synthase 1	0.68	1.11	-0.3	0.09	-0.24	0.03	-1.4	-1.1		
At2g39800	delta 1-pyrroline-5-carboxylate synthetase A / (P5CS1)	2.28	2.23	0.93	0.51	0.24	0.09	-0.9	-1.3		
At1g58520	early-responsive to dehydration ERD4	0.79	1.3	-0.2	-1.9	-0.35	-0.66	-1.3	-1.1		
At3g03470	cytochrome P450 89A2	2.42	1.59	0	0	-1.86	-2.87	1.12	0.21		
At5g10930	CBL-interacting protein kinase 5 (CIPK5)	1.59	1.49	0.22	0.56	-1.44	-1.03	-0.7	-0.4		
At1g20160	subtilisin-type protease	1.17	1.02	0.03	-0.7	-1.65	-1.75	-0.2	-0.4		
At1g78850	curculin-like (mannose-binding) lectin family protein	1.54	1.71	-1.9	-2.6	-0.38	-0.02	-0.4	-1.2		
At5g58770	dehydrodolichyl diphosphate synthase	1.95	2.63	-1.4	-1.4	0.35	-1.18	-0	-0.2		
At3g14067	subtilase family protein, cucumisin-like serine protease	1.88	1.87	-1	-1.5	-0.41	-0.88	-0.4	-0.7		
At1g48320	thioesterase family protein	1.6	1.61	-1.8	-2.2	0.43	-0.56	0.56	0.33		
At3g56240	copper homeostasis factor / copper chaperone (ATX1)	1.23	0.75	-1.3	-2.1	0.59	-0.51	0.07	-0.3		
At2g33380	calcium-binding RD20, induced by abscisic acid	3.89	3.76	0.06	0.27	-1.23	-1.35	-1.5	-1.5		
At1g51140	basic helix-loop-helix (bHLH) family protein	1.83	2.6	0.56	-0.9	-1.11	-2.49	-1	-1.2		
At4g15440	hydroperoxide lyase (HPL1)	1.15	1.08	0	-2	-1.96	-1.04	-1.7	-1.3		
At4g21960	peroxidase 42 (PER42) (P42) (PRXR1) (Atperox P42)	1.03	1.15	-1	-1.1	0.34	0.44	-2	-2.2		
At4g30450	glycine-rich protein	1.5	1.35	-1.1	-1	-0.87	-1.4	-0.2	-0.3		
At1g02205	CER1 protein identical to maize gl1 homolog (glossy1)	0.97	2.37	-1.2	-0.8	-2.03	-1.5	0.69	0.2		
At4g33550	LTP/protease inhibitor/seed storage	2.78	2.6	-0.6	-1.9	-1.06	-1.55	-1.3	-1.9		
At5g23750	remorin family protein	1.53	0.93	-1.6	-0.7	-1.14	-1.12	-1.6	-1.1		
At1g32900	starch synthase	1.34	0.93	-1.1	-1.1	-1.08	-1.54	-1.5	-1.4		

Table 3A: Genes induced specifically by drought

		DROUGHT		DREB1A		HARDY		SHINE	
Name	**Gene Description**	127	128	193	195	129	130	126	131
At4g34000	ABA-responsive elements-binding factor (ABF3)	2.84	2.99	0.3	0.59	0.31	-0.37	-0.35	-0.26
At5g66400	dehydrin (RAB18)	2.64	2.35	0.46	1.29	0.66	0.47	0.82	-0.73
At1g72770	protein phosphatase / PP2C P2C-HA (AtP2C-HA)	2.58	2.73	0	0.18	-0.08	-0.28	0.06	-0.08
At4g26080	protein phosphatase 2C / abscisic acid-insensitive 1 (ABI1)	2.03	1.98	0.71	0.17	0.08	-0.14	0.33	0.76
At1g73500	mitogen-activated protein kinase kinase (MAPKK), (MKK9)	1.57	1.57	0.37	0.31	0.59	0.13	-0.17	-0.03
At1g17550	protein phosphatase 2C-related / PP2C-related	1.3	1.29	-0.45	-0.17	-0.62	-0.16	0.12	-0.14
At3g05640	protein phosphatase 2C, PP2C	1.21	1.35	-0.46	0.06	-0.76	0.14	-0.24	0.21
At5g10480	protein phosphatase-like, (PAS2) PEPINO/PASTICCINO2	1	1.95	-0.25	0.27	-0.09	0.61	0.32	0.23
At5g15500	ankyrin repeat family protein	4.7	5.38	0	1.08	0.01	0.05	-1.19	-0.04
At5g47640	CCAAT-box binding transcription factor (AHAP3)	2.32	2.88	0.71	-0.26	0.01	-0.59	-0.41	-0.56
At1g07590	pentatricopeptide (PPR) repeat DNA-binding protein	1.87	1.37	0	0.77	1.48	-0.32	0	-0.06
At3g01470	homeobox-leucine zipper protein 5 (HAT5)/ATHB-1	1.8	1.19	0.41	0.08	-0.4	-0.74	0.3	0.29
At3g52800	zinc finger (AN1-like) family protein	1.44	1.33	0.68	0.11	0.25	-0.35	0.34	0.21
At3g09770	zinc finger (C3HC4-type RING finger)	1.37	1.03	0.16	0	0.07	0.09	-0.22	0.14
At2g22430	homeobox-leucine zipper protein ATHB-6	1.1	1.08	-0.56	-0.52	0.55	0.43	0.21	-0.02
At1g21410	F-box family protein, SKIP2-like	1.77	1.54	0	0.46	-0.07	-0.44	-0.46	-0.36
At5g24120	RNA polymerase sigma subunit SigE (sigE)	2.04	1.71	0.39	0.18	-0.23	-1.07	-0.35	-0.37
At5g47120	Bax inhibitor-1 (BI-1) (AtBI-1).	1.31	1.02	0.07	-0.29	-0.19	-0.48	0.67	0.55
At4g35090	catalase 2	1.55	1.11	0.33	0.23	0.25	-1.02	-0.36	-0.47
At1g33660	L-ascorbate peroxidase, cytosolic	1.45	1.28	0.23	0.27	0.72	-0.31	-0.39	-0.17
At1g07890	L-ascorbate peroxidase 1, cytosolic (APX1)	1.37	1.53	0.52	0.41	0.5	-0.09	-0.41	-0.2
At5g43850	acireductone dioxygenase (ARD/ARD')	1.44	1.18	0.71	-0.26	-0.06	-0.83	-0.52	-0.63
At3g23920	beta-amylase/ 1,4-alpha-D-glucan maltohydrolase,	2.03	1.58	0.16	0.06	-0.21	-0.36	-0.3	-0.56
At4g01070	UDP-glucoronosyl/UDP-glucosyl transferase	1.84	1.1	-0.24	-0.37	-0.44	-1.15	-0.15	-0.32
At3g17000	ubiquitin-conjugating enzyme, (NCUBE1)	1.78	1.44	-0.18	-0.22	0.08	-0.18	0.35	0.11
At4g15530	pyruvate phosphate dikinase family protein contains	1.75	1.18	0.06	-1.17	-0.11	-2.02	0.18	-0.23
At3g17790	acid phosphatase type 5 (ACP5)	1.7	1.23	3.61	0	0.17	-1.02	0.44	0.03
At5g15450	heat shock protein 100/ HSP100/ClpB	1.16	1.17	0.18	-0.04	0.07	-0.4	-0.37	-0.38
At5g51070	ATP-dependent Clp protease (ERD1)	1.37	1.08	0.33	-0.52	0.57	-0.42	0.64	0.43
At4g16190	papain-like cysteine proteinase	1.36	1.36	0.54	0.36	0.09	-0.23	-0.12	-0.11
At4g01610	cathepsin B-like cysteine protease	1.57	1.04	0.84	0.24	0.64	-0.01	0.58	0.61
At5g60360	cysteine proteinase / AALP protein (AALP)	1.69	1.35	0.59	0.18	0.36	-0.54	0.28	0.11
At1g78670	gamma-Glu-X carboxypeptidase	1.64	1.25	0.25	-0.42	0.06	-0.74	-0.04	0.19
At1g07600	metallothionein-like protein 1A (MT-1A) (MT-Q)	1.55	1.17	-0.53	0.86	1.38	-0.53	0.23	-0.08
At2g43570	chitinase class IV	3.46	2.08	0	-2.74	-0.49	-3.41	1.88	0
At1g19660	wound-responsive family protein	1.52	1.2	-0.06	-0.42	0.53	-0.36	0.06	0.04
At4g28240	wound-responsive protein-related	1.28	1.07	-0.27	-0.83	1.27	0.2	-0.03	-0.42
At3g44870	S-adenosyl-L-methionine:carboxyl methyltransferase	1.51	1.23	-0.13	-1.32	0.1	-0.93	0.17	0.2
At4g21980	Microtubule associated protein 1A/1B	1.5	1.24	-0.21	0.57	0.71	-0.09	0.52	0.26
At1g08650	phosphoenolpyruvate carboxylase kinase	1.49	1.21	0.27	-0.02	0	-0.55	-0.21	-0.35
At4g34050	caffeoyl-CoA 3-O-methyltransferase	1.49	1.32	-0.05	-0.32	-0.44	-0.34	-0.09	-0.46
At1g22930	T-complex protein 11	1.46	1.13	-0.19	-0.56	0.13	-0.27	0.41	-0.13
At1g63010	SPX (SYG1/Pho81/XPR1) domain-containing protein	1.45	1.14	0.76	-1.1	-0.86	-0.51	-0.28	-0.5
At3g51000	epoxide hydrolase, alpha/beta fold family	1.45	1.06	0.22	0.16	0.27	-0.77	0.06	-0.05
At4g15490	UDP-glucoronosyl/UDP-glucosyl transferase	1.44	1.04	-0.01	-0.07	-0.04	-0.5	-0.21	-0.55
At3g19030	phosphoserine aminotransferase	1.32	1.26	-0.01	-0.22	0.53	-0.03	0.15	0.4
At1g56700	Pyrrolidone-carboxylate peptidase (PGP-I)	1.3	1.08	-0.05	-0.57	0.36	-0.3	0.05	-0.06
At2g23840	HNH endonuclease domain-containing protein	1.29	1.18	-0.37	-1.21	1.05	0.17	0.03	-0.14
At3g01390	vacuolar ATP synthase subunit G 1 (VATG1)	1.27	1.69	-0.22	0.08	-0.68	0.02	0.08	0.26
At1g52870	peroxisomal membrane protein-related	1.25	1.23	-0.42	-1.13	0.39	-0.4	0.11	0.2
At1g21750	Protein disulfide isomerase (PDI)	1.19	1.2	0.6	0.15	-0.13	-0.17	1.07	0.5
At1g70580	glutamate:glyoxylate aminotransferase 2 (GGT2)	1.18	1.43	0.29	-0.23	-0.06	-0.79	-0.38	-0.75
At2g39000	GCN5-related N-acetyltransferase (GNAT) family protein	1.14	1.92	0.34	0.3	0.88	0.22	0.21	-0.03
At4g32770	tocopherol cyclase / vitamin E deficient 1 (VTE1)	1.11	1.02	-0.28	-0.46	-0.06	-0.46	-0.3	-0.43
At4g22240	plastid-lipid associated protein PAP2	1.04	1.31	0.45	0.27	0.25	-0.22	-0.22	-0.26
At1g28960	coenzyme A diphosphatase	1.02	1.43	-0.42	-1	0.69	0.31	0.55	0.41
At1g68570	proton-dependent oligopeptide transport (POT)	2	1.5	1.09	-2.43	-0.45	-0.66	-0.51	-0.63
At1g67300	hexose transporter	1.49	1.47	0.33	-2.25	-0.31	-0.36	0.11	-0.47
At3g07700	ABC1 family protein	1.32	1.09	0.14	-0.42	-0.08	-0.82	0.28	0.2
At1g55910	zinc transporter ZIP2	1.02	1.1	0.85	-1.32	0.54	-0.03	0.53	0.9

Table 3B: Genes repressed under drought in interaction to drought stress resistance

		DROUGHT		DREB1A		HARDY		SHINE	
Name	**Gene Description**	127	128	193	195	129	130	126	131
At5g62920	two-component responsive regulator (ARR6)	-2.09	-2.5	2.83	0.87	1.35	0.6	0.78	0.95
At2g21660	glycine-rich RNA-binding protein (GRP7)	-1.82	-1.86	2.79	2.53	1.17	1.82	-0.12	-0.58
At1g15360	AP2 transcription factor	-0.63	-1.26	0.87	-0.1	-0.1	-0.63	4.66	4.36
At1g08930	early-responsive to dehydration stress protein (ERD6)	-1.16	-1.27	-0.21	-0.25	0.34	0.07	0.95	1.23
At3g22120	LTP / protease inhibitor/seed storage	-1.43	-1.74	0.15	1.17	0.92	1.9	-0.27	-0.55
At3g49940	lateral organ boundaries domain protein (LBD38)	-1.66	-1.63	0.81	-0.89	1.02	0.96	0.38	0.11
At2g29980	omega-3 fatty acid desaturase (FAD3)	-1.81	-1.52	-0.51	-0.2	0.91	1.66	0.24	0.53
At4g32280	auxin-responsive AUX/IAA family protein	-2.57	-1.52	0	0	1.38	1.09	-0.9	-0.93
At3g04910	protein kinase family protein	-1.61	-1.62	0.78	1.05	0.28	0.96	-0.08	-0.18
At3g54400	aspartyl protease	-1.36	-1.49	1.51	2.44	0.14	1.38	-0.9	-0.6
At2g25900	zinc finger (CCCH-type)	-2.11	-1.23	-4.86	0	-0.59	-0.98	-0.12	-0.4
At2g27840	histone deacetylase-related	-1.39	-1.27	0.96	0.89	0.54	0.11	-1.1	-0.15
At5g54630	zinc finger protein C2H2 type	-1.02	-1.1	-0.45	-0.34	-0.26	-0.14	-0.42	-1.13
At1g32540	zinc finger protein, Lsd1 like	-1.6	-1.89	1.44	-0.01	0.79	0.44	-0.4	-0.28
At1g10470	two-component responsive regulator (ARR4)	-1.01	-1.25	-0.28	-0.89	0.21	-0.3	0.23	0.27
At5g47210	nuclear RNA-binding protein	-1.04	-1	0.07	0.19	0.22	0.11	0.21	-0.04
At1g49600	RNA-binding protein 47 (RBP47)	-1.22	-1.7	0.11	-0.8	0.2	0.27	0.35	0.1
At4g12730	fasciclin-like arabinogalactan-protein (FLA2)	-1.45	-1.35	0.88	0	-1.65	-0.69	-0.37	-0.57
At3g18680	aspartate/glutamate/uridylate kinase	-1.21	-1.18	0.32	0	-1.11	-3.01	-0.42	0.02
At4g19120	early-responsive to dehydration stress protein (ERD3)	-1.82	-1.01	0.75	1.03	1.16	1.37	-1.44	-1.29
At5g20630	germin-like protein (GER3)	-5.41	-3.14	-0.84	-0.76	-0.45	2.06	-2.35	-1.18
At2g37640	expansin (EXP3)	-1.7	-1.44	-0.29	0.48	-0.64	-0.43	-1.62	-1.46
At1g33811	GDSL-motif lipase/hydrolase family protein	-1.32	-1.82	-0.17	-0.28	-0.81	0.33	-2.23	-1.62
At5g15230	gibberellin-regulated protein 4 (GASA4)	-1.31	-1.63	-0.26	0.39	0.05	0.44	-1.55	-1.25
At1g74670	gibberellin-responsive protein similar to GASA4	-2.74	-2.77	-4.35	-4.02	1.28	1.03	0.86	0.79
At4g23060	calmodulin-binding protein	-1.12	-1.88	-1.3	-1.47	1.09	0.93	-0.01	-0.37
At5g20250	raffinose synthase	-1	-1.62	-2.5	-2.72	-0.22	-0.75	1.8	1.63
At4g38860	auxin-responsive protein	-1.9	-1.88	-1.33	-0.73	0.32	0.16	0.51	0.33
At1g69530	expansin, putative (EXP1)	-3.24	-2.53	-1.87	-2.71	0.47	0.62	0.39	0.74
At1g12560	expansin, putative (EXP7)	-1.21	-1.31	-0.75	-1.39	0.38	0.32	0.71	0.84
At1g72610	germin-like protein (GER1)	-2.27	-2.19	-1.54	-1.27	-0.15	-0.06	0.71	0.3
At5g22920	zinc finger (C3HC4-type RING finger)	-1.65	-1.44	-2.76	-2.89	0.13	-0.09	0.72	0.8
At1g68520	zinc finger (B-box type)	-1.27	-1.68	-1.62	-2.73	0.5	-0.08	0.26	0.18
At5g07580	ethylene-responsive element-binding protein, AtERF5-like	-1.14	-1.24	-1.55	-2.67	0.19	-0.31	-0.62	-0.8
At5g28770	bZIP transcription factor similar to opaque-2	-1.91	-2.17	-2.25	-1.57	-0.5	-0.42	0.27	0.13
At5g61590	AP2 transcription factor	-1.05	-1.48	-2.89	-2.64	-0.05	-1.36	-0.05	-0.2
At2g02450	no apical meristem (NAM) family	-1.06	-1.18	-1.21	-0.7	-0.22	-0.16	-0.5	-1.12
At4g36540	basic helix-loop-helix (bHLH)	-2.22	-1.7	-2.52	-2.68	0.16	0.02	0.1	0
At2g18300	basic helix-loop-helix (bHLH)	-2.8	-3.06	-1.85	-2.04	-0.31	-0.66	-0.06	-0.22
At3g44490	histone deacetylase-related	-1.28	-0.59	-1.06	-1.36	-0.31	-0.15	-0.56	-0.15
At5g61650	cyclin family protein similar to cyclin 2	-1.35	-0.85	-1.41	-2.18	-0.11	-0.12	-0.39	0.37
At5g02760	protein phosphatase 2C / PP2C	-2.44	-3.06	-4.1	-1.06	0.05	0.04	-0.71	-0.68
At3g13690	protein kinase	-1	-1.32	-1.37	-0.87	-0.07	-0.63	-0.58	-0.42
At1g13110	cytochrome P450 71B7 (CYP71B7)	-1.02	-1.39	-2.11	-1.91	-0.43	-0.66	-0.3	-0.21
At3g49260	calmodulin-binding protein	-1.6	-2.13	-1.66	-1.12	-1.39	-0.32	-0.33	-0.42
At1g80440	kelch repeat-containing F-box family protein	-1.61	-2.25	-1.14	-1.85	0.18	-1.75	-0.24	-0.01
At4g22690	cytochrome P450, flavonoid 3',5'-hydroxylase Hf1	-2.05	-0.94	-2.82	-1.71	-1.03	-0.44	-0.18	-0.25
At2g38120	amino acid permease (AUX1)	-1.84	-2.13	-1.51	-1.27	-0.84	-0.11	-0.53	-0.37
At2g40610	expansin (EXP8)	-3.18	-3.86	-4.26	-3.47	0.01	0.11	-1.42	-0.8
At5g25190	ethylene-responsive element-binding protein	-3.59	-4.27	-2.45	-3.89	0.81	0.21	-0.72	-1.08
At4g01460	basic helix-loop-helix (bHLH) protein	-1.22	-1.33	-3.2	-2.15	-0.23	0.4	-1.79	-0.8
At5g65730	xyloglucan:xyloglucosyl transferase	-1.01	-1.6	-1.89	-1.08	-1.05	-0.81	-0.41	-1.02
At3g16240	delta tonoplast integral protein (delta-TIP)	-2.05	-1.89	-1.33	-2.14	-1.38	-0.78	0.61	0.51
At5g48490	LTP/ protease inhibitor/seed storage	-2.33	-2.32	-3.61	-3.84	-2.57	-1.8	-0.73	-0.4
At5g49730	ferric reductase-like	-3.37	-1.35	-1.72	-1.28	-1.99	-1.06	-0.58	-0.74
At1g66700	S-adenosyl-L-methionine:carboxyl methyltransferase	-1.62	-1.1	-2.67	-3.28	-0.87	-1.49	-0.33	-1.03
At4g39640	gamma-glutamyltranspeptidase	-2.75	-1.61	-3.11	-1.75	-1.61	-0.87	0.17	-0.31
At5g44020	acid phosphatase class B	-6.56	-5.57	-3.24	-1.88	-1.81	-0.78	-0.23	-0.55
At5g08330	TCP family transcription factor	-2.07	-1.89	-2.09	-1.41	-1.18	-0.92	-0.22	0.03
At5g39860	bHLH DNA-binding protein	-2.34	-1.85	-2.88	-3.62	-1.6	-2	-1.95	-1.39

Table 4A: The DREB1A induced regulon in interaction to drought resistant genotypes

		DREB1A		HARDY		SHINE		DROUGHT	
Name	**Gene Description**	193	195	129	130	126	131	127	128
At2g38530	lipid transfer protein 2 (LTP2)	3.82	5.47	2.14	1.97	0.94	1.72	0.94	0.87
At4g12480	lipid transfer protein (LTP) family protein identical to pEARLI 1	5.66	5.7	3.15	1.14	3.28	4.26	1.16	-1.58
At4g12470	lipid transfer protein (LTP) family protein similar to pEARLI 1	2.71	4.56	3.44	1.29	0.99	1.48	0.06	-0.67
At3g47540	chitinase, similar to basic endochitinase CHB4	1.69	0.65	3.59	2.75	1.28	0.77	0.15	0.63
At2g24560	GDSL-motif lipase/hydrolase family protein	7	7.39	3.43	3.7	-0.01	0.34	-0.15	0.23
At3g53990	universal stress protein (USP)	4.06	3.69	1.78	1.2	0.19	0.17	0.03	0.13
At1g08570	thioredoxin family protein	4.21	4.36	1.63	1.44	0.01	-0.01	0.67	0.44
At2g02100	plant defensin-fusion protein, putative (PDF2.2)	3.7	3.86	2.34	4	-0.36	-0.2	-1.11	-0.29
At1g30360	early-responsive to dehydration stress protein (ERD4)	2.77	3.63	2.76	2.27	0.45	0.12	-0.36	-0.48
At1g54410	dehydrin family protein	1.78	2.96	2.47	1.41	-0.12	-0.49	0.18	0.12
At2g26580	YABBY transcription factor	1.77	2.06	1.28	1.7	-0.42	0.09	0.19	-0.1
At5g14800	pyrroline-5-carboxylate reductase	1.17	1.16	0.63	1.01	0.32	0.2	0.06	-0.4
At5g07030	aspartyl protease	1.13	2.07	1.65	2.1	0.34	-0.48	-0.33	-0.19
At2g28550	AP2 domain-containing transcription factor RAP2.7	1.63	0.76	1.3	1	-0.29	-0.64	-0.09	-0.06
At1g30820	CTP synthase / UTP--ammonia ligase	0.99	1.39	0.98	1.37	0.35	0.42	-0.36	0.63
At3g16400	jacalin lectin protein similar to myrosinase-binding protein	2.38	2.28	2.47	2.11	-0.49	-0.95	0.13	1.12
At1g04240	indoleacetic acid-induced protein 3 (IAA3)	2.56	2.2	2.51	2.12	-0.39	-1.1	-0.44	-0.62
At3g05880	hydrophobic protein (RCI2A) /(LTI6A)	2.84	4.04	1.87	1.4	-0.44	-0.59	0.05	0.86
At3g09540	pectate lyase family protein	1.74	1.87	2.34	2.55	-0.88	-0.66	-0.75	-0.49
At5g07010	steroid sulfotransferase	2.51	2.01	4.65	4.71	-0.94	-0.63	-0.14	0
At2g42530	cold-regulated protein (cor15b)	3.92	5.93	0.99	1.24	-0.87	-1.29	-0.02	0.62
At4g30650	hydrophobic protein, similar to RCI2A / LTI6A	3.11	6.7	3.91	4.34	-1.58	-1.13	-0.29	0.27
At5g07000	steroid sulfotransferase	0.95	1.25	3.23	4.1	-1.55	-1.39	-0.37	0.49
At2g46600	EF-hand Ca2+-binding protein	1.78	1.2	1.26	-0	0.67	1.04	1.35	-0.26
At2g41540	NAD-dependent glycerol-3-phosphate dehydrogenase	1.28	0.8	-0.05	1.55	2.37	2.7	-1.53	-0.17
At1g08830	copper/zinc superoxide dismutase (CSD1)	2.02	2.42	0.26	0.37	1.46	1.55	0.15	-0.1
At1g02930	glutathione S-transferase	2.55	0.74	1.11	-1.1	1.94	2.69	-0.19	0.68
At2g33830	dormancy/auxin associated protein	2.05	0.91	-0.61	0.17	0.84	0.98	-0.91	-0.76
At2g28190	copper/zinc superoxide dismutase (CSD2)	1.74	2.25	-0.36	0.57	0.94	1.15	-0.77	-0.62
At2g23340	AP2 domain-containing transcription factor, putative	3.53	2.34	-0.82	-0.1	-0.21	-0.33	0.2	0.04
At3g11200	PHD finger family protein	1.89	1.01	-0.3	0.02	0.16	0.14	-0.12	0.11
At2g47890	zinc finger (B-box type) family protein	1.51	1.44	-0.85	0.48	-0.09	0.18	0.04	-0.03
At3g14200	DNAJ heat shock protein	1.36	1.1	0.81	0.97	0	-0.49	0.09	-0.07
At5g61810	calcium-binding EF-hand domain protein	2.36	2.76	-0.08	0.07	-0.41	-0.38	0.78	0.61
At1g43890	Ras-related GTP-binding protein (RAB1Y)	1.69	1.16	0.42	-0.1	0.19	-0.01	0.64	0.46
At2g30740	serine/threonine protein kinase, Pto kinase interactor 1 (Pti1)-like	1	2.07	0.26	-0.1	0.07	-0.05	0.38	-0.31
At3g52470	harpin-induced family protein / HIN1	2.03	1.4	-0.17	-0.3	0.35	0	0.8	0.63
At1g54290	eukaryotic translation initiation factor SUI1	1.96	1.78	1.13	0.49	-0.12	-0.18	0.82	0.87
At4g35300	hexose transporter	1.19	1.24	-0.26	-0.2	-0.67	-0.79	0.64	0.93
At5g64080	LTP/ protease inhibitor/seed storage protein	1.92	2.37	-0.17	-0.3	-0.9	-0.49	-0.26	-0.35
At2g38870	protease inhibitor	1.84	2.68	-0.61	-0.5	-0.16	-0.24	0.22	-0.28
At2g40880	cysteine protease inhibitor / cystatin	1.33	1.17	0.49	-0.5	0.2	0.13	0.14	-0.29
At5g47040	Lon protease homolog 1	1.58	1.63	-0.55	-0.1	0.27	0.21	-0.23	0.13
At3g55610	delta 1-pyrroline-5-carboxylate synthetase B / P5CS B (P5CS2)	2.46	3.77	-0.39	-0	-0.21	-0.21	-1.07	0.26
At2g43620	chitinase, similar to basic endochitinase CHB4	3.55	5.64	-0.87	0.97	0.84	0.5	-0.4	0.62
At1g10760	starch excess protein (SEX1)	2.86	3.02	0.35	-0.1	-0.35	-0.23	0.62	0.44
At5g24300	starch synthase	1.08	1.27	0.29	0.23	-0.33	-0.3	-0.03	0.04
At4g17090	beta-amylase (CT-BMY)	2.44	2.22	-0.47	-0.7	-0.23	-0.35	-0.05	-0.6
At4g23600	coronatine-responsive tyrosine aminotransferase	2.67	2.4	-0.02	1.12	-0.52	-0.64	0.03	0.89
At1g29390	stress-responsive protein, cold acclimation WCOR413-like	1.95	3.1	0.45	0.67	-0.28	-0.31	0.13	0.45
At5g14570	nitrate transporter protein AtNRT2:1 like	1.85	1.47	0.58	0.29	-0.53	-0.19	0.43	0.62
At3g11670	digalactosyldiacylglycerol synthase 1 (DGD1)	1.63	1.71	-0.04	0.08	-0.23	-0.27	0.56	0.37
At3g14650	cytochrome P450	1.79	1.41	-0.08	0.78	-0.55	0	0.21	-0.22
At3g14640	cytochrome P450	1.23	1.41	-0.02	0.13	-0.59	-0.22	0.26	0.26
At3g14690	cytochrome P450	1.19	1.22	0.37	0.43	-0.08	0.03	0.2	0.44
At1g35720	annexin 1 (ANN1)	2.09	2.4	-0.24	-0.3	-0.74	-1.08	2.2	0
At2g22170	PLAT/LH2 (Polycystin-1, Lipoxygenase)	1.01	2.35	-0.66	0.11	-1.04	-0.98	-0.38	-0.49
At4g37870	phosphoenolpyruvate carboxykinase	1.31	2.24	-0.16	0.56	-0.89	-1.16	0.57	0.33
At4g03210	xyloglucan:xyloglucosyl transferase	1.42	2.44	-1.46	-0.4	-1.54	-1.34	-0.69	-0.99
At4g25480	DRE-binding protein (DREB1A) / (CBF3)	8.2	8.12	-1.97	-0.6	-1.02	-1.42	0.73	0.15

Table 4B: The DREB1A repressed regulon in interaction to drought resistant genotypes

		DREB1A		HARDY		SHINE		DROUGHT	
Name	**Gene Description**	193	195	129	130	126	131	127	128
At3g15540	indoleacetic acid-induced protein 19 (IAA19)	-3.24	-2.79	0.17	0.41	1.25	1.29	-0.75	-0.14
At4g14365	zinc finger (C3HC4-type RING finger)	-2.12	-3.39	-0.49	-1.2	0.95	1.37	0.25	0.64
At4g08850	leucine-rich repeat / protein kinase protein	-1.48	-1.86	-0.76	-1	1.14	0.81	0.51	-0.14
At3g14310	pectin methylesterase	-1.19	-1.67	1.27	1.23	0.39	0.21	0.77	0.11
At4g16780	homeobox-leucine zipper protein 4 (HAT4) / Athb-2	-2.22	-0.89	0.71	1.21	-0.1	0.04	-0.03	-0.27
At2g43010	basic helix-loop-helix protein 9 (bHLH9)	-1.08	-1.26	-0.16	-0.4	-0.55	-0.31	-0.21	-0.16
At5g11060	homeobox protein knotted-1 like 4 (KNAT4)	-1.22	-2.16	-0.45	-0.3	-0.27	-0.17	-0.16	0.35
At4g14400	ankyrin repeat protein	-1.24	-3.82	-0.55	-1.6	-0.39	0.03	0.91	0.45
At4g32040	homeobox protein knotted-1 like 5 (KNAT5)	-1.33	-1.65	0.11	-0.3	0.4	0.03	0.88	0.39
At5g05090	myb family transcription factor	-1.56	-1.01	-0.24	-0.5	0.03	-0.14	-0.09	0.04
At1g68190	zinc finger (B-box type) family protein	-2.37	-1.68	-0.18	0.22	-0.13	-0.2	-0.79	-0.56
At1g22300	14-3-3 protein GF14	-2.43	-1.32	-0.2	-0.5	-0.12	-0.18	-0.24	-0.1
At5g45110	ankyrin repeat family protein	-1.49	-2.19	-0.66	-0.9	0.73	0.2	-0.49	-1.06
At3g56400	WRKY family transcription factor WRKY4	-1.58	-1.91	-0.59	-0.9	0.49	0.67	0.47	0.5
At1g22590	MADS-box protein AGL87	-0.68	-3.26	-0.62	-0.8	0.1	0.45	-1.01	-0.58
At2g47060	Pto kinase interactor 1 (Pti1)-like	-2.17	-1.27	0.04	0.09	0.08	0.25	-0.39	-0.09
At1g21270	wall-associated kinase 2 (WAK2)	-1.58	-3.2	-0.3	-0.5	0.27	0.75	-0.05	-0.19
At3g45640	mitogen-activated protein kinase / MAPK (MPK3)	-1.42	-1.27	0.09	-0.2	-0.18	-0.26	-0.1	0.06
At3g17510	CBL-interacting protein kinase 1 (CIPK1)	-2.24	-1.94	0.16	-0.1	-0.46	-0.53	-0.23	-0.04
At1g01140	CBL-interacting protein kinase 9 (CIPK9)	-1.19	-1.73	-0.35	-1.1	0.07	-0.08	1.07	0.15
At5g44420	plant defensin protein, (PDF1.2a)	-4.58	-4.71	-0.02	-0.2	0.9	0.94	0.97	-2.29
At1g75830	plant defensin-fusion protein, (PDF1.1)	-4.84	-2.82	0	-0.3	0.85	0.67	1.44	-2.13
At2g26010	plant defensin-fusion protein, (PDF1.3)	-3.31	-1.56	0.26	-0.1	0.64	0.43	1.08	-0.65
At5g44430	plant defensin-fusion protein, (PDF1.2c)	-3.71	-1.15	0.09	-0.2	0.49	0.52	1.35	-1.95
At1g51680	4-coumarate--CoA ligase 1	-1.38	-1.38	-1.15	-0.6	0.03	-0.48	-0.75	-0.2
At3g53260	phenylalanine ammonia-lyase 2 (PAL2)	-1.96	-1.64	-0.07	-0.2	0.02	-0.36	-0.22	-0.44
At2g40540	potassium transporter, AtKT2p	-1.61	-1.73	-0.6	-0.4	-0.34	-0.27	-0.08	-0.25
At1g01790	K+ efflux antiporter, (KEA1)	-1.25	-1.33	-0.17	-0.2	-0.48	-0.53	-0.02	0.27
At1g12110	nitrate/chlorate transporter (NRT1.1) (CHL1)	-1.96	-2.36	-0.59	-0.4	-0.1	-0.01	-0.44	-0.68
At5g07200	gibberellin 20-oxidase	-1.82	-1.14	0.66	0.18	-0.17	-0.17	-0.62	-0.06
At4g39400	brassinosteroid insensitive 1 (BRI1)	-1.29	-1.3	0.12	0.5	0.24	0.04	-0.39	-0.7
At3g23030	indoleacetic acid-induced protein 2 (IAA2)	-1.84	-1.45	0.97	0.38	-0.34	-0.51	-0.05	0.1
At5g43700	indoleacetic acid-induced protein 4 (IAA4)	-1.56	-1.04	-0.11	0.57	-0.55	-0.38	-0.72	-0.43
At3g11930	universal stress protein (USP) ER6 like	-1.27	-1.58	0.44	0.43	0.85	0.53	-0.07	-0.31
At1g18020	12-oxophytodienoate reductase, OPR1 like	-2.18	-4.1	-0.24	-0.4	0.31	0.26	-0.22	-0.62
At1g17990	12-oxophytodienoate reductase OPR1-like	-2.54	-2.44	-0.16	-0.4	-0.36	0.28	-0.02	-0.37
At2g29170	short-chain dehydrogenase/reductase (SDR)	-1.17	-1.74	-0.09	-0.7	-0.82	-0.5	-0.83	-0.77
At5g49630	amino acid permease 6 (AAP6)	-2.04	-1.48	-0.78	-0.9	-1.51	-1.26	0.56	-0.33
At1g78020	senescence-associated protein similar SAG102	-1.25	-1.67	-0.47	-1.6	-0.97	-1.19	0.32	0.32
At1g04250	indoleacetic acid-induced protein 17 (IAA17)	-1.39	-1.71	-0.26	-0.7	-1.56	-1.36	-0.94	-0.43
At2g01420	auxin transport protein, similar to PIN7	-1.64	-1.29	-0.29	-0.2	-1.51	-1.08	-0.3	-0.68
At4g23220	protein kinase	-1.88	-2.07	-1.39	-1.8	1.69	1.95	1.09	0.37
At5g49360	glycosyl hydrolase family 3 protein	-3.61	-3.64	-2.8	-1.3	1.77	1.48	0.17	-1.08
At1g64400	long-chain acyl-CoA synthetase	-1.29	-0.89	-0.67	-1.5	1.32	1.52	-0.1	0.73
At1g01120	fatty acid elongase 3-ketoacyl-CoA synthase 1 (KCS1)	-1.53	-0.94	-1.02	-0.6	0.9	0.77	0.24	0.72
At1g72150	SEC14 / phosphoglyceride transfer family protein	-1.54	-1.83	-1.29	-1.2	0.77	0.38	-0.9	-0.68
At1g05010	1-aminocyclopropane-1-carboxylate oxidase / ACC oxidase	-1.75	-1.65	-1.21	-1.4	0.67	0.25	-0.67	-0.46
At2g46450	cyclic nucleotide-regulated ion channel (CNGC12)	-1.79	-5.07	-1	-1.7	-0.05	0.31	0.89	0.08
At3g45140	lipoxygenase (LOX2) i	-2.59	-3.07	-1.89	-1.8	-0.38	-0.81	0.95	0.35
At4g31500	cytochrome P450 83B1 (CYP83B1)	-3.25	-4.68	-0.93	-1.4	-0.38	-0.98	0.49	-0.26
At4g38690	1-phosphatidylinositol phosphodiesterase	-2.23	-1.2	-1.51	-1.5	-0.28	-0.98	0.47	0.06
At3g43800	glutathione S-transferase	-1.57	-1.26	-0.86	-1.8	0.15	-0.1	0.14	-0.21
At1g19350	brassinosteroid signalling regulator, similar to BZR1	-2.18	-1.62	-0.96	-1.1	-0.06	-0.22	0.11	0.31
At4g23810	WRKY family transcription factor AR411	-2.62	-3.82	-1.56	-1.8	0.41	-0.26	-0.46	-0.19
At1g64200	vacuolar ATP synthase subunit E	-2.45	-1.49	-1.44	-1.5	-1.14	-1.25	0.96	-0.91
At4g39940	adenylylsulfate kinase 2 (AKN2)	-1.21	-1.79	-1.01	-1.5	-1.45	-1.87	1.55	0.48
At2g38750	annexin 4 (AnnAt4)	-2.28	-2.52	-1.51	-1.4	-1.87	-2.72	1.03	-0.4
At5g50950	fumarate hydratase	-2.66	-3.52	-1.86	-2.1	-1.02	-1.02	-0.97	0.44
At1g54040	kelch repeat-containing protein	-2.19	-1.56	-2.06	-1.8	-2.59	-2.18	0.49	0.25
At1g45201	lipase class 3 family protein	-2.35	-1.45	-1.41	-1.9	-1.26	-1.65	0.47	-0.45

Table 5: The SHINE regulon in interaction to HARDY

		SHINE		HARDY		DREB		DROUGHT	
Name	**Gene Description**	126	131	129	130	193	195	127	128
At1g15360	AP2 transcription factor SHINE1	4.66	4.36	-0.1	-0.63	0.87	-0.1	-0.63	-1.26
At3g48740	nodulin MtN3 family protein	1.74	1.31	1.11	0.59	-0.39	-0.61	0.6	-0.14
At3g50060	myb family transcription factor	1.22	0.63	0.67	1.21	0.59	0	0.36	0.04
At3g28910	myb family transcription factor (MYB30)	1.33	1.33	0.43	0.63	0.06	-1.2	-0.21	-0.79
At5g62470	myb family transcription factor (MYB96)	1.31	1.1	-0.06	0.3	0.02	-0.23	-2.05	0.73
At5g25390	AP2 domain-containing transcription factor, RAP2.4	3.67	3.94	0.32	0.27	-0.88	-0.18	0.96	0.19
At1g05805	basic helix-loop-helix (bHLH) family protein	1.24	0.61	0.02	-0.34	-0.02	-1.29	-0.23	-0.56
At2g30250	WRKY family transcription factor	1.09	1.38	-0.02	-1.24	4.67	-3.98	1.08	0.14
At2g41170	F-box family protein; similar to SKP1 interacting partner 2 (SKIP2)	2.23	2.21	-1.42	0.89	0	0	-0.11	0.88
At1g80460	glycerol kinase (ATP:glycerol 3-phosphotransferase, Glycerokinase, GK)	1.03	0.88	0.11	-0.18	0.33	-0.1	0.32	0.16
At1g49430	long-chain acyl-CoA synthetase (MF45P)	1.08	1.26	-0.06	0.61	0.44	1.35	-0.31	1.11
At1g01610	phospholipid/glycerol acyltransferase family protein	1.49	1.15	0.3	0.32	-0.51	-0.72	0.14	0.66
At5g57800	WAX2, similar to CER1 and maize glossy1	1.06	1.17	-1.1	-0.55	-0.83	-0.13	0.5	0.82
At3g10570	cytochrome P450 77A3	2.6	2.71	-0.84	-0.22	-1.25	1.17	-0.92	0.55
At4g01850	S-adenosylmethionine synthetase 2 (SAM2)	1.2	0.92	-0.16	0.35	0.12	0.34	0.33	0.56
At3g47480	calcium-binding EF hand family protein	2.08	3.01	1.55	-2.08	0	-5.18	0.91	0.48
At1g79160	phosphopantetheine binding, ACP binding	0.85	1.11	-0.34	-0.33	0.12	0.46	0.16	-0.41
At2g21140	proline-rich protein 2, hydroxyproline-rich glycoprotein	2.83	2.55	-0.68	-0.06	-0.28	0.69	-0.58	-0.63
At4g14130	xyloglucan:xyloglucosyl transferase, (XTR7)	2.39	3.44	-1.22	0.51	0.37	0.74	-0.82	0.97
At3g13790	beta-fructosidase (BFRUCT1) / cell wall invertase	2.05	3.39	1.35	0.54	3.85	-0.73	0.13	-0.57
At2g26440	pectinesterase family protein	1.2	1.31	0.2	-0.49	-0.13	-2.99	0.84	0.69
At2g05540	glycine-rich protein	2.02	2.3	-0.76	0.58	0.4	2.73	2.14	0.15
At1g76930	proline-rich extensin-like family protein	1.79	2.51	-0.45	0.53	0	0	0.42	-0.06
At1g02920	glutathione S-transferase,	1.47	1.8	1.18	-0.57	0.99	-1.01	-0.62	0.46
At4g02520	glutathione S-transferase, putative	1.22	1.78	1.09	-0.19	0.15	-2.25	0.63	0.39
At4g30270	MERI-5 protein (MERI5B) / xyloglucan endo-1,4-beta-D-glucanase (SEN4)	1.49	0.88	-1.06	-2.09	0.94	0.77	1.38	-0.53
At1g12570	glucose-methanol-choline (GMC) oxidoreductase	2.78	2.27	-1.44	-0.94	-4.04	2.86	-0.33	0.91
At3g57240	beta-1,3-glucanase (BG3)	2.58	3.14	-0.93	-1.91	0	-0.68	0.13	0.08
At2g28630	beta-ketoacyl-CoA synthase family protein	1.27	1.58	-1.44	-1.13	0	0	-0.56	-0.56
At1g56120	leucine-rich repeat / protein kinase	0.73	1.26	-1.05	-1.99	0.23	-1.88	0.97	0.65
At4g30660	Hydrophobic protein RCI2A (LTI6A)	-1.21	-1.4	1.59	1.16	-0.37	0.89	-0.26	-0.4
At3g14210	myrosinase-associated, Lipase/Acylhydrolase with GDSL-like motif	-1.07	-1.1	1.76	1.32	-1.28	-0.58	-0.46	-0.9
At2g43100	aconitase	-1.05	-1.5	-0.23	-1.79	0.69	0.73	0.63	-0.62
At2g30860	glutathione S-transferase	-1	-1.5	-0.16	-1.16	0.2	-0.02	1.25	0.5
At1g78370	glutathione S-transferase	-1.21	-1.6	-0.1	-1.09	0.57	0.82	0.68	-0.21
At1g17200	integral membrane family	-1.24	-1	0.18	-0.04	-0.04	-0.23	-0.14	-0.02
At4g36360	beta-galactosidase / lactase	-1.4	-1.6	-0.51	0.35	-0.95	-0.12	-0.62	-0.28
At4g14040	selenium-binding protein, (SBP56)	-1.68	-1.6	-0.58	-0.88	-0.05	-0.13	1.07	0.41
At3g55130	ABC transporter, similar to breast cancer resistance protein 1 BCRP1	-1.96	-2.7	-0.2	-0.19	0.4	-0.75	0.22	-0.27
At1g55260	LTP/ protease inhibitor/seed storage	-2.17	-2.2	-0.19	-0.38	0.29	-0.11	0.03	0.56
At3g62820	invertase/pectin methylesterase inhibitor f	-2.37	-2.3	-0.81	-0.43	0.06	-0.09	0.61	0.46
At1g64390	endo-1,4-beta-glucanase / cellulase	-1.7	-1.3	0.33	1.31	1.53	-1.18	-0.93	-0.96
At1g72970	glucose-methanol-choline (GMC) oxidoreductase	-2.84	-3.1	-1.6	-0.38	-0.73	0.44	-1.02	-0.45
At1g62180	5'-adenylylsulfate reductase 2, chloroplast (APR2) (APSR)	-1.22	-0.7	-0.64	-0.78	0.12	-0.06	-0.04	-0.32
At5g21100	L-ascorbate oxidase	-1.04	-1.3	-0.96	-0.77	-0.79	-0.85	0.66	0.31
At5g45950	GDSL-motif lipase/hydrolase	-2.21	-2	-1.04	-0.92	-1.28	-0.41	0.25	1.29
At5g26000	glycosyl hydrolase / Myrosinase	-1.19	-1.3	-1.52	-1.23	1.03	-0.46	-0.36	-0.23
At1g65860	flavin-containing monooxygenase / FMO	-1.49	-1.6	-1.19	-1.87	-0.36	-0.22	1.35	-0.14
At3g19710	branched-chain amino acid aminotransferase (BCAT4)	-1.63	-2.4	-1.02	-2.37	0.5	0.17	1.49	0.09
At1g74090	Flavonol 4'-sulfotransferase (F4-ST)	-1.06	-1.3	-0.81	-1.17	0.07	0.45	0.67	0.1
At1g18590	Flavonol 4'-sulfotransferase (F4-ST)	-1.31	-1.4	-0.88	-1.25	-0.5	-0.82	1.39	0.09
At1g62560	flavin-containing monooxygenase / FMO	-2.07	-2.7	-1.07	-1.68	-0.24	-0.71	1.43	0.33
At2g46650	cytochrome b5	-1.21	-1.9	-0.78	-1.75	-0.41	-0.61	1.13	0.07
At1g16410	cytochrome P450	-1.98	-2.2	-1.32	-1.79	-0.03	-0.93	0.41	-0.09
At3g54600	DJ-1 family protease	-1.11	-1.3	-1	-1.2	-0.85	-0.58	0.59	-0.22
At3g58990	aconitase	-1.99	-2.2	-1.47	-2.18	-0.5	-1.27	0.93	-0.14
At4g12030	bile acid:sodium symporter family protein	-1.55	-2.3	-0.69	-1.31	-0.31	-0.88	-0.08	-0.26
At4g37980	mannitol dehydrogenase (ELI3-1)	-2.34	-2.1	-1.53	-0.82	-0.49	-0.51	-0.39	0.56
At1g70830	Pathogenesis-related protein Bet v I family	-2.11	-2.1	-1.08	-1.7	0.04	0.28	0.63	0.08
At1g24020	Pathogenesis-related protein Bet v I family	-4.26	-4.2	-2.18	-1.32	-0.75	-0.87	-0.5	-0.09

Table 6: HARDY specific regulon

		HARDY		SHINE		DREB1A		DROUGHT	
Name	**Gene Description**	129	130	126	131	193	195	127	128
At2g36450	AP2 TF DREB1B like	5.83	5.72	0.35	0	0	-0.32	0.19	-0.5
At2g45820	DNA-binding protein, Remorin	1.23	0.61	-0.06	-0.33	0.58	0.3	0.32	-0.18
At2g46970	basic helix-loop-helix (bHLH) protein, (PIL1)	2.97	3.48	0.81	-0.06	-0.71	3.99	-0.73	0.31
At3g61460	zinc finger (C3HC4-type RING finger) / (BRH1)	1.32	0.99	0.21	0.33	-1.19	0	0.18	-0.18
At5g06710	homeobox-leucine zipper protein 14 (HAT14)	1.25	1.35	-0.69	-0.58	-0.24	0.2	-0.37	-2.41
At3g60530	zinc finger (GATA type)	1.19	1	-0.1	0.09	2.05	0	0.48	0.24
At5g10860	CBS domain-containing protein	1.2	1	0.63	0.54	0.88	0.8	0.25	-0.04
At1g60140	trehalose-phosphatase	2.2	1.76	0.61	0.7	-0.76	1.58	0.41	0.18
At1g09350	galactinol synthase	2.51	0.84	-0.53	0.07	-2.33	0	-0.51	-1.12
At2g41700	ABC transporter	1.47	0.76	0.93	0.69	1.23	-0.01	0.37	0.04
At2g41180	sigA-binding protein-related	1.7	1.01	-0.1	0.13	1.18	0.57	0.66	0.96
At2g46690	indole-3-acetic acid induced protein ARG7 like	1.65	1.17	-0.27	-0.31	0.22	-0.58	0.73	0.72
At1g15550	gibberellin 3 beta-hydroxylase (GA4)	2.57	2.38	0.14	-0.2	0	0	-0.37	-0.33
At2g21010	C2 domain-containing protein	1.21	1.47	-0.16	-0.22	0.37	0.53	0.01	0.15
At2g20990	Ca2+-dependent lipid-binding protein (CLB1)	0.82	1.55	-0.04	-0.22	0.31	0.87	-0.32	0.01
At2g27030	calmodulin-2/3/5 (CAM5) (TCH1) thaliana}	1.41	1.48	-0.4	0.05	2.19	-0.3	-0.44	-0.44
At2g02130	plant defensin-fusion protein, (PDF2.3)	1.97	2.23	-0.03	-0.34	0.34	2.13	-0.64	-0.51
At5g05170	cellulose synthase	0.72	1.1	0.21	0.06	-0.15	0	-0.02	0.23
At2g03440	Early nodulin 12B precursor (N-12B) like	1.39	1.12	-0.07	-0.23	0.23	0.57	-0.19	-0.08
At3g50440	hydrolase, similar to ethylene-induced esterase	1.26	0.73	-0.54	-0.49	-0.01	-0.31	-0.16	0.32
At2g40300	ferritin	1.38	0.79	-0.53	0.74	0.8	-0.06	0.01	0.86
At1g73480	hydrolase, alpha/beta fold family	1.11	1.51	-0.52	-0.58	-1.16	2.43	0.78	0.57
At5g44130	fasciclin-like arabinogalactan-protein	1.69	1.33	0.8	0.43	-0.06	0.25	-0.61	-0.72
At5g54580	RNA recognition motif (RRM)-containing protein	1.26	0.65	0.32	0.04	0.52	0.58	0.33	0.27
At5g11790	N-myc downstream regulated gene, alpha/beta hydrolase	1.02	0.96	-0.44	-0.42	0.1	0.58	-0.16	0.08
At3g06035	GPI anchored protein	1.73	1.09	-0.54	-0.45	0.85	0.52	0.19	-0.16
At2g36460	fructose-bisphosphate aldolase	1.66	1.56	-0.06	-0.09	0.79	0.51	0.06	0.03
At4g15450	early-responsive to dehydration stress ERD7-like	1.38	1.73	0.23	-0.15	0.77	0	0.77	0.39
At3g50970	dehydrin xero2 (XERO2) / (LTI30)	1.39	1.14	0.23	-2.46	8.89	0	-1.06	2.31
At1g09570	phytochrome A (PHYA)	1.12	1.48	0.73	0.53	-0.32	0.6	0.04	-0.7
At1g78450	SOUL heme-binding protein	2.76	2.65	-0.46	-0.86	-0.99	1.01	-0.42	-0.78
At5g05590	phosphoribosylanthranilate isomerase 2 (PAI2)	1.18	0.81	0.01	0.13	1.71	0.03	0.05	-0.24
At5g12250	tubulin beta-6 chain (TUB6)	0.81	1.71	-0.42	-0.5	-0.51	-0.2	-0.63	0.42
At1g69040	ACT domain containing protein (ACR4)	1.04	0.86	-0.03	0.25	-0.19	-0.03	-0.66	-0.42
At1g63980	D111/G-patch domain-containing protein	0.59	1.08	-0.09	0.02	0.36	0.72	-0.25	-0.19
At5g59690	histone H4 jambhiri GI:16797797	1.83	0.7	0.2	0.18	-0.01	1.06	-0.27	-0.54
At5g46160	ribosomal protein L14 family protein	1.17	0.93	-0.07	0.11	0	0.23	-0.3	-0.57
At1g09690	60S ribosomal protein L21 (RPL21C)	1.08	0.7	0.42	0.58	-0.09	1.14	-0.54	-0.62
At1g33140	60S ribosomal protein L9 (RPL90A/C)	1.08	0.66	0.17	0.54	0.51	0.64	-0.48	-0.46
At4g33490	nucellin protein	1.55	1.83	-0.78	-0.61	-1.13	2.47	-0.1	-0.61
At4g31550	WRKY family transcription factor	-1.01	-0.59	0.54	0.32	-0.67	-0.65	-0.23	0.26
At5g54610	ankyrin repeat protein	-0.6	-1.95	-0.15	1.24	0.41	-2.71	0.86	0.64
At4g35610	zinc finger (C2H2 type)	-0.82	-3.92	-0.06	-0.72	0	-0.23	0.12	-0.28
At1g78600	zinc finger (B-box type)	-1.08	-0.72	0.21	-0.09	-0.21	0.06	0.7	0.33
At1g23390	kelch repeat-containing F-box family protein	-1.1	-1.38	0.13	-0.18	0.85	-2.05	-0.77	-0.58
At4g00970	protein kinase family protein	-1.06	-1.05	-0.51	-0.81	-5.96	0	-0.77	-0.7
At2g18730	diacylglycerol kinase	-0.88	-1.28	-0.61	-0.16	-0.11	-0.93	0.23	-0.11
At5g66040	senescence-associated, ketoconazole resistant protein	-0.6	-1.15	-0.56	-0.77	0.22	0.35	0.54	0.16
At5g65040	senescence-associated protein-related	-1.23	-0.72	0.33	0.44	0.28	-0.6	-0.98	-0.63
At1g71695	peroxidase 12 (PER12) (P12) (PRXR6)	-0.87	-1.32	-0.51	-0.53	-0.27	-0.14	-0.2	-0.18
At2g06050	12-oxophytodienoate reductase (OPR3) /(DDE1)	-1.01	-0.72	-0.02	-0.38	-0.49	0	-0.23	0.05
At1g55920	serine O-acetyltransferase	-1.06	-0.86	-0.54	-0.76	-0.36	-0.89	-0.02	-0.08
At4g00430	plasma membrane intrinsic protein	-1.18	-0.64	-0.27	-0.21	-0.44	-0.25	0	0.55
At3g47960	proton-dependent oligopeptide transport (POT) family protein	-1.24	-1.08	-0.6	-0.48	0.23	-0.45	0.87	0.31
At2g47440	DNAJ heat shock domain-containing protein	-1.27	-0.87	-0.24	-0.58	-0.6	-0.23	-0.55	-0.66
At2g43120	Pirin	-1.34	-1.42	0.27	-0.05	-0.54	-0.14	-0.05	-0.05
At4g35100	plasma membrane intrinsic protein (SIMIP)	-1.53	-0.82	-0.47	-0.1	-1.78	0	-0.92	-0.86
At5g02230	haloacid dehalogenase-like hydrolase family protein	-0.88	-1.13	-0.87	-0.89	0.06	-0.21	0.27	0.39
At5g10180	sulfate transporter	-1.06	-1.54	-0.61	-0.7	-0.43	-1.69	-0.19	0.08
At2g33530	serine carboxypeptidase S10 family protein	-0.68	-1.13	0.53	0.5	-0.61	-0.66	0.68	0.22

Chapter 4

Expression of the Arabidopsis *SHINE* gene in rice for drought resistance

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ABSTRACT

The SHINE clade of Arabidopsis AP2/ERF transcription factors increase epicuticular wax, alter epidermal properties and confer drought resistance in Arabidopsis. As rice has very low wax content, we introduced the Arabidopsis *SHINE2* gene under control of the constitutive CaMV 35S promoter into rice by Agrobacterium mediated transformation. From among 15 independent transformants, no obvious epicuticular wax or leaf phenotype was observed. However the rice transformants displayed changes in cuticular and epidermal properties, with increased cuticular permeability and reduction in stomatal density. We designed a drought resistance test similar to that used in Arabidopsis, and demonstrated that the rice *SHINE2* overexpressors were more resistant to drought than untransformed rice. We identified a number of *CER1*-like genes with predicted protein products similar to the Arabidopsis CER1 protein. This CER1 protein is required for wax biosynthesis and is induced by *SHINE* overexpression in Arabidopsis. However none of these *CER1*-like putative downstream genes were induced in the rice *SHINE2* overexpressor plants suggesting that wax biosynthesis in rice is not under control of the SHINE clade and wax synthesis is not a requirement for altering the cuticular and epidermal properties that are responsible for the drought resistance.

INTRODUCTION

Drought is one of the most important constraints for stable rice production in many rice producing areas of the world (Herdt, 1991). Drought stress can cause severe damage at any stage of rice growth and development, which leads to yield loss (Zhang and Luo, 1999). The scarcity of water internationally (IWMI 2000, Gleick, 1993) justifies the importance of innovative breeding efforts for drought resistance in rice.

Drought resistance improvement is probably one of the most difficult tasks for the rice breeders. The difficulty comes from the diversity and the unpredictability of drought conditions in the fields, and from the diversity of drought resistance strategies developed by the plants that may be targeted and used as selection criterion (This and Teulat-Merah, 1999). However, substantial progress on the unraveling of genetic pathways of drought resistance mechanisms in the model plant Arabidopsis helps to better understand general drought resistance engineering and to define strategies for rice improvement.

One important achievement is discovery of genes encoding CBF/DREB proteins, a member of AP2/ERF transcription factors that regulate the expression of a number of downstream genes conferring drought/salt/cold stress resistance (Liu et al., 1998; Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Haake et al., 2002; Maruyama et al., 2004). Constitutive overexpression of the Arabidopsis *CBF*/*DREB* genes in canola resulted in increased freezing tolerance (Jaglo et al., 2001) and drought tolerance. Recently, it was shown that overexpression of *CBF3*/*DREB1A* in transgenic rice elevated tolerance to drought and high salinity (Oh et al., 2005). It was also demonstrated that CBF3/DREB1A activated a smaller number of target genes in rice than in Arabidopsis, indicating that CBF3 cannot recognize the *cis* elements in the promoter region of certain stress-related rice genes.

The genotypic variation for drought resistance in rice has been attributed to epicuticular wax (EW) content and mostly to long chain alkanes (O'Toole and Cruz, 1983; Haque et al., 1992). Recently, it was suggested that a cuticular skin composed of highly structured waxy domains and not a high wax load is responsible for water transport (Kerstiens, 1996). Furthermore, a wide chain length distribution of aliphatic wax constituents, contributing to an amorphous structure, was suggested as the main determinant of barrier properties. However, rice has been documented to have a very low level of EW (O'Toole et al., 1979). Thus increasing levels of EW would be expected to provide an effective protective

layer against environmental stresses like drought. A number of genes involved in EW have been identified from Arabidopsis, like the *CER1* gene involved in formation of alkanes (Aarts et al, 1995) and the *CER6* (*CUT1*) gene (Fiebig et al., 2000; Millar et al., 1999) involved in elongation of the carbon backbone in Very Long Chain Fatty Acid synthesis. Our microarray study demonstrated that *CER1* and *CER6* (*CUT1*) are upregulated by drought treatment in Arabidopsis (Chapter 3), indicating their possible role in drought resistance mechanisms.

Recently, independently, Aharoni et al. (2004) and Broun et al. (2004) identified and characterized the SHINE (WIN) clade of AP2/ERF transcription factors that regulate the expression of a number of wax biosynthesis genes. Overexpression of *SHINE1 (SHN1)* in Arabidopsis increased epicuticular wax deposition, elevated cuticle permeability, decreased stomatal index, and enhanced significant drought resistance and recovery (Aharoni et al., 2004). Our recent data showed that some stress-related genes, like *RD22*, LEA, calnexin, calreticulin are also upregulated in the Arabidopsis *SHN1* overexpressor (Chapter 3), indicating the role of the SHINE clade in drought/salt tolerance mechanisms.

In this study, we have generated transgenic rice plants constitutively expressing one of the Arabidopsis *SHINE* genes (*SHN2)* and characterized their phenotypes. We discuss the similarity in overexpression phenotypes and drought resistance improvement between Arabidopsis and rice, and thus the conservation in function of this transcription factor.

MATERIALS AND METHODS

Sequence analysis

Identification of putative orthologs of *CER1* in the rice genome was conducted using a BlastP algorithm (Altschul et al., 1990) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments and phylogenetic analysis were performed using CLUSTAL-X 1.81 with default settings (Thompson et al., 1997). We used the neighbour joining method for calculating the phylogenetic tree and 1000 bootstrap replicates (recommended by the program). The GENEDOC (Nicholas et al., 1997) and TreeView (Page, 1996) programs were used for editing the alignment and drawing of the phylogenetic tree, respectively.

Generation of plant transformation constructs

SHINE2 (*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 from 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 chimaeric CaMV 35S-hygromycin phosphotransferase-tNos for selection during transformation. A fragment encompassing the upstream region of *OsCER1#10* (Os10g33250) was amplified (using *pfu* DNA polymerase) from young leaf genomic DNA of rice cv. Nipponbare. Oligonucleotides OsCER1#10PF (5'- CACCGGTTTAACGCCGAAACTTG - 3') and OsCER1#10PR (5'- GGTAGACGATCTATGGGTGCAGA - 3') were used to amplify a 0.8 kb fragment containing the upstream region of *OsCER1#10*. The amplified fragment was introduced to the *EcoRV*-digested pBlueScript SK+ vector and subsequently sequenced from both sides before digestion and ligation to the binary vector. The promoter::GUS construct was assembled by ligating the upstream region of *OsCER1#10* (*Hin*dIII-*Bam*HI fragment) and the GUS-tNOS cassette (*Bam*HI-*Eco*RI fragment) derived from the pBI121 (Clontech, Palo Alto, CA) in between the *Hin*dIII and *Eco*RI sites of pMOG22.

Plant transformation and growth conditions

Agrobacterium-mediated transformation of *Oryza sativa* ssp. *japonica* cv. Nipponbare, plant regeneration and growth were performed according Greco et al. (2001). The *Agrobacterium* strain AGL-1 was employed. Regenerated transgenic plantlets were transferred to the greenhouse and grown in hydroponic culture with a regime of 12 hours light, 28^{0}C, 85% relative humidity and 12 hours dark, 21^{0}C, 60% humidity.

Expression analysis

Total RNA for RT-PCR was isolated from young leaves derived from 4-week-old transgenic and wild-type (cv. Nipponbare) plants using the Trizol reagent as described by the manufacturer (Invitrogen, Life technologies, Carlsbad, CA). 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). The cDNA was diluted 50 times and used for amplification using specific oligonucleotides for the actin gene to equalize the concentrations of the cDNA samples. Subsequently, the diluted cDNA was used to perform a PCR reaction using specific oligonucleotides designed to amplify the *SHN2* gene and three putative *OsCER1* genes. Oligonucleotides AP69 (5'-CGGATCCATGGTACATTCGAGGAAGTTCCG –3') and AP70 (5'-CGAGCTCTCAATCCAATTCAGCAACTCC –3') were used to amplify the *SHN2* gene, oligonucleotides OsCER1#2aF (5'- CGGTATTATTGCGTGAAAGAGG -3') and OsCER1#2aR (5'- CGTAGAACACCAACCCATTGAA -3') to amplify *OsCER1#2a*, oligonucleotides OsCER1#2bF (5'- GCAACCAAACCACACACAGCTA -3') and OsCER1#2bR (5'-TGTGCAGCCAGTAGTAGAGGAA -3') to amplify *OsCER1#2b*, oligonucleotides OsCER1#10F (5'-GCTGCAGTGGTTGTCAACAGTA -3') and OsCER1#10R (5'- CCATATCAAGCACCTTGTCACC -3') to amplify *OsCER1#10*. The reaction conditions for PCR included a denaturing step of 95°C for 3 min, followed by 35 cycles of 1 min at 95°C, 1 min at 55°C, and 1.5 min at 72°C, ending with an elongation step of 5 min at 72°C. For the control PCR with actin oligonucleotides, 30 amplification cycles were used

Chlorophyll leaching assay and stomata analyses

For chlorophyll leaching assays, the second fully expanded leaf from the top of the main tiller from both the *SHN2* overexpressor and wild-type 4 weeks old plants were cut and weighed to get equal weight (0.25g) and put in tubes containing 20 ml of 80% ethanol at room temperature (gently agitating in the dark). After 1 hour incubation the pictures were taken.

For stomatal density measurements we used the second fully expanded leaf from the top of the main tiller from 4 weeks old *SHN2* overexpressors and wild-type plants. For each

genotype four different plants were used to generate imprints of their abaxial surface. A xylene-thermocol mixture, made by dissolving thermocol in xylene until the solution became viscous, was applied uniformly on the abaxial surface of the leaves and allowed to dry. 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 40X magnification using a light microscope (Zeiss). Numbers of stomata were counted per mm^2.

GUS staining and microscopy

Tissues from various organs either from hydroponic grown plants or seedlings grown on filter paper moistened with 1/2MS were analyzed for their GUS expression patterns. The GUS solution contained 100Mm 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 0.5 mM each of potassium ferri/ferrocyanide. Samples were vacuum-infiltrated and incubated at 37°C for 16 to 24 h and depleted from chlorophyll 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.

Drought resistance experiment

For the rice drought resistance experiments, soil mixture comprised 1 part of sand and perlite and 2 parts of compost [a mixture made up of 25% clay and 75% turf with EC=1 (NPK); Hortimea, Netherlands]. The 7-days-old seedlings were transferred into soil at density of five plants per 4 cm pot in a tray with 51 pots (Aracon containers, BetaTech, Belgium). Nutrients (Hydroagri, Rotterdam, The Netherlands; 2.6 EC) were supplied 3 days after transplanting and at 7 days after transplanting the plants were subjected to drought for 9 days by transferring the pots to dry trays (after drying each pot from outside). Everyday in drought, the plants were moved within the tray to nullify pot position effects. Subsequently, plants were rehydrated and observed for recovery after one week. This experiment was conducted to compare drought resistance between wild-type and 35S::*SHN2* plants.

RESULTS

Transformation of an Arabidopsis SHN gene overexpression construct in rice

To analyze the effect of overexpression of the Arabidopsis *SHINE2* (*SHN2*) gene in rice, we introduced the gene controlled by the CaMV 35S promoter into rice by means of an *Agrobacterium*-mediated embryogenic callus transformation. Fifteen independent transgenic lines were generated. Different from the overexpression of the *SHN2* gene in Arabidopsis that displayed a very clear phenotype, including twisted and downward folded leaves with a brilliant, shiny green leaf surface and increased epicuticular wax, none of the rice transformants revealed any obvious leaf wax increase or plant phenotype. RT-PCR analysis, however, confirmed high level expression of the *SHN2* gene (Figure 1A). A line with high expression and enough seed was used for further experimentation.

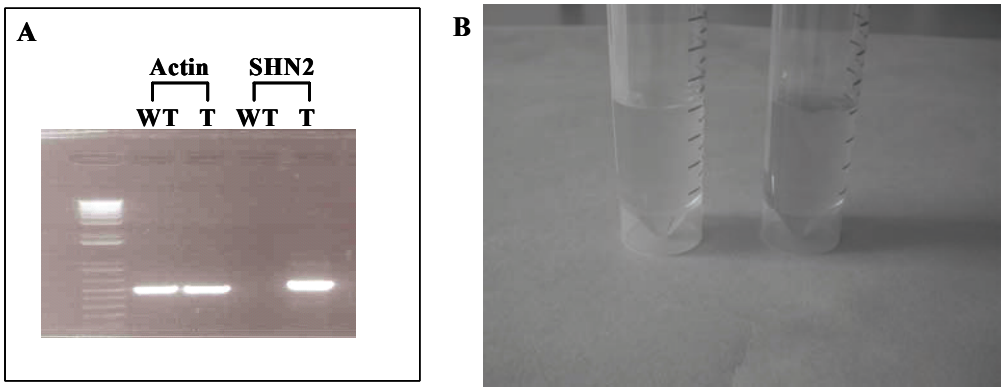


Figure 1. Expression of *SHN2* and alteration in cuticle permeability

A) RT-PCR experiment using oligonucleotides for amplification of *SHN2* gene in 35S::*SHN2*. Expression of *SHN2* is strong in leaves of 35S::*SHN2* transformants, while no expression is detected in the wild-type plant. Amplification of the actin gene was used as a control for presence and levels of cDNA.

B) Chlorophyll extracted in 80% ethanol for 1 h from second fully expanded leaves of *SHN2* overexpressor (right tube) and wild-type (left tube).

Epidermis and cuticle phenotype of rice SHN2 overexpressing plants

To investigate whether the *SHN2* overexpressor cuticular membrane properties were altered, we conducted a chlorophyll leaching experiment in which the second fully expanded

leaf from the top of the main tiller from both *SHN2* overexpressor and wild-type plants were submerged in 80% ethanol for 1 hour. Chlorophyll was observed to be extracted much faster from leaves of *SHN2* overexpressors as compared to the wild type (Figure 1B); therefore, the higher elution of chlorophyll from *SHN2* overexpressor leaves indicated an increase in cuticle permeability as suggested before for Arabidopsis (Aharoni et al., 2004).

We tested whether other features of epidermal cell differentiation were also altered by the overexpression of *SHN2*. Stomatal density on the abaxial side of the 35S::*SHN2* leaves was reduced to 3/4th compared with wild-type leaves (Table 1).

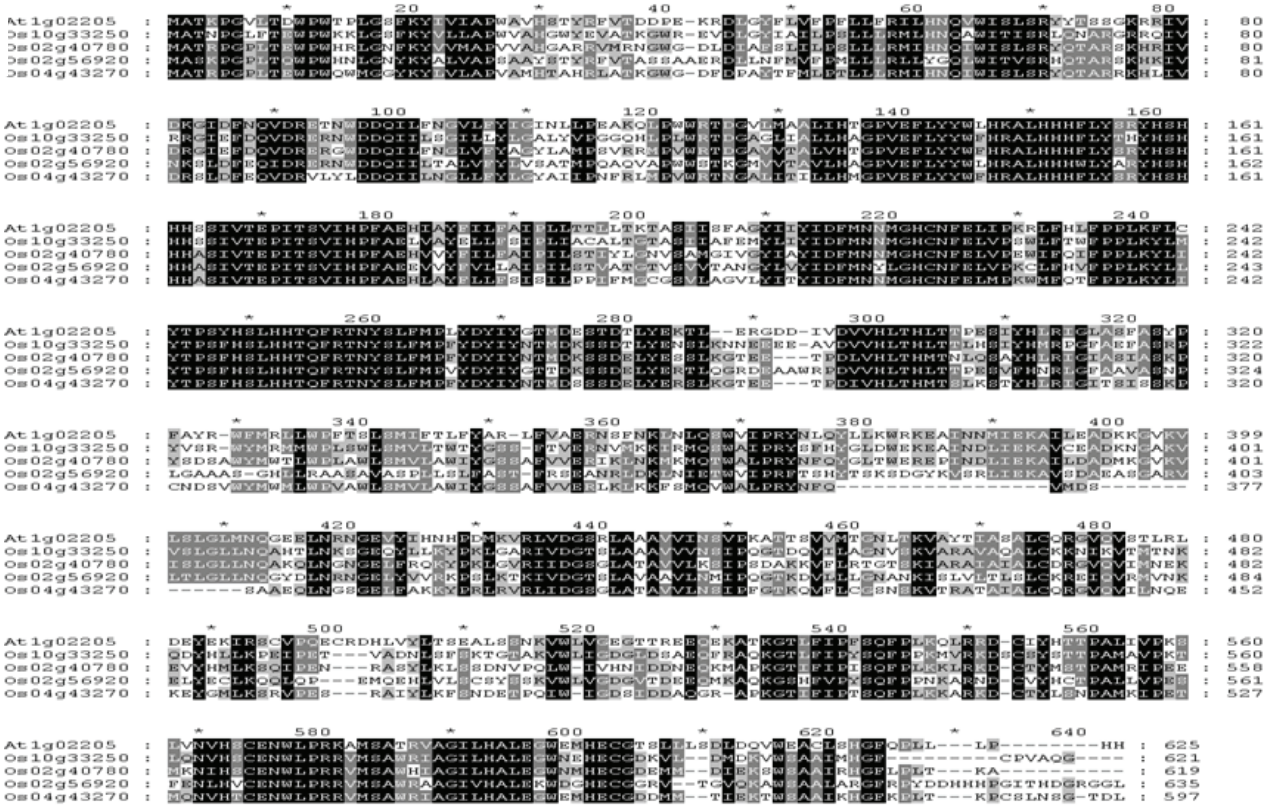
Table 1: Comparison of stomatal density of 35S::*SHN2* and wild-type leaves

	Stomatal density (cells/mm^2 ± SD)
Wild-type	40.62 ± 3.61
35S::*SHN2*	29.69 ± 3.12

Expression analysis of putative target genes of SHN in rice

Over-expression of the *WIN1/SHN1* in transgenic Arabidopsis plants activated the expression of a number of genes which are known to be involved in wax biosynthesis, like *CER1, KCS1,* and *CER2* (Broun et al., 2004; Aharoni et al. unpublished data). In this study, we searched the putative homologs of *CER1* in rice and tested their expression in 35S::*SHN2* transgenic rice. We searched the rice genome database for proteins with amino acid sequences similar to the Arabidopsis CER1 protein, and found four genomic clones with predicted amino acids that showed high homology (accession numbers AAP54228, BAD21579, BAD22402 and CAE03390, respectively). We named these four genes *OsCER1 (#10), OsCER1 (#2a), OsCER1 (#2b)* and *OsCER1 (#4)* based on their chromosomal position. *OsCER1 (#10), OsCER1 (#2a), OsCER1 (#2b)* and *OsCER1 (#4)* contained an open reading frame of 621, 619, 635 and 597 amino acids, respectively (Figure 2A). These proteins are 50.9-58.4 % similar to the Arabidopsis protein and 46.8-70.8 % similar to each other (Figure 2B).

A



B

	Percent identity			
	Os10g33250	Os02g40780	Os02g56920	Os04g43270
At1g02205	58.4	56.2	53.3	50.9
Os10g33250		63.0	53.6	57.3
Os02g40780			53.0	70.8
Os02g56920				46.8

Figure 2. The putative rice OsCER1 protein family

A) Sequence alignment of the Arabidopsis CER1 and the four rice OsCER1 proteins. Black background indicates 100% conservation, gray is 80%, and light gray is 60% conservation.

B) Percent identity of the CER1 protein members

In order to know if the expression of these genes is induced in the *SHN2* overexpressor, we conducted RT-PCR using cDNAs from young leaves of the wild type and the *SHN2* overexpressor. In the wild type, only *OsCER1 (#2a)* was expressed at a very low level (Figure 3D). The size of the RT-PCR product is seen smaller than that derived from the

DNA control due to presence of an intron in the fragment amplified by the primers. There is no induction of expression of this gene in the *SHN2* overexpressor.

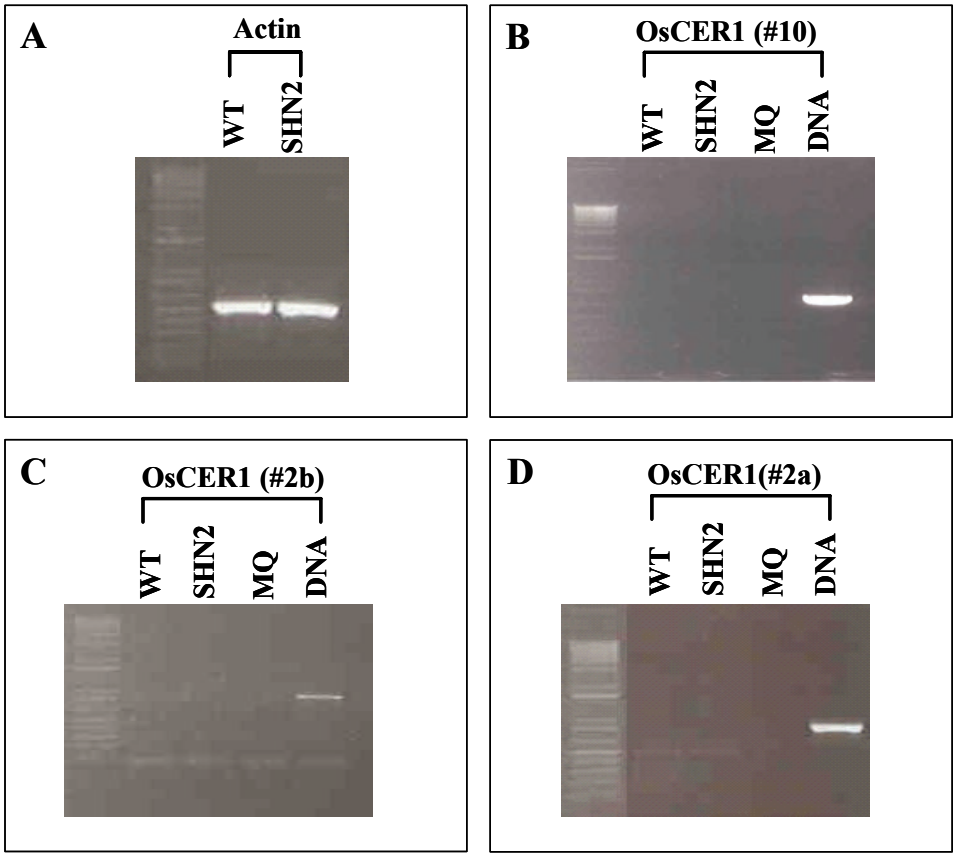


Figure 3. Expression of the three *OsCER1* genes in the 35S::*SHN2* line and the wild type RT-PCR experiments using oligonucleotides for amplification of the actin gene (A), *OsCER1#10* (B), *OsCER1#2b* (C), and *OsCER1#2a* (D). Among the three putative *OsCER1* genes, only *OsCER1#2a* expression can be detected at low level but with no alteration in expression level between 35S::*SHN2* line and wild-type.

We took the rice gene which is most similar to Arabidopsis *CER1* (*OsCER1 (#10))* and studied its expression by making a promoter-GUS fusion construct and transformed it into rice. In the young leaf, this gene was expressed very specifically in the guard cells (Figure 4A and 4B), explaining our failure to detect this low expression by RT-PCR. We also detected the expression of this gene in the anther and pistil (Figure 4C), but we didn't find GUS expression in the pollen. This gene was also expressed in the root hairs of young seedling (Figure 4D).

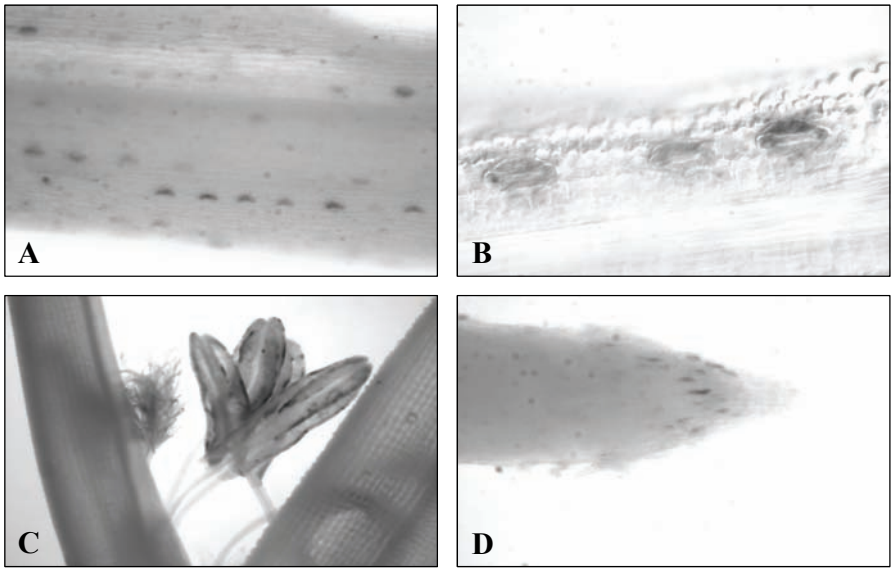


Figure 4. Expression patterns of *OsCER1#10* detected in transgenic rice *OsCER1#10* promoter::GUS lines. (A) and (B) Guard cells of a young leaf; (C) Anther and pistils; (D) Root hairs of young seedling

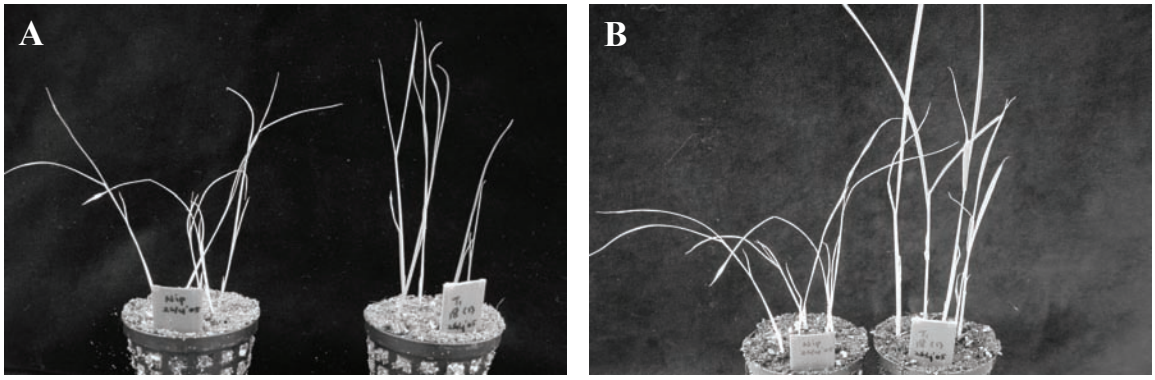


Figure 5. Drought resistance experiment with 35S::*SHN2* line and the wild type.
A) Fourteen days old seedlings (5 seedlings per pot) of either wild-type (left) or progeny of 35S::*SHN2* line (right) were exposed to dehydration stress by withholding water for 9 days.
B) The seedlings were watered and their appearance after a week (recovery) is displayed. There is a clear difference between wild-type and 35S::*SHN2*, in which there is 100% recovery of the overexpression line which turned into light green and no recovery of the wild-type was visible.

Drought resistance of SHN overexpressing rice plants

The rice transformants overexpressing the *SHN2* gene were used in a pot assay in the greenhouse for drought resistance according to Aharoni et al. (2004). Whereas wild-type plants did not recover from the dehydration treatments longer than 9 days and completely dried out, all seedlings derived from line expressing the gene recovered after rehydration to become greener and stronger (Figure 5).

DISCUSSION

It was previously reported that transgenic Arabidopsis overexpressing the *SHINE1*, *SHINE2*, or *SHINE3* genes showed an identical visual phenotype, including the brilliant, shiny green color of both rosette and cauline leaves, leaf curling, reduction in trichome number, and altered petal structure and silique length (Aharoni et al., 2004). We deduced that these three SHN clade members are redundant in their function and probably interact with the same target genes. In order to examine the conservation of functions of the SHN clade between Arabidopsis and rice we overexpressed the Arabidopsis *SHINE2* (*SHN2*) gene in rice. Our results showed that all rice transformants revealed no obvious leaf wax increase or plant phenotype. However, we detected other major effects like increased cuticle permeability and reduced stomatal density, which are similar to the Arabidopsis activation tag *shine* mutant and the *SHINE1* (*SHN1*) overexpressor (Aharoni et al., 2004).

In Arabidopsis, overexpression of the *SHN1*/*WIN1* gene increases the total leaf wax 6-fold and contributes to a layer of wax crystals that give the leaf a shiny appearance. As rice has got a very low leaf wax level, even lower than Arabidopsis, probably other genes in the pathway are lacking and thus overexpression of SHN might not cause an increase in wax. We, therefore, investigated if potential target genes of SHN in the epicuticular wax (EW) pathway were affected. Two distinct Arabidopsis EW genes *CER1* and *KCS1* are induced by SHN1/WIN1. Among the many EW genes from Arabidopsis, it is not straightforward to identify orthologs based on sequence similarity alone. Thus orthologs of Arabidopsis *KCS1*/*CER6* genes are not easy to identify without any expression studies. However we could identify a set of 4 rice genes from the genome sequence, with their predicted protein sequences similar to that of the Arabidopsis CER1 protein. We took the promoter of the rice

gene most similar to *CER1* and studied its expression in rice using a *GUS* reporter gene. The expression level was very low, and localized at the stomata in the leaf. We then studied the expression of three rice *CER1*-like genes by RT-PCR and could not detect any increase in transcription in the rice transgenic plants overexpressing the Arabidopsis *SHN2* gene. In general, expression of the three *OsCER1*-like genes studied was around the limits of detection by the RT-PCR experiments in the wild-type plants.

Our results show that the rice *CER1*-like genes are not induced by *SHN2* overexpression. This might be a reason why no leaf wax is observed in the *SHN2* plants. Thus there is another level of regulation of EW genes in rice that probably limits the expression of EW accumulation, both in wild-type and in the *SHN* overexpressor. Comparative microarray experiments of *SHN* overexpression Arabidopsis and rice would reveal if there are further differences in regulation of downstream genes.

At the limits of our detection, overexpression of Arabidopsis *SHN* in rice does not increase the leaf EW or induce downstream target genes involved in EW biosynthesis. Neither does it cause a change in leaf morphology like curling. However it does cause a change in cuticular properties like permeability and reduction in stomatal density. Thus we might conclude that changes brought about in the cuticular and epidermal properties are independent of the epicuticular wax level or genes involved in that process. In other words the expression of *SHN* in rice is able to dissect and distinguish between the cuticular and epicuticular changes.

Overexpression of the Arabidopsis *SHN* gene in rice confers drought resistance. This is probably due to the effect of reduced water loss related to a lower stomatal density. In contrast to Arabidopsis that has an accompanying EW increase, the experiments in rice demonstrate that it is the altered epidermal properties that are more important in the drought resistance phenotype.

The SHN clade of transcription factors has conserved functions in affecting the epidermal properties and providing drought resistance. As they function between Arabidopsis and rice, it suggests that they will function between dicots and monocots and be applicable to a wide range of crop plants for providing drought resistance. This drought resistance is probably not dependent on the leaf epicuticular wax and thus EW and the leaf phenotype does not need to be modified in order to provide drought resistance in crop plants. The SHN clade

thus provides an additional tool in increasing drought resistance and can be used along with other genes having complementary resistance mechanisms.

A considerable effort has been made in developing transgenic rice lines tolerant to drought stress. Overexpression of genes encoding enzymes responsible for the accumulation of some compatible solutes such as proline, glycinebetaine, trehalose, and polyamine enhanced drought tolerance in rice (Zhu et al., 1998; Sawahel, 2003; Garg et al., 2002; Jang et al., 2003; Capell et al., 2004). These compounds serve as osmoprotectants and, in some cases, stabilize biomolecules under stress conditions (Yancey et al., 1982). Improved tolerance of rice to drought stress has also been demonstrated by introducing specific genes encoding late embryogenesis abundant (LEA) proteins from barley and wheat (Xu et al., 1996; Cheng et al., 2002; Rohila et al., 2002; Babu et al., 2004). These proteins were thought to improve performance of rice plants by protecting cell membranes from injury under drought stress (Babu et al., 2004).

However, plant drought resistance is a complex trait that involves multiple physiological and biochemical mechanisms and regulation of numerous genes. Engineering a single pathway or mechanism may not be adequate to confer drought resistance in the field (Ingram and Bartels, 1996). Overexpression of genes encoding regulatory proteins that can regulate the expression of many of stress-resistance genes involved in different pathways or mechanisms might be more promising to get plants tolerant to the field drought conditions. Overexpression of a rice gene encoding a calcium-dependent protein kinase (CDPK) conferred drought 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.

Recently, it was reported that overexpression of Arabidopsis genes encoding DREB1A/CBF3 and ABF3 proteins enhanced drought 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 (Chapter 3), suggesting the potential to activate complementary stress resistance pathways.

One important criterion in the overexpression of regulatory proteins to get stress tolerant plants is that the transgenic plants should not show growth retardation under normal conditions. 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 et al., 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).

In conclusion, overexpression of SHN in rice provides a complementary mechanism to that of other available genes and also generates plants that are not compromised in plant performance. Field testing of these overexpression genotypes in normal and drought environments will be able to validate their use in making a drought resistant rice crop.

ACKNOWLEDGEMENTS

We thank Nayelli Marsch-Martinez for critical reading of the manuscript, John Franken for assistance with microscope photograph.

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Chapter 5

Overexpression phenotypes of the rice and Arabidopsis SHINE AP2/ERF transcription factors reveal a conserved function

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ABSTRACT

The conserved domains of the Arabidopsis SHINE clade of AP2/ERF transcription factors were used to identify two rice homologs (OsSHN) from the genome sequence. The function of the *OsSHN1* gene was characterized by expression in Arabidopsis. Over-expression of *OsSHN1* in transgenic Arabidopsis revealed similar phenotypes compared to the overexpression of the Arabidopsis *SHN* genes, including enhancement in cuticle permeability, decrease in stomatal index, and increase in drought resistance. In the *OsSHN1* overexpressor we showed the induction of some adaptive drought responsive genes that are downstream targets of the Arabidopsis *SHN* genes. This indicated that *OsSHN1* has functional similarity to the Arabidopsis SHN clade. *OsSHN1* is, therefore, potentially useful for producing drought resistant monocot plants. We also showed that overexpression of a chimeric OsSHN1 repressor suppressed the expression of *CER1,* yielding plants with a glossy green stem and semi sterile phenotype. This supports the hypothesis that SHN proteins have a function in regulating Arabidopsis wax biosynthesis.

INTRODUCTION

The AP2/ERF transcription factor family has been found throughout the angiosperms in both monocots and eudicots. They are involved in key developmental steps, such as flower organogenesis or seed development, and in many stress responses (Riechmann and Meyerowitz, 1998). The conserved domain that defines this family was first described in the homeotic gene *APETALA2* (Jofuku et al., 1994), and in ethylene-responsive element binding proteins (EREBPs) from tobacco (Ohme-Takagi and Shinshi, 1995). Sakuma et al. (2002) divided the 145 members of AP2/ERF family, found in Arabidopsis, into five subfamilies: AP2, RAV, DREB, ERF, and others. Fourteen proteins have two AP2 domain repetitions and belong to the AP2 subgroup. The RAV transcription regulators (six members) have 2 different DNA-binding domains, AP2/ERF and B3. The DREB and ERF subfamilies (121 proteins) have a single AP2 domain and a conserved WLG motif. The four AP2/ERF members of the other subfamily have a single AP2 repetition but lack the WLG motif.

DNA binding specificity has been shown for members of the AP2, RAV, ERF, and DREB subfamilies. The only member of the AP2 subfamily with a characterized binding sequence is the Arabidopsis AINTEGUMENTA (ANT) protein. The two AP2 domains in ANT selectively bind the consensus sequence gCAC(A/G)N(A/T)TcCC(a/g)ANG(c/t) (Nole-Wilson and Krizek, 2000; Krizek, 2003). The Arabidopsis RAV1 transcription factor can bind a bipartite recognition sequence with the B3 and AP2 domain recognizing the sequences CACCTG and CAACA, respectively (Kagaya et al., 1999). Several ERF proteins bind the GCC box (AGCCGCC) (Ohme-Takagi and Shinshi, 1995; Buttner and Singh, 1997; Zhou et al., 1997; Hao et al., 1998; Fujimoto et al., 2000; Hao et al., 2002). DREB proteins are known to bind the dehydration response element and C-repeat which share the CCGAC motif (Yamaguchi-Shinozaki and Shinozaki, 1994; Baker et al., 1994; Stockinger et al., 1997).

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). Stress induced genes identified by different criteria have been named *rd* (responsive to dehydration), *erd* (early responsive to dehydration), *cor* (cold-regulated), *lti* (low-temperature induced) and *kin* (cold-inducible). Some of these genes respond to multiple stresses, e.g. dehydration-inducible genes respond to cold stress and vice

versa, suggesting parallel induction mechanisms. The promoters of stress responsive genes contain a cis-acting dehydration-responsive element (DRE) (Yamaguchi-Shinozaki and Shinozaki, 1994) or similar the low-temperature C-repeat (CRT) element (Baker et al., 1994), suggesting a convergence in the signal transduction pathway between dehydration and cold stress. This also explains cross-tolerance between different stresses as was shown by overexpression of the AP2/EREBP 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).

The orthologous genes of Arabidopsis *CBF*/*DREB* have been found in most crop plants examined so far (Jaglo et al., 2001; Choi et al., 2002; Gao et al., 2002; Shen et al., 2003; Dubouzet et al., 2003). Overexpression of the Arabidopsis *CBF*/*DREB* genes in canola increased freezing and drought tolerance (Jaglo et al., 2001). 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 many plants.

Recently, Aharoni et al. (2004) identified and characterized the SHINE clade of ERF subfamily and showed that overexpression of either one of them will cause a major effect on the plant surface. Such plants displayed a brilliant, shiny green leaf surface with increased epicuticular wax compared with the leaves of wild-type plants. In a similar study, Broun et al. (2004) showed that a number of genes, like *CER1*, *KCS1*, and *CER2*, which are known to be involved in wax biosynthesis were induced in plants overexpressing *WIN1*/*SHN1*. Overexpression of the *SHN* genes also increased cuticle permeability, altered leaf and petal epidermal cell structure, trichome number, and branching as well as the stomatal index (Aharoni et al., 2004). Interestingly, *SHN* overexpressors displayed significant drought resistance (Aharoni et al., 2004).

Rice is not only an important crop but also a model monocot crop (Dubouzet et al 2003). It is important to identify and characterize the *SHN* homologs in rice to assess the conservation in function to their counterparts in Arabidopsis, in order to identify rice genes that can be used to improve drought resistance. In this study, we isolated one rice *SHN* homolog and analyzed its function in Arabidopsis. We discuss the similarity in overexpression phenotypes and drought resistance improvement between Arabidopsis *SHN* and rice *OsSHN1* genes.

MATERIAL AND METHODS

Plant material and drought resistance experiment

All Arabidopsis plants were grown in the greenhouse at around 22°C and were in the ecotype Wassilewskija (Ws). For the drought resistance experiments, soil mixture comprised 1 part of sand and perlite and 2 parts of compost [a mixture made up of 25% clay and 75% turf with EC=1 (NPK); Hortimea, Netherlands]. Seeds were sown (after 3 nights at 4°C) at density of six plants per 4-cm pot in a tray with 51 pots (Aracon containers, BetaTech, Belgium). Nutrients (Hydroagri, Rotterdam, The Netherlands; 2.6 EC) were supplied 10 days after germination and at two weeks after germination the plants were subjected to drought (for 13, 14, 15 or 16 days) by transferring the pots to dry trays (after drying each pot from outside). Every 2 days in drought, the plants were moved within the tray to nullify pot position effects. Subsequently, plants were rehydrated and observed for recovery after one week. The experiment was conducted to compare drought resistance between wild-type, 35S::*AtSHN1*(#2-2), 35S::*OsSHN1*(#1) and 35S::*OsSHN1*(#16) plants.

Sequence analysis

Putative orthologs of *SHN* in the rice genome were identified using a BlastP algorithm (Altschul et al., 1990) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). 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 et al., 1997) and TreeView (Page, 1996) programs were used for editing the alignment and drawing the phylogenetic tree, respectively.

Generation of plant transformation constructs, transgenic Arabidopsis and transgenic rice

Fragments encompassing the full length coding region and the upstream region of *OsSHN1* were amplified (using *pfu* DNA polymerase) from young leaf genomic DNA of rice cv. Nipponbare. Oligonucleotides OsSHN1F (5'-AATAAGGATCCATGGTACAGCCAAAGAAG-3') and OsSHN1R (5'-AATAAGTCGACTCAGATGACAAAGCTACC-3') were used to amplify the 0.76 kb fragment containing the full length coding region of *OsSHN1*. The pair of oligonucleotides introduced *BamH*I and *Sal*I restriction sites to the amplified fragments at their 5' and 3', respectively, which were utilized for ligation. Oligonucleotides OsSHN1F (as described above) and OsSHN1::SRDXR (5'–CGTCGACTCAAGCGAAACCCAAACGGAGTTCTAGATCCAGATCCAGGATGACAA AGCTACCCTCTCCCTCTC-3') were used to amplify the 0.8 kb fragment containing a chimeric fusion of the full length coding region of OsSHN1 and SRDX (LDLDLELRLGFA) at the 3' end. Oligunucleotide OsSHN1::SRDXR introduced a *Sal*I restriction site to the amplified fragment at its 3'. OsSHN1PF (5'-AATAAAAGCTTACTCAGCAGTGAGCACACACC–3') and OsSHN1PR (5'-AATAAGGATCCCCAGCAGTGGAGAGATTC -3') were used to amplify the 1.38 kb fragment containing the upstream region of *OsSHN1* and introduced *Hin*dIII and *Bam*HI restriction sites to the amplified fragment at the 5' and 3'. 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. The overexpression and chimeric repressor constructs were assembled by multi-point ligations, in which the individual fragments (promoter, *OsSHN1* gene or *OsSHN1::SRDX* 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). A CaMV35S terminator fragment was obtained as a 0.21 kb *Sal*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 chimaeric CaMV 35S-hygromycin phosphotransferase-tNos for selection during transformation. The promoter::GUS construct was assembled by ligating the upstream

region of *OsSHN1* (*Hin*dIII-*Bam*HI fragment) and the GUS-tNOS cassette (*Bam*HI-*Eco*RI fragment) derived from the vector pBI121 (Clontech, Palo Alto, CA) in between the *Hin*dIII and *Eco*RI sites of pMOG22. PCR, restriction digestions, plasmid DNA isolation and gel electrophoresis were performed using standard protocols. The overexpression and chimeric repressor constructs were introduced into Arabidopsis using the floral dipping transformation method (Clough and Bent, 1998). The seeds were plated on one-half-strength Murashige and Skoog medium (1/2MS; Murashige and Skoog, 1962) and 15g/L sucrose. Seedlings selected on 20 mg/l hygromycin were subsequently transferred to the greenhouse.

The promoter::GUS construct was introduced into rice cv. Nipponbare according Greco et al. (2001). The *Agrobacterium* strain AGL-1 was employed. Regenerated transgenic plantlets were transferred to the greenhouse and grown in hydroponic culture with a regime of 12 hours light, 28^0C, 85% relative humidity and 12 hours dark, 21^0C, 60% humidity.

For growing progeny seeds, the seeds were dehusked, surface-sterilized (1 min in 70% ethanol, followed by 20 min in 1% NaOCl, and four rinses with sterile water) and soaked in sterile MilliQ water. After germination the seedlings were transferred to filter paper moistened with 1/2MS and grown in a climate chamber under long-day conditions (16 h light, 8 h dark, 28^0C) for about two weeks, before being transferred to the greenhouse.

Gene expression analyses

For RNA isolation, rosette leaves of 35S::*OsSHN1*(#16) and wild type plants; and stems of 4 week old 35S::*OsSHN1SRDX*(#5) and wild type plants were used. Total RNA for Reverse Transcriptase-PCR (RT-PCR) was isolated using the TrizolReagent 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 (RACTP1, 5'-GCGGTTTTCCCCAGTGTTGTTG -3' and RACTP2, 5'-TGCCTGGACCTGCTTCATCATACT -3') to equalize the concentrations of the cDNA samples. Subsequently the diluted cDNA was utilized to perform a PCR reaction using specific oligonucleotides. Oligonucleotides OsSHN1F and OsSHN1R (see above) were used

to amplify OsSHN1 on rosette samples and OsSHN1::SRDX on stem samples; oligonucleotides AtCER1F (5'- TGGTCGACGGCAGTAGATTA -3') and AtCER1R (5'- TTACCTCACAGGAGTGGACA -3') to amplify At1g02205 (*AtCER1*), oligonucleotides KCS1F (5'- CGGTTCTAGACGAAGTGCAG -3') and KCS1R (5'- GCATTACCGGTCATCTCCTC 3') to amplify At1g01120 (*KCS1*), oligonucleotides RD22F (5'- GAGGATGGTTACGGAGGCAA -3') and RD22R (5'- GGTTCCAAGCTGAGGTGTTC -3') to amplify At5g25610 (*RD22*). The reaction conditions for PCR included a denaturing step of 95°C for 3 min, followed by 25 cycles of 1 min at 95°C, 1 min at 55°C, and 1.5 min at 72°C, ending with an elongation step of 5 min at 72°C.

Chlorophyll leaching assay and stomata analyses

For chlorophyll leaching assays, roots and inflorescence stems of 4 week old 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). Four hundred microliter were removed from each sample after 15, 30, and 45 min. Absorbance of each sample was measured at 664 and 647 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 micromoles chlorophyll = 7.93 (A_{664}) + 19.53 (A_{647}).

For stomatal density, pavement cell density and stomatal index measurements we used similar size and age mature green rosette leaves, derived from 6 weeks old plants of wild type and 35S::*OsSHN1* line #16. Two leaves from four different plants (from each of the two genotypes) were used to generate imprints of their abaxial surface. A xylene-thermocol mixture, made by dissolving thermocol in xylene until the solution becomes viscous, was applied uniformly on the abaxial surface of the leaves and allowed to dry. 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 40X magnification using a light microscope (Zeiss). Numbers of epidermal pavement cells and stomata were counted per mm^2 (two different regions per leaf) and stomatal index was calculated (Mishra, 1997).

GUS staining and microscopy

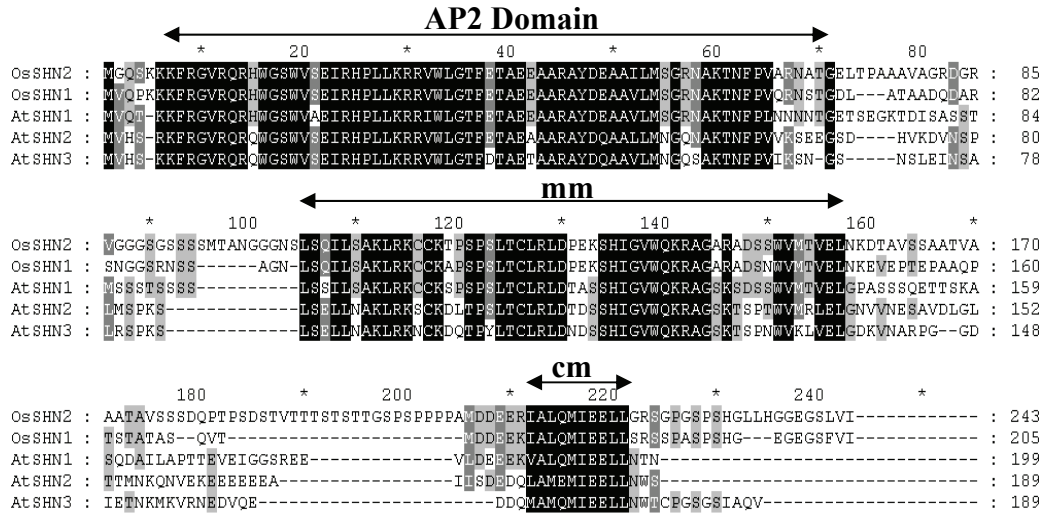
Tissues from various organs either from hydroponic grown plants or seedlings grown on filter paper were analyzed for their GUS expression patterns. The GUS solution contained 100Mm sodium phosphate buffer, pH 7.0, 0.5 mg/ml 5-bromo-4-chloro-3-indolyl β-D glucuronic acid (X-Gluc, Duchefa, The Netherlands), 0.1% Triton, and 0.5 mM each of potassium ferri/ferrocyanide. Samples were vacuum-infiltrated and incubated at 37°C for 16 to 24 h and depleted of chlorophyll in 70% ethanol. Observations were conducted under the binocular (WILD M3Z of Heerbrugg Switzerland, type-S) and a RS Photometrics CoolSNAP camera (MediaCybernetics®) was used to take the digital images, with the corresponding CoolSNAP software.

RESULTS

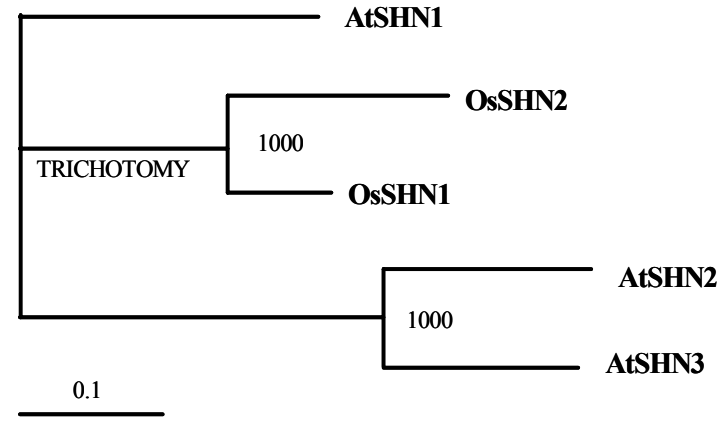
Identification of rice OsSHN homologs of the SHINE clade

Comparison of the amino acid sequences of three similar Arabidopsis SHN-related proteins (Aharoni et al, 2004) revealed high similarity in the central portion (middle 'mm') and C termini ('cm') as well as the AP2 DNA-binding domains. Using these consensus domains ('mm', 'cm' and 'AP2') to screen the sequence databases, members of the SHN clade of proteins could be defined as those that show high similarity to the Arabidopsis SHN proteins in these conserved domains. We searched the rice genome database for proteins with amino acid sequences similar to the SHN protein conserved regions, and found two genomic clones with predicted amino acids that showed high homology in these conserved regions (accession number BAD15859 and BAD35470). We named these two genes *OsSHN1* and *OsSHN2*. *OsSHN1* and *OsSHN2* contained an open reading frame of 205 and 243 amino acids, respectively (Figure 1A). These proteins are 43.2-62.4 % similar to the Arabidopsis proteins and 68.3 % similar to each other (Figure 1B and 1C). The *OsSHN1* and *OsSHN2* DNA sequences display 80.2–86.4 % identity with 20-25 nucleotide stretches of identity in the conserved protein domains (Figure 1D, 1E, and 1F).

A



B



C

Percent identity

	OsSHN2	AtSHN1	AtSHN2	AtSHN3
OsSHN1	68.3	62.4	52.2	52.7
OsSHN2		51.4	43.2	43.2
AtSHN1			53.8	52.8
AtSHN2				72.5

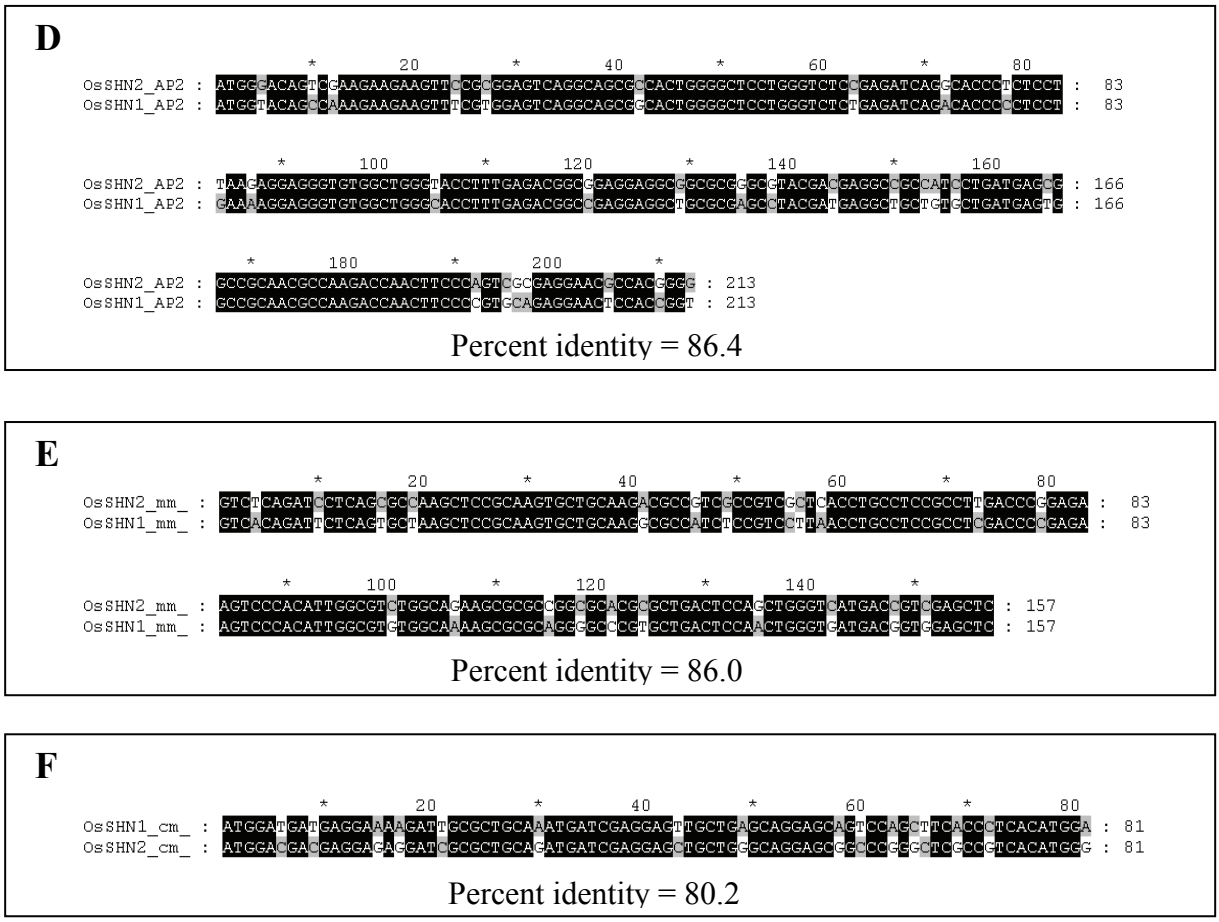


Figure 1. The SHINE Clade of the Arabidopsis and Rice AP2/ERF Transcription Family

(A) Sequence alignment of the three Arabidopsis and the two rice SHN proteins. All five proteins contain a single AP2 domain at their N termini, a conserved middle domain (termed mm), and a conserved C-terminal domain (termed cm). Black background indicates 100% conservation, gray is 80%, and light gray is 60% conservation.

(B) Phylogenetic analysis of the SHN clade protein members. The scale bar of 0.1 is equal to 10% sequence divergence. Bootstrap values are given for nodes and are considered as a value for significance of the branches. Values higher than 850 are likely to be significant.

(C) Percent identity of the SHN clade protein members.

(D) Nucleotide identity of the AP2 domain of rice SHN members.

(E) Nucleotide identity of the mm domain of rice SHN members.

(F) Nucleotide identity of the cm domain of rice SHN members.

Overexpression of OsSHN1 in Arabidopsis results in a phenotype similar to SHN

The genomic sequence encompassing the coding region of *OsSHN1* was used for overexpression (using the 35S CaMV promoter) and transformation of this gene in Arabidopsis plants. Interestingly, plants overexpressing *OsSHN1* showed an identical visual phenotype to the one obtained when overexpressing the Arabidopsis *SHN1* gene, including the brilliant, shiny green color of both rosette and cauline leaves, leaf curling, and altered silique length (Figure 2A and 2B).

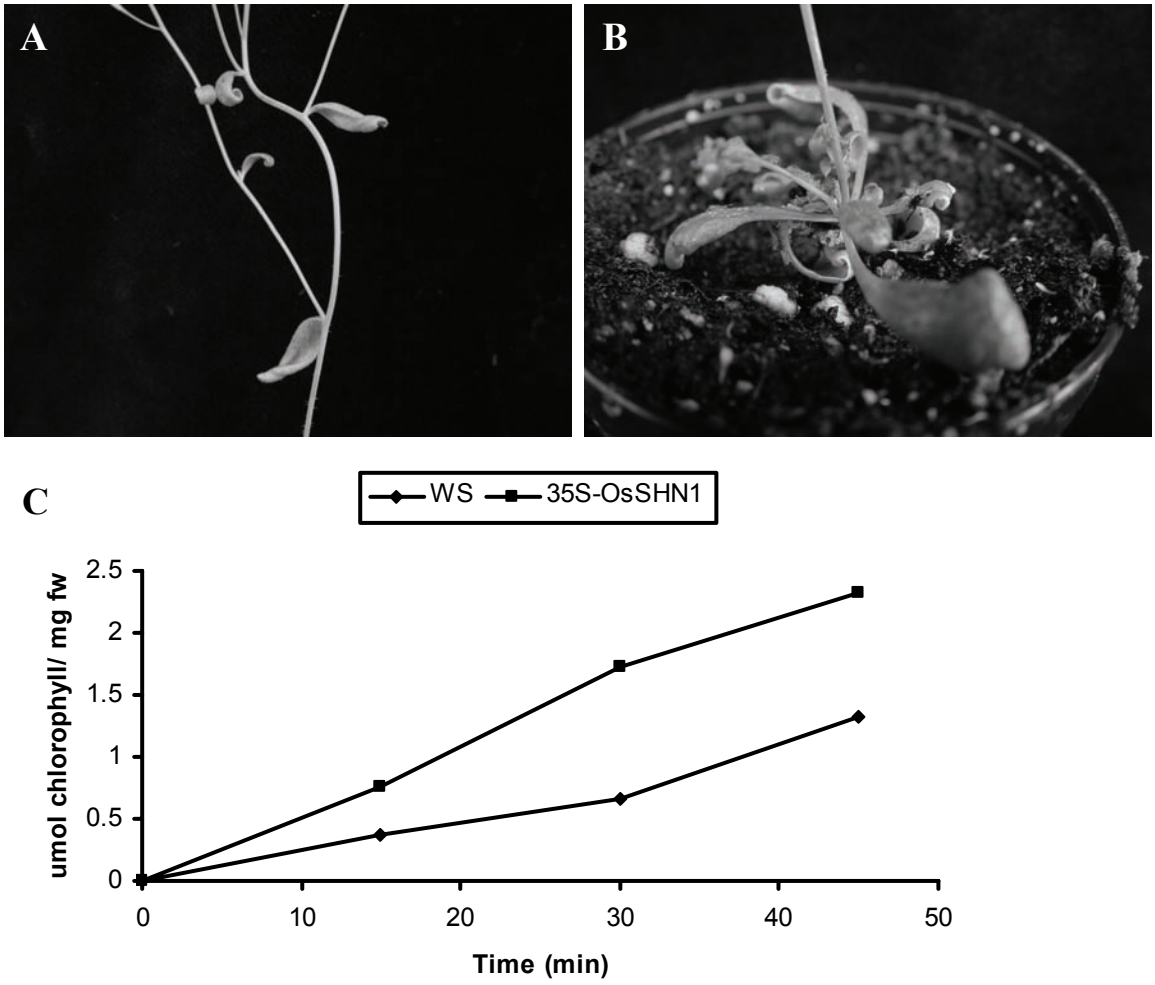


Figure 2. The 35S::*OsSHN1* Plants Phenotype and Surface Permeability

(A) Folded and twisted cauline leaves derived from a 35S::*OsSHN1* line.

(B) Brilliant and shiny green rosette leaves derived from a 35S::*OsSHN1* line.

(C) Chlorophyll leaching assays with mature rosette leaves of 35S::*OsSHN1* and wild-type immersed in 80% ethanol for different time intervals. The results are derived from three independent experiments.

To investigate whether the cuticular membrane properties of the *OsSHN1* overexpressor were altered, we conducted a chlorophyll leaching experiment in which rosette leaves from both *OsSHN1* overexpressor and wild-type plants were submerged in 80% ethanol for different time periods, and the chlorophyll concentration in the solution was determined. Chlorophyll was extracted much faster from leaves of the *OsSHN1* overexpressor as compared to the wild type; therefore, the higher elution of chlorophyll from *OsSHN1* overexpressor indicates an increase in cuticle permeability to organic solvents (Figure 2C).

We tested whether two other features of epidermal cell differentiation were also altered by overexpression of *OsSHN1*. Both of pavement cell density and stomatal density on the abaxial side of the *OsSHN1* overexpressor was reduced compared with wild-type leaves. Calculating the stomatal index revealed that it was reduced by 40.56 % in the *OsSHN1* overexpressor leaves compared with the wild-type (Table 1).

Table 1. Stomatal Density, Pavement Cell Density, and Stomatal Index of Mature 35S::*OsSHN1* and Wild-Type Rosette Leaf Blades

Plant Line	Stomatal Density (cells/mm^2 ± SD)	Pavement Cell Density (cells/mm^2 ± SD)	Stomatal Index
Wild Type	25.39 ± 3.59	83.20 ± 10.13	30.55 ± 2.88
35S::*OsSHN1*	10.94 ± 3.61	59.38 ± 9.41	18.16 ± 4.52

Overexpression of chimeric OsSHN1 repressor in Arabidopsis reduces epicuticular wax

To assess the role of the OsSHN1 protein as a transcriptional activator in wax biosynthesis we modified OsSHN1 protein to be a chimeric repressor (OsSHN1-SRDX) by fusing it to the EAR repression domain (Hiratsu et al., 2003) and overexpressed it in Arabidopsis. Previous studies (Aharoni et al., personal communication) using RNAi constructs of the Arabidopsis *SHN* genes did not reveal a mutant phenotype, therefore, this alternative option was taken to avoid functional redundancy. Forty-five primary transformants were generated from the transformation experiments. From these, eighteen primary transformants showed a loss-of-function mutant stem phenotype with reduced epicuticular wax (glossy green stem) (Figure 3A and 3B).

Some of the primary transformants did not set seed showing very short empty siliques indicating sterility. Some of the sterile primary transformants were covered in a plastic bag for a few days during flowering and showed good seed set, indicating a conditional semi male-sterile phenotype as is seen for some Arabidopsis *cer* mutants lacking wax in the pollen coat (Aarts et al., 1995). We also found some primary transformants that had flat siliques that is due to change in structure of the silique replum and valves making the silique more extended laterally. The glossy green stem phenotype was not very obvious on primary transformants transferred to the greenhouse from selection media primarily due to the thin stem structure. The T2 progeny, however, revealed the glossy thinner stems inherited as a dominant allele (about 3/4 progeny). The *OsSHN1-SRDX* overexpressors also showed smaller rosette leaves and shorter siliques in the progeny.

The expression of OsSHN1 and OsSHN1-repressor effects the expression of the downstream regulated Arabidopsis CER1 gene

We conducted RT-PCR experiments to check expression of the introduced *OsSHN1* and *OsSHN1*-repressor genes. High levels of expression were obtained as seen in Figure 3C and 3D. To investigate whether the OsSHN1 has the same downstream target genes as that as the Arabidopsis SHN1 we conducted RT-PCR for the *CER1* gene and other candidate downstream genes using leaf rosette RNA samples from both the *OsSHN1* overexpressor and wild-type plants. We found that the *CER1* gene was significantly overexpressed in the 35S-OsSHN1 plants (Figure 3D). In addition the *RD22* gene also shows a slight increase in expression in OsSHN1.

To check whether downregulation of the *CER1* transcript is responsible for the glossy '*cer'* stem phenotype we conducted RT-PCR for the *CER1* gene using stem RNA samples from both 35S:*OsSHN1-SRDX* and wild-type plants. We found that the *CER1* gene was partially repressed in the 35S:*OsSHN1-SRDX* plants (Figure 3C). Using RT-PCR for actin as control in repeated experiments, the RT-PCR of the *CER1* gene consistently gave these changes in levels of expression in the 35S::*OsSHN1-SRDX* plants.

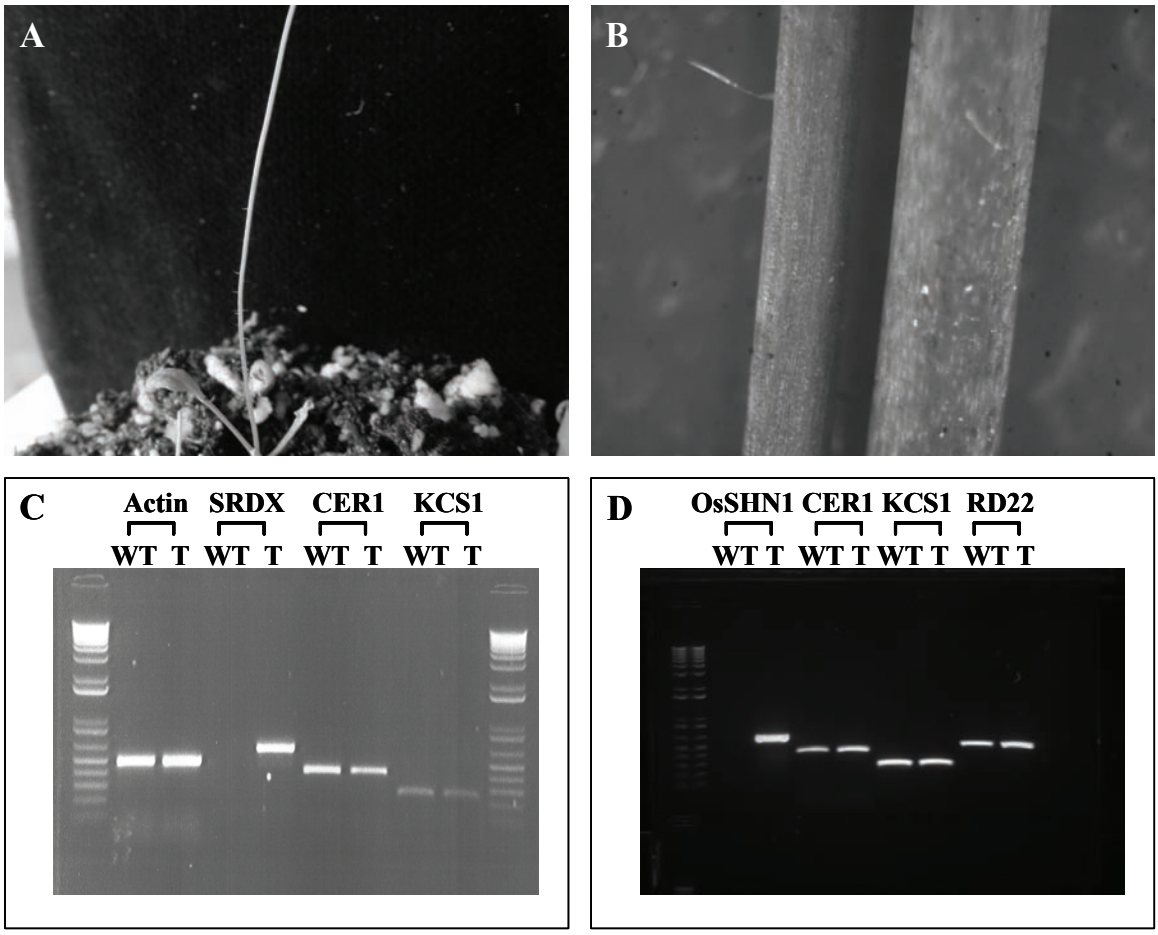


Figure 3. The Chimeric *OsSHN1*-Repressor Phenotype and Expression of Downstream Regulated Genes

A) Glossy green stem of a 35S::*OsSHN1-SRDX* line.

B) Mature stems of the wild-type plant (ecotype Wassilewskija [WS]) and the 35S::*OsSHN1-SRDX* on the right and left, respectively. Note the characteristic wax-less and thinner appearance of the 35S::*OsSHN1-SRDX* stem.

C) RT-PCR experiment using oligonucleotides for amplification of the putative downstream regulated genes in 35S::*OsSHN1-SRDX*. Expression of *CER1* is clearly suppressed in the stem of 35S::*OsSHN1-SRDX*, whereas only slight suppression could be detected for *KCS1*.

D) RT-PCR experiment using oligonucleotides for amplification of the putative downstream regulated genes in 35S::*OsSHN1*. Expression of *CER1* and *RD22* is induced in rosette leaves of 35S::*OsSHN1*, whereas no induction could be detected for *KCS1*.

Drought resistance by overexpression of the rice OsSHN1 in Arabidopsis

The Arabidopsis transformants overexpressing the *OsSHN1* gene (#1 and #16) together with the Arabidopsis *AtSHN1* overexpression line were used in a pot assay for drought resistance as described earlier (Aharoni et al., 2004). Whereas wild-type plants did not recover from the dehydration treatments longer than 13 days and completely dried out, all seedlings derived from lines expressing the *OsSHN1* gene recovered after rehydration to become greener and stronger (Figure 4). The drought resistance revealed in this test is equivalent to that shown by the Arabidopsis *SHN1* gene.

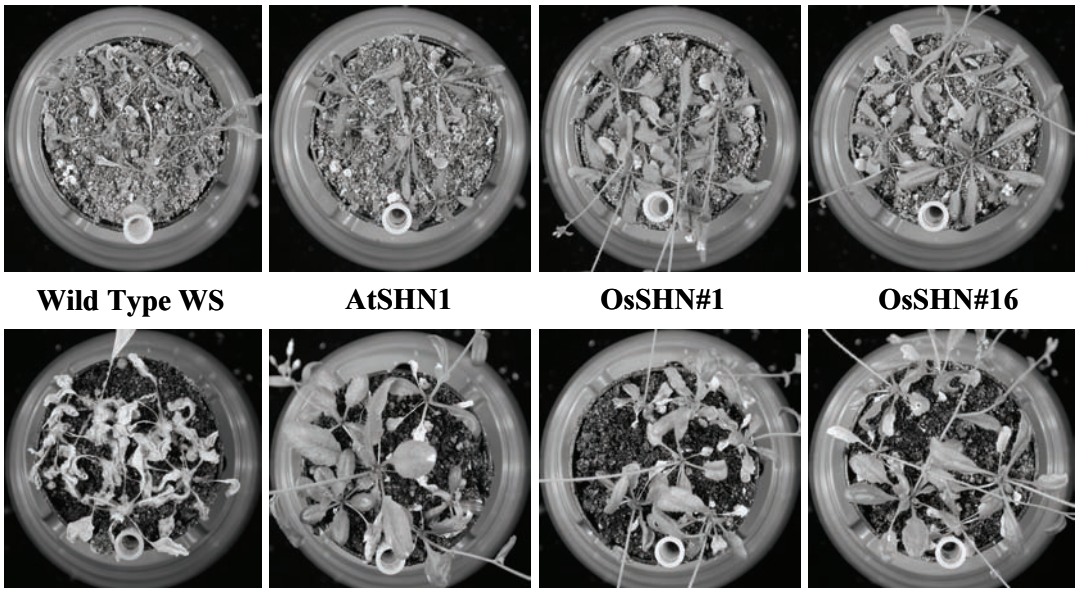


Figure 4. Drought Resistance Experiment with 35S::*OsSHN1* Lines.
Fifteen days old seedlings (6 seeds sown per pot) of either wild-type, progeny of two 35S::*OsSHN1* lines (#1 and #16) and the Arabidopsis SHN1 overexpressor, were exposed for a period of 13 days of dehydration as shown in the top row. Subsequently, plants were watered and their appearance after a week (recovery) is displayed in the bottom row. The 35S::*OsSHN1* and 35S::*AtSHN1* plants show 100% recovery and wild-type 0% recovery.

Spatial and temporal expression of OsSHN1 in rice

In order to examine the role and expression of *OsSHN1* in plants we generated a construct, which linked 1.38-kb DNA sequences upstream of the predicted ATG codon of the gene to the *β-glucuronidase* (*GUS*) reporter gene. In rice plants the *GUS* expression was detected in the germinating seedling (especially roots) and in the immature anther of the pre-

exsertion panicle (Figure 5). In addition wounded leaf tissue also displayed induced GUS expression. In Arabidopsis, the GUS expression of the OsSHN1 promoter was also seen in young seedlings and root tips and also in wounded stem tissue of older plants. The expression of the OsSHN1 thus is similar to the Arabidopsis SHN genes that display expression in protective tissue, e.g. after wounding and young tissue.

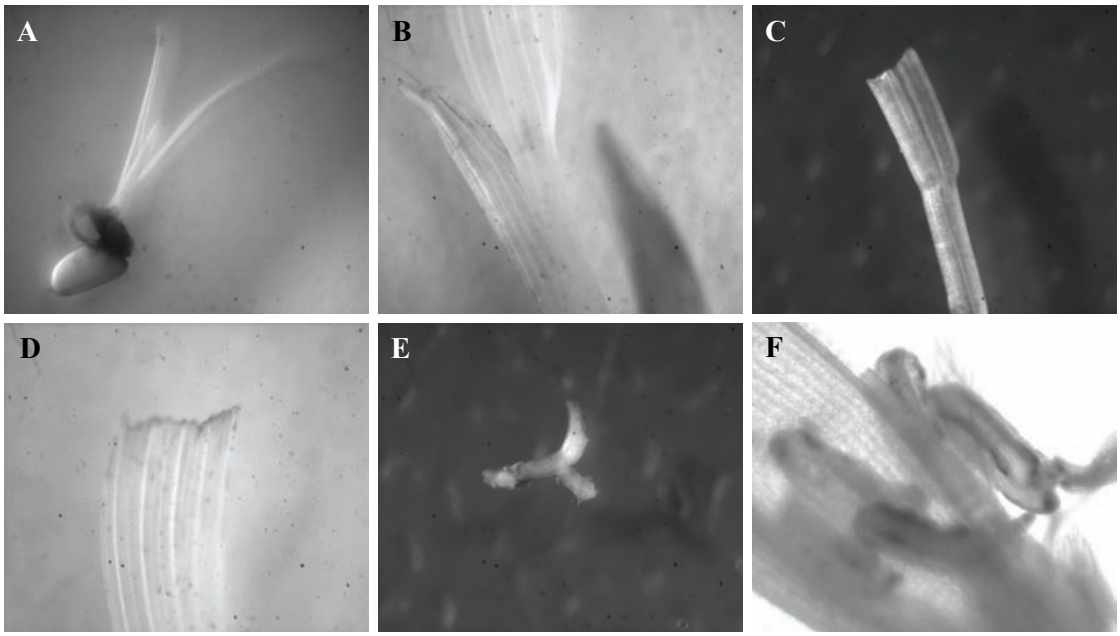


Figure 5. Expression Patterns of *OsSHN1* Detected in rice *OsSHN1* promoter::GUS Lines
(A) (B) (C) Leaf of young seedling
(D) Cut leaf of young seedling
(A) Root of young seedling
(B) Immature anther of pre-exserting panicle

DISCUSSION

We isolated one rice *SHINE* homolog, *OsSHINE1*, to assess and compare the function of Arabidopsis and rice SHINE clade members. Previously, we reported high similarity in the amino acid sequence of Arabidopsis SHN1, SHN2, and SHN3 and rice OsSHN1, most notably outside the AP2 domain (Aharoni et al., 2004). Recently, with a more complete rice genome sequence available, we identified another related rice AP2/EREBP protein sequence in the public database (OsSHINE2). This protein has also the complete mm domain that is only present in the SHINE clade. Comparing the gene structure among the three Arabidopsis and two rice proteins, all of them have two exons and a single intron. The intron of the three Arabidopsis genes is located 80 bp from the ATG start codon, whereas in their two rice counterparts the intron is positioned 83 bp from start codon. This is due to the additional amino acid lysine at position 5 of the rice proteins. The DNA sequence between the rice genes also shows significant similarity, suggesting that a gene duplication in rice or an ancestor gave rise to the two genes, rather than conserved sequences between dicots and monocots. This is supported by sequence similarities between the Arabidopsis genes that ranges between 80-87% in conserved regions. Independent gene duplication events from a common ancestor, in different plant lineages, thus seem to be the manner of evolution of this clade of transcription factors.

Previously, we also reported that plants overexpressing *SHN1*, *SHN2*, or *SHN3* showed an identical visual phenotype (Aharoni et al., 2004). On overexpressing the *OsSHN1* in Arabidopsis, we also obtained the same phenotype, including the more brilliant, shiny green color of rosette and cauline leaves, leaf curling, and altered silique length. We also detected increased cuticle permeability in the *OsSHN1* overexpressor, as demonstrated by the increased rate of chlorophyll leaching when immersed in ethanol, similar to the Arabidopsis activation tag *shn* mutant and the *SHN1* overexpressor.

Over-expression of the *WIN1/SHN1* in transgenic Arabidopsis plants activated the expression of a number of genes which are known to be involved in wax biosynthesis, like *CER1, KCS1,* and *CER2* (Broun et al., 2004, Aharoni et al., unpublished data). In this study we also found that the expression of *CER1* was induced in 35S::*OsSHN1* overexpressor, as shown by RT-PCR. This indicated a conserved regulation of downstream regulated genes involved in the lipid biosynthesis between Arabidopsis and rice SHN clade members.

In our previous study we could not obtain loss-of-function phenotypes for any of the three *SHN* genes when attempting to obtain plants silenced for *SHN1, SHN2, and SHN3* using the RNA interference approach. In another similar study on *WIN1*/*SHN1*, Broun et al. (2004) could not obtain fully silenced transgenics and also did not identify knockout lines in their > 100,000 T-DNA lines collection. The unavailability of loss-of-function mutants made the interpretation difficult of the true biological role of the SHN proteins. In this study, we used chimeric repressor silencing technology (Hiratsu et al., 2003) to overcome the probable redundancy problem between SHN clade members. We found that plants overexpressing the chimeric OsSHN repressor showed a loss-of-function mutant stem phenotype with reduced epicuticular wax (glossy green stem). Expression analysis by RT-PCR showed that the *CER1* gene was repressed in the 35S:*OsSHN1-SRDX* plants. These results support the idea that SHN proteins are regulators of Arabidopsis wax biosynthesis (Broun et al., 2004; Aharoni et al., 2004).

Over-expression of the *SHN1* in transgenic Arabidopsis plants resulted in higher resistance to drought, probably related to the reduced stomatal density (Aharoni et al., 2004). Over-expression of *OsSHN1* in transgenic Arabidopsis also enhanced drought resistance. It is probable that the reduction in the number of stomata that we also found in the *OsSHN1* overexpressors is responsible for this drought resistance. But we also found that overexpression of *OsSHN1* induced expression of *RD22*, a gene responsive to dehydration stress (Yamaguchi-Shinozaki and Shinozaki, 1993), as detected by RT-PCR. This indicated that other mechanisms are also probably involved in the enhancement of drought resistance by *OsSHN1* overexpression. In our microarray data, *RD22* is one of many abiotic stress-inducible genes up-regulated in transgenic *35S::SHN1* Arabidopsis (Chapter 3). It was reported previously that MYC and MYB recognition sites in the *RD22* promoter region function as cis-acting elements in the drought- and ABA-induced gene expression of *RD22* (Iwasaki et al., 1995, Abe et al., 1997). Based on our results, we propose that there is another cis-acting element in the *RD22* promoter region as binding site of SHN protein clade members. On the other hand, it is also possible that alteration in stomatal index or cuticular permeability has an effect on ABA signaling, that in turn enhances the expression of ABA-inducible genes.

The expression of *OsSHN1* in rice, as detected by the GUS expression pattern, is in the young seedling, mainly in the leaf and root, and in the immature anther of the pre-

exsertion panicle. We could not detect expression of *OsSHN1* in other tissue or stage. Thus *OsSHN1* is expressed in young tissue with a role in protective functions. These specific expression patterns correspond to publicly available rice MPSS data (Meyers and Wang, 2004). Interestingly, expression of *OsSHN1* is induced upon wounding. Among the three Arabidopsis *SHN* genes, *SHN3* is induced by wounding, suggesting a comparable role to *OsSHN1* in wounded tissue. The expression pattern of the *OsSHN2* gene would be interesting to compare to the patterns of the rest of the SHINE clade.

The many functions of the SHINE clade in Arabidopsis and rice seem to be conserved as revealed by overexpression phenotypes of the individual genes in Arabidopsis. However, the different expression patterns suggest different specific roles of the genes in various organs or induced conditions. In support of the proposed general role of the SHINE clade in plant protective functions involved in formation of protective layers (Aharoni et al., 2004), the clade members reveal specific roles that are reflected by the pattern of expression in the plant.

ACKNOWLEDGMENTS

We would like to thank N. Marsch-Martinez, S. Dixit and S. de Folter for helpful discussions.

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Chapter 6

Activation tagging using the *En-I* and *Ac-Ds* maize transposon systems in rice

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ABSTRACT

A collection of transposon *Ac*/*Ds* and *En*/*I* activation tag starter lines is being developed in rice that will contribute to the development of a rice activation tag population for identification and characterization of drought resistance genes in rice. Molecular analyses of *Ac*/*Ds* system revealed high transpositional activity, with 67% of the T_0 primary transformants showing ongoing active transposition. Independent transposition frequency varied between 33-85% among different independent transformants. Mobilization of *I* element in somatic sectors was demonstrated by sequencing of excision products in T_0 primary transformants, but Southern analysis indicated low transposition frequency of the *En*/*I* system. The genotypes produced here containing the actively transposing *Ac*/*Ds* system can be used to develop an extensive activation tag population of rice plants.

INTRODUCTION

The rice genome has been sequenced and provides us a resource to identify genes of biological interest and agricultural application (Goff et al., 2002; Yu et al., 2002; http://rgp.dna.affrc.go.jp/). Bioinformatics methods are being used to annotate the genes based on gene prediction programs and similarity to known genes. However, the association of the rice gene sequence to its precise function in the plant has to be made by experimental approaches.

Knockout mutagenesis of rice genes would provide us with a clue to their phenotype in a classical loss-of function approach. Classical knockout mutants or genetic variants can be obtained by natural variation, chemical mutagenesis or insertional mutagenesis. Insertional mutagenesis loss-of-function approaches in rice are presently focused on the generation of large populations of insertions, obtained by T-DNA, rice retrotransposons or maize *Ac*/*Ds* elements (Hirochika et al., 2004). More emphasis is now being placed in reverse genetics approaches where the rice DNA, flanking the insertion sites, is isolated, sequenced and compared to the rice genome. This approach uses Flanking Sequence Tags (FSTs) that can be used to place inserts on the rice genome sequence. This provides us with a map of rice genes with the associated insertions that enables one to search for insertion mutants in any gene of interest (http://orygenesdb.cirad.fr/).

The analysis of Arabidopsis genome by mutagenesis approaches has shown us that the functions of most genes cannot be revealed by loss-of-function approaches alone. This is mainly due to the high frequency (about 2/3rd) of genes that are redundant and thus do not display a mutant phenotype unless the duplicate/redundant genes are mutated too. On the other hand, activation tagging using a gain-of-function approach has been very productive in revealing mutant phenotypes of genetic/functional redundant genes or those that have small effects when mutated. Though, the analysis of developmental biological processes have been served well by analysis of single and multiple knockout mutants, analysis of genes involved in stress responses have been mainly amenable using gain-of-function approaches. Thus the first genes conferring stress tolerance, e.g. *DREB1*/*CBF* have been identified by overexpression of stress-induced AP2/ERF transcription factors in plants and examining the phenotypes in replicated stress tests (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999). These

reasons suggest that an activation tagging strategy would be useful to identify genes for stress tolerance in rice.

Activation tagging can be carried out by providing the insertion sequence with a strong enhancer (e.g. the constitutive CaMV 35S enhancer) that can activate genes at a distance and independent of orientation. Thus, in Arabidopsis activation tagging has been approached using either T-DNA carrying a 35S enhancer core or a maize transposon engineered to have a 35S enhancer (Weigel et al., 2000; Marsch-Martinez et al., 2002). The use of the transposon activation tag was shown to confer a ten times higher frequency of tagged dominant mutants, probably due to the simple structure of the insertion activation tag. The transposon system uses a method to mobilize the transposon containing the enhancer into different parts of the genome, and then segregate out the transposase responsible for the mobilization and thus generate transposon activation tags (ATag) that are stable in their genomic positions (Marsch-Martinez et al., 2002).

In rice, maize transposons belonging to the *Ac/Ds* and *En/I(Spm)* systems have been modified to be used as mutagens for rice (Greco et al., 2003; Greco et al., 2004), but not yet been demonstrated for activation tagging. The *Ac-Ds* transposon system comprises of the transposase *Ac* and a mobile component *Ds*, where the *Ac* is often modified to be immobile and under control of a strong promoter. The *Ds* component often contains a marker for selection (e.g. *BAR* gene conferring Basta herbicide resistance) and other components, e.g. a multiple 35S enhancer for activation tagging. To be able to segregate the immobile *Ac* transposase on the T-DNA and the transposed *Ds* bearing the Activation tag enhancer, an extra segregation marker (e.g. green fluorescent protein, GFP) is provided on the T-DNA that enables the selection of T-DNA and the *Ds* element separately. In a similar way to *Ac-Ds* the *En* (transposase)- *I* (mobile, ATag) transposon constructs are made. The mobile *I* and *Ds* transposons constructed for Activation tagging are termed *I*ATag and *Ds*ATag. In this paper, we developed *Ac-Ds* and *En-I* transposon tagging constructs for activation tagging, generated transformants in rice and analyzed transposition parameters to provide a resource to make large populations for activation tagging in rice.

MATERIALS AND METHODS

Vector construction

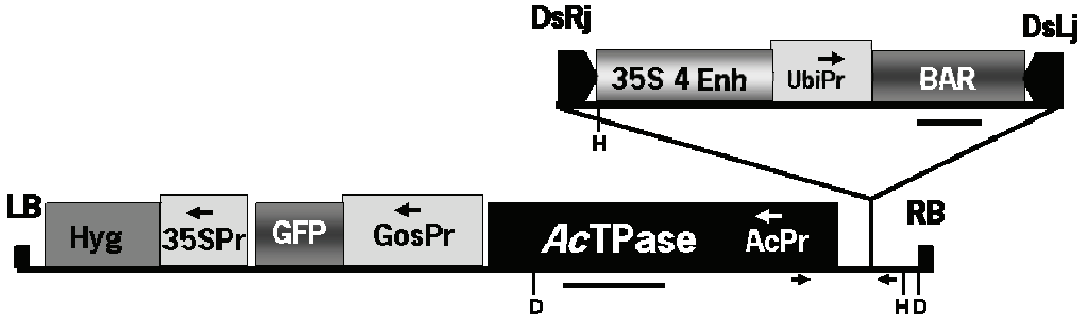
The immobilized *En* transposase driven by the CaMV 35S promoter was obtained from Aarts et al. (1995). The left (*I*-Lj) ends of the mobile *I*/*dSpm* element was derived from *Spm-I*6078, which was originally cloned from the *A1* locus of maize (Schwarz-Sommer et al., 1985), extends from position 1 up to the *Xba*I site at position 812. The right (*I*-Rj) ends of the mobile *I*/*dSpm* element was derived from *En*-1, which was originally cloned from the *Waxy* locus of maize (Pereira et al., 1985; GenBank Accesion M25427), extends from the *Nla*III site at position 7647 to the end of the element at position 8287.

The construct was made in a multiple fragment ligation from the components: (1) *Xho*I-*Not*I fragment containing the immobilised CaMV 35S-*En* transposase; (2) *Not*I-*Kpn*I fragment containing Gos2 promoter-*sGFP(S65T)*-RBSC terminator cassette originally derived from pBS-GOS/GFP (Langeveld, pers.comm.); (3) *Kpn*I-*Sst*I fragment containing the *I*-Rj fused to an intron-less Ubiquitin promoter- *BAR –Nos* terminator cassette originally derived from pAHC25 (Christensen and Quail, 1996); (4) *Sst*I-*Bam*HI fragment containing a tetramer of CaMV 35S enhancer (twice –392 to –90 and –526 to –90; Odell et al., 1985) fused to the *I* -Lj.

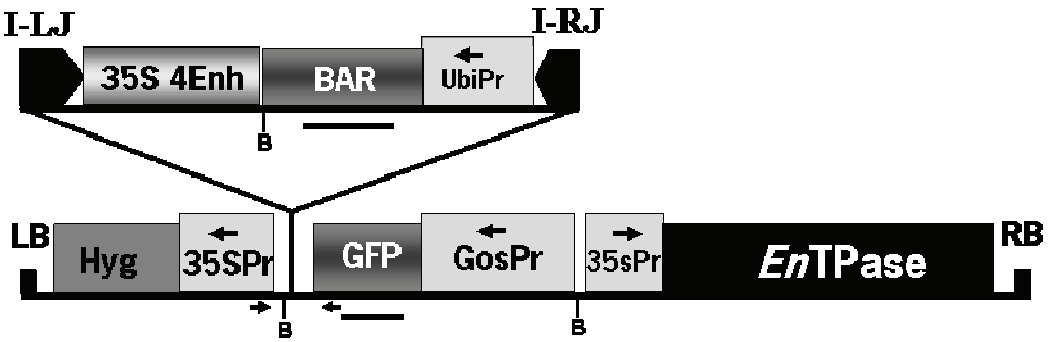
For the *Ac*-*Ds* construct, the immobile transposase extending from the *Bst*NI site at position 939 of *Ac* (Genbank Accession X05424) till the end of the element was obtained from a derivative of pKU2 (Baker et al., 1987). This *Ac* fragment was fused to *Ac* promoter fragment and subsequently excised as a unique *Apa*I-*Not*I fragment. The 5' terminus of *Ac* till the *Bal*I site at position 252 (*Ds* left junction) was fused to a intron-less maize Ubiquitin promoter-*BAR*-*Nos* terminator cassette, originally derived from pAHC25 (Christensen and Quail, 1996), and subsequently excised as a unique *Hin*dIII-*Spe*I fragment. A 0.94 kb fragment extending up to the *Bgl*II site in the first exon of the Ubiquitin promoter was used (Christensen and Quail, 1996). The 3' terminus of *Ac* from the *Pac*I site at position 4302 till the end (*Ds* right junction) was fused to a tetramer of CaMV 35S enhancer (twice –392 to –90 and –526 to –90; Odell et al., 1985), and subsequently excised as a unique *Spe*I-*Apa*I fragment. The humanized red-shifted GFP (sGFPS65T; Chiu et al. 1996) used as transposase

selection marker was derived as *Not*I-*Xho*I Gos2 promoter- *GFP* – RBSC terminator fusion originally derived from pBS-GOS/GFP (Langeveld, pers.comm.)

The constructs were assembled by multi-point ligation in the binary vector pMOG22 (Zeneca-MOGEN, Leiden, The Netherlands) containing the hygromycin phosphotransferase (HPT) gene for selection of plant transformants.



Ac-DsATag-Bar_gosGFP



En-IATag-Bar_gosGFP

Figure 1. Activation tagging constructs of the *Ac-Ds* and *En-I* transposon systems.
The marker genes (dark shaded) BAR (Basta resistance), Hyg (hygromycin resistance), GFP (green fluorescent protein) are driven by the promoters (light shaded) 35S and Gos with the arrows showing the direction of transcription. The positions of primers used for the EDS PCR are shown by arrows below the construct. For the Southern blots the restriction enzyme sites H (*Hind*III) and D (*Dra*I) used are shown, with the probes for hybridization shown as thick lines below the constructs. Other abbreviations are: RB and LB are the T-DNA right and left borders. Transposon termini *Ds*/*I* are labeled as LJ and RJ (left junction, right junction). The TPase denotes the *Ac/En* transposase and the 35S 4Enh denotes the tetramer of the 35S enhancer.

Plant transformation and growth conditions

Agrobacterium-mediated transformation of *Oryza sativa* ssp. *japonica* cv. Nipponbare, plant regeneration and growth were performed following as described in Greco et al. (2001b). The *Agrobacterium* strain AGL-1 was employed. Regenerated transgenic plantlets were transferred to the greenhouse and grown in hydroponic culture with a regime of 12 hours light, 28^0C, 85% relative humidity and 12 hours dark, 21^0C, 60% humidity.

DNA isolation and molecular analysis of transposition

Genomic DNA was isolated from leaves of plants according to Pereira and Aarts (1998). Regenerated transformants were first checked by PCR using hygromycin phosphotransferase (HPT) specific primers according to Greco et al. (2001b) to confirm the presence of the T-DNA. Excision events of *En-I* transformants were monitored by amplification of the empty donor site (EDS) using a primer annealing to the *sGFP-S65T* gene (sGFP-S65T forward: 5'-CGTCTATATCATGGCCGACAAGC-3') in combination with a primer annealing to the 35S promoter (35S-reverse: 5'-GATGAGACCTGCTGCGTAAGCC-3'). An amplification product is detected only if *I*/*dSpm* excised from the T-DNA. To confirm excision, the EDS fragments were cloned in pGEM-T Easy (PROMEGA) and sequenced.

The full donor site (FDS) corresponding to *I*/*dSpm* at its original position in the T-DNA was also examined. The FDS-left junction was amplified using primers from the 5'region of *I*/*dSpm* (*I*/*dSpm* forward: 5'-AAGCGTCGGTTTCATCGGGAC-3') and the 35S promoter (see above).

Excision events of *Ac-Ds* transformants were monitored by amplification of the EDS using a primer annealing to the RB-pMog22 (RB-pMOG22 forward: 5'-GGAAACGACAATCTGATCTCTAGG-3') in combination with a primer annealing to the *Ac* promoter (*Ac*-prom reverse: 5'-CTCAGTGGTTATGGATGGGAGTTG-3'). An amplification product is detected only if *Ds* excised from the T-DNA. To confirm excision, the EDS fragments were cloned in pGEM-T Easy (PROMEGA) and sequenced.

Excision and reinsertion events on *En-I* and *Ac-Ds* transformants were monitored by Southern analysis according to Greco et al. (2001b). For *En-I* transformants, about 2 μg of *Bam*HI –digested DNA from different regenerant lines were used. The same blots were

hybridized with different probes, after stripping of the first probe by boiling in 0.1% SDS. Excision events were monitored by hybridization with the *sGFP-S65T* gene (Fig. 1), which is expected to detect a 3.6 kb EDS fragment in case of excision and/or a 5.8 kb FDS fragment in case of *I*/*dSpm* still residing in the donor T-DNA. Transposed *I*/*dSpm* elements were expected to be detected as fragments hybridizing to a BAR probe with a minimum size of 2.5 kb.

To detect excision on *Ac-Ds* transformants, DNAs from different regenerant lines were digested by *Dra*I and the blot was hybridized with a 1.6 kb fragment from the 5' region of *Ac* (Fig 1), which is expected to detect a 3.8 kb EDS fragment in case of excision and/or 7.6 kb FDS fragment in case of *Ds* still residing in the donor T-DNA. To detect reinsertion of the *Ds* element, the DNAs were digested by *Hin*dIII and the blot was hybridized with the BAR probe (Fig. 1). Transposed *Ds* elements were expected to be detected as fragments hybridizing to a BAR probe with a minimum size of 3.6 kb.

RESULTS

Transformation of the transposon activation tags in rice

Transformation of rice cultivar Nipponbare resulted in 16 *Ac-Ds*ATag and 3 *En-I*ATag independent transformants. Each transformed callus line produced a number of regenerants that were also grown to plants as these would provide independent regenerants per callus that would be useful to study transposition parameters. The plants were grown to maturity in the greenhouse and used for molecular analysis. The progeny of some were screened at seed level for GFP to check for efficacy of the GFP marker that showed expected segregation ratios. Figure 2 shows a germinated seed with a high level of GFP activity. This is also visible under a hand held light source with spectacles bearing filters to observe GFP.

Excision of DsATag in rice

A number of the transformants containing the Ac-DsATag-BAR_gosGFP were analyzed for presence of different parts of the construct, e.g. *Ds* LJ and RJ, GFP and BAR. The transformants were then screened with primers to reveal the Empty Donor Site (EDS) indicative of transposon excision. A number of regenerated plants from different calli showed a few different EDS fragments based on sequencing as shown in Figure 3, indicating excision

of *Ds* from within the construct. The footprints left behind after excision were as expected, with small deletions or nucleotide changes at the target site, proving excision of the *Ds*ATag construct at the molecular level. However, PCR based screening for EDS in all plants was not very indicative as there were also other artefact fragments observed due to the repeats within the constructs.

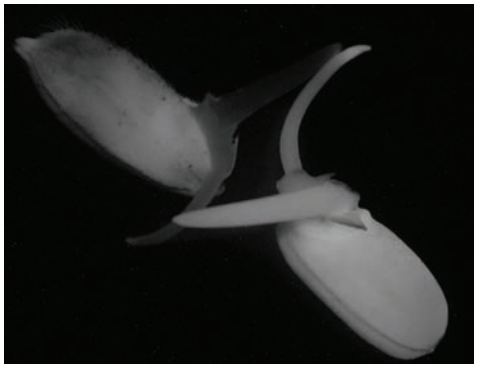


Figure 2. GFP activity of a transposon marker for segregation.
Young germinated seedling showing GFP activity that can be used for screening for T-DNA containing and free plants before transfer to the greenhouse.

To reproducibly screen for excision in a large number of plants Southern blot analysis was chosen. The DNA from 15 independent transformed calli with a total of 24 regenerants was analyzed by restricting with *Dra*I that cuts in the polylinker near the T-DNA RB and another site at position 3622 bp in *Ac*, as shown in Figure 1. The blot was hybridized with an internal *Ac* probe (*Hin*dIII 1.6 kb fragment) that reveals a 7.6 kb Full Donor Site (FDS) fragment and a 3.8 kb EDS in some plants. The results of the hybridization for 24 regenerants from 15 independent transformed calli (numbers shown in blot) are shown in Figure 4. At least 10 transformed lines out of the 15 display a clearly visible EDS indicative of excision. In some plants no FDS is visible, e.g. line 3.1E, suggesting early excision in the regenerant, but with an FDS present in another regenerant 3.1D from the same callus. Other lines with difference in EDS:FDS intensities are lines 18 and 21. The difference in relative intensity in the plants suggests that *Ds*ATag excision is variable and late in most of the plants as the FDS is visible.

FDS

TGGGGCGCGTTGCGTGACC GCGTGACCCGGCCGCGCGGATCC

EDS 22_Pl2

TGGGGCGCGTTGCGTGACGCCGTGACCCGGCCGCGCGGATCC

EDS 22_Pl37

TGGGGCGCGTTGCGTGAC GGCCGTGACCCGGCCGCGCGGATCC

EDS 22_Pl23f

TGGGGCGCGTTGAGTGACACCGTGACCCGGCCGCGCGCCGGGATCC

Figure 3. Excision of *Ds* from the activation tag construct.

DNA sequencing of the EDS fragments in transformants reveals excision of *Ds*. The FDS sequence is shown above with the 8-nucleotide target site duplication flanking the *Ds* insertion site (indicated by the gap between the underlined nucleotides). The EDS in different plants show a footprint as changes in nucleotide sequence along with the absence of the *Ds* element.

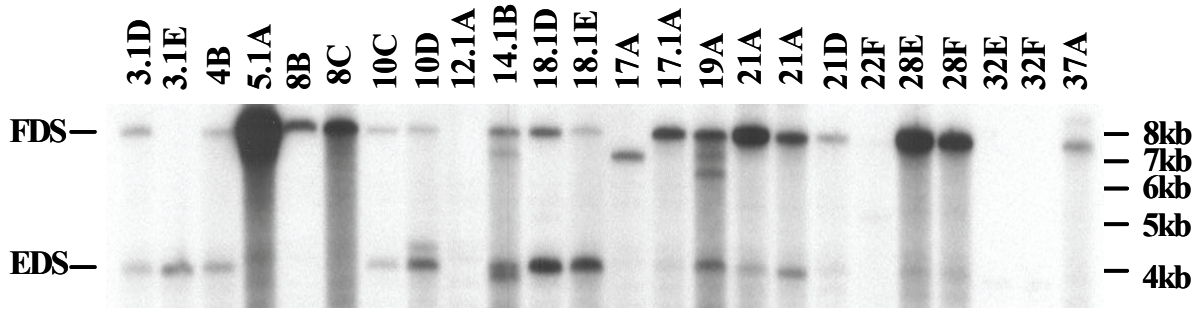


Figure 4. Excision of *Ds* revealed by blot hybridization.

DNA from 24 *Ds*ATag transformants was digested with *Dra*I and the blot hybridized with an *Ac*-transposase probe that reveals a 7.6-kb FDS and a 3.8-kb EDS.

Transposition of DsATag in rice

Important parameters of transposition are the timing, re-insertion frequency and the frequency of independent transposition. These parameters were studied by DNA blot hybridization of 48 regenerants from 16 independent transformed calli. The results of 15 regenerants of 5 transformed lines are shown in Figure 5. The blot contains DNA from the transformants restricted with *Hin*dIII that cuts once within the *Ds*ATag (Figure 1), and hybridization with a BAR probe reveals insertions in the genome. A common band visible is that of the FDS, with the *Hin*dIII site very close to the transposon end, thus most new insertions having reinsertion fragments that are larger than this 3.6 kb FDS fragment.

In Figure 5, five transformed lines are shown (lines 3, 4, 8, 10, 14) with a few regenerants per line in order to compare the transposition pattern. Each plant shows a number of transposed *Ds*ATag inserts as revealed by differently sized fragments. The insertion positions differ between plants in a line indicating transposition later in the callus or in the regenerated plants. In addition the intensity of the fragments with lower than single copy insertion intensity and variable intensity also indicate later transposition events. Late transpositions are more likely to give independent transpositions in the progeny.

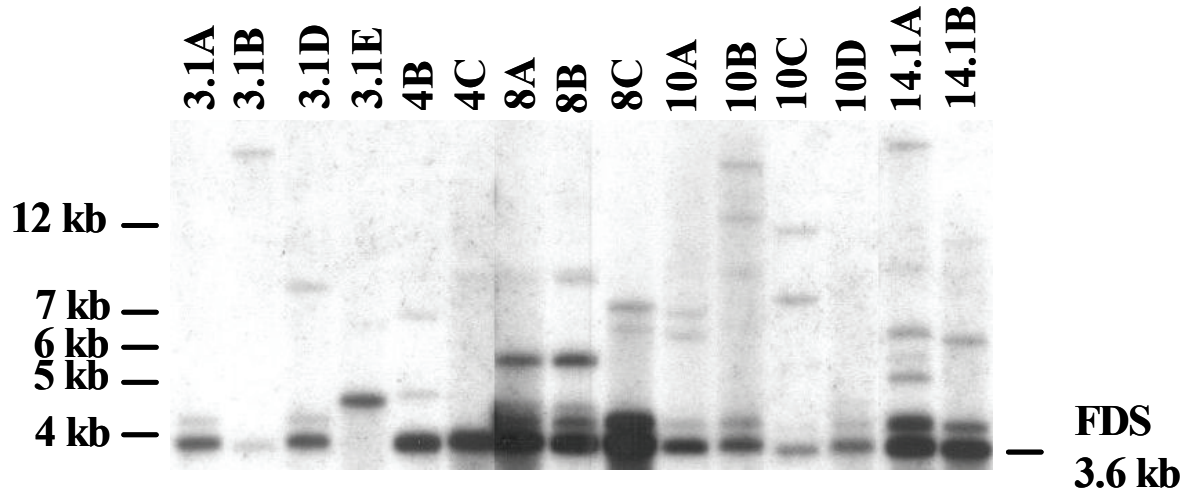


Figure 5. Transposition of a *Ds*ATag in rice.
Southern blot hybridization of five transformed lines, restricted with *Hin*dIII and hybridized to a BAR gene probe. The common 3.6 kb fragment is the FDS fragment of *Ds*ATag at its original position in the T-DNA, the other fragments of variable size and intensity are transposed *Ds*ATag insertions in the rice genome.

To calculate the frequency of independent transposition events as a parameter for tagging, we determined the Independent Transposition Frequency (ITF) as described before in Arabidopsis and rice (Aarts et al., 1995; Greco et al., 2001b). For this the Southern blots were evaluated for the number of unique inserts in a family of T0 regenerants from an independent transformed line. Each plant has a number of reinsertions after excision suggesting a high level of excision. The variable intensity of the reinsertion fragments indicates that transposition is still going on in the plant. The frequency of unique to total inserts in a family indicated the ITF. The high ITF has been seen before in primary transformants with the *Ac-Ds* systems (Greco et al., 2003), often due to high transposition in the early callus phase.

Table 1. Independent transposition frequency (ITF) of *Ac-Ds*ATag transformants

	Transformed line							
	3.1	4	8	10	14.1	18.1	21	22
Total number of plants	4	2	3	4	2	5	3	5
Plants with uniqe inserts	4	2	2	4	2	1	3	3
Total number of inserts	6	5	11	11	11	3	13	7
Uniqe inserts	5	4	6	8	7	1	11	4
ITF[a]	83%	80%	55%	73%	64%	33%	85%	57%

[a]The ITF is calculated as the percentage of unique inserts amongst the total number of inserts

Excision analysis of En-IATag transformants

A number of transformants were obtained with the *En-I*ATag construct and transferred to the greenhouse for further analysis and seed set. DNA isolated was used for Southern blot hybridization and PCR analysis. PCR for the excision produced EDS fragments that were cloned and sequenced. EDS fragments were sequenced from 2 plants and revealed 5 different EDS sequences as shown in Figure 6. These EDS fragments displayed the footprints characteristic of excision and indicated frequent and late excision in the plants.

FDS

GGCCGGATCTACCTCA TATATACGAATC

EDS Pl1_Cl1, Pl3_Cl1, Pl3_Cl2

GGCCGGATCTACCT_____ACGAATC

EDS Pl1_Cl2

GGCCGGATCTACCTCA_____CGAATC

EDS Pl1_Cl3, Pl1_Cl4

GGCCGGATCTACCT____ATATACGAATC

EDS Pl3_Cl3

GGCCGGATCTACCTCA___TATACGAATC

EDS Pl3_Cl4

GGCCGGATCTACCT_____ATACGAATC

Figure 6. Excision of *I*ATag revealed by sequencing of the EDS fragments.
DNA sequencing of the EDS fragments in transformants reveals excision of *I*ATag. The FDS sequence is shown above with the 3-nucleotide target site duplication flanking the *I*ATag insertion site (indicated by the gap between the underlined nucleotides). The EDS in different plants show a footprint as changes in nucleotide sequence along with the absence of the *I*ATag element

DNA from a number of regenerants was used for Southern blot hybridization experiments to reveal transposition. The DNA was restricted with *Bam*HI that cuts within the *I*ATag element (Figure 1), the blot hybridized to a GFP gene probe to reveal excision as shown in Figure 7. The expected sizes of the FDS is 5.8 kb and for EDS 3.6 kb. Three transformed lines (1, 2, 3) with two regenerants each are shown. Line 2 shows truncation as multiple fragments but none of expected size observed. Lines 1 and 3 show s distinct FDS fragment and a low intensity EDS fragment of 3.6 kb. This low intensity indicates late

excision, confirming the results of the EDS fragment sequencing. A fragment larger than the expected FDS was due to partial digestion with the *Bam*HI enzyme. The same blot was hybridized with a *BAR* gene probe to reveal *I*ATag reinsertion, but no distinct reinsertion fragments were observed, probably due to low intensity below detection, and suggesting late or multiple transposition.

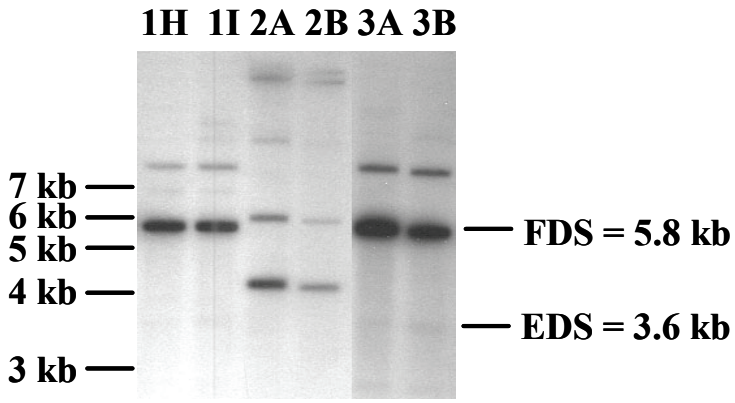


Figure 7: DNA blot hybridization showing excision of *I*ATag in rice.
DNA from 6 *En-I*ATag regenerants of three transformed lines was digested with *Bam*HI and the blot hybridized with a GFP probe that reveals a 5.8-kb FDS and a 3.6-kb EDS of low intensity in lines 1 and 3.

DISCUSSION

Transposition of the *Ac-Ds* system has been shown in rice many times previously but no effective system for activation tagging has been described (Hirochika et al., 2004). Similarly the *En-I* system has recently been shown to work in rice (Greco et al., 2004) albeit at a low frequency. In this manuscript we developed vectors for activation tagging with the two systems using the concept of two components (transposase and mobile element) in one vector (Greco et al., 2001a). This two component system is useful for plants that cannot be crossed easily, as is for rice in greenhouse conditions in Europe. The two component constructs also allow rapid evaluation of the construct and transposition parameters.

We demonstrate here that the *Ac-Ds*ATag construct displays active transposition of the *Ds*ATag insert in rice. This is in contrast to earlier efforts from our lab, where we found constructs with many repeats (of 35S Enhancer and transposon ends) were very unstable and did not give transformants with complete inserts in them. The conformations of the vectors we chose here have minimal repeats, we used the Gos promoter to drive the GFP, the 35S promoter for hygromycin and Ubiquitin promoter for BAR. In addition we chose the orientations so that the transposon ends do not form adjacent inverted repeats. This is in particular with the *Ds* construct that has the homologous *Ds*-LJ and *Ac*-promoter separated in the construct. In earlier constructs we observed that when the homologous transposon ends were adjacent and in a repeat orientation to each other, there was no transposition detectable, probably because of silencing due to the inverted repeat ends. For the *En-I* system we use the 35S-*En* transposase that does not have the *I*-transposon homologous termini. In an earlier version of the *En-I* constructs (Greco et al., 2004) we had a smaller ~200bp fragment of the *I*-LJ, to minimize homology to the *En*-transposase part. That construct did not reveal high transposition, so we have increased the *I*-LJ to about 800bp in the construct reported here. This should have all the cis-sequences necessary of *I*-element transposition. However, this introduces more homology to the *En*-transposase gene fragment used and might affect transposition. In fact though we observe excision with the *En-I*ATag constructs with many different excision events per plant, the total level of excision estimated by Southern blot hybridization was not more than 10%. Thus, the total level of excision containing cells is still low and probably can be improved by reducing the part homologous and repeated between the *En* and *I* components.

The strategy for use of the vectors is to produce transformants and use the immediate progeny or advanced generations to select for transposed ATag inserts that are stable, due to segregation of the transposase. To segregate the transposase, we have employed a GFP marker on the T-DNA that can be used for screening for GFP+ progeny that contain the transposase and GFP- progeny that lack the transposase. We show that the GFP can be used at freshly germinated seed to screen for fluorescence. The mobile *I/Ds*ATag transposon also contains a *BAR* gene that confers Basta resistance that can be screened for using spraying with the herbicide or even by painting on the leaves. The GFP+BAR+ progeny of transformants contain both the transposase and the mobile transposons, and can thus be used to propagate the lines before selection of transposition. To select for stable transposition, the GFP- seedlings can be transferred to the greenhouse and sprayed with Basta to select the BAR+ seedlings. Thus the GFP-BAR+ seedlings selected are new transposants of the *Ds/I*ATag elements. A population of these stable ATag inserts can be thus made in a high throughput way in the greenhouse and screened for mutant phenotypes. At present a small collection is being made to identify stable transposants and calculate efficiency of generating dominant mutants.

An activation tag collection in rice has been generated using the *Agrobacterium tumefaciens* T-DNA as a tag (Jeong et al., 2002). Out of ten lines investigated four were found to activate the gene near the tetrameric enhancer at a range of 1.5-4.3 kb. Dominant mutations were recovered at a frequency of 0.3%. The current population size of this collection is 50 000 activation lines (Hirochika et al., 2004). However, the use of T-DNA has the disadvantage of the creation of complex integration patterns and associated methylation of the enhancer sequences that reduce the frequency of stable and active activation tag inserts (Chalfun-Junior et al., 2003). In addition, because rice transformation is absolutely dependent on tissue culture, a high degree of background mutations due to somaclonal variation is expected for T-DNA insert collections, that have to be screened for segregation of mutant and phenotype.

To make a population of activation tag inserts to saturate the genome, a lesser number would be required than that required for knockout mutants. The 35S enhancer of the activation tag has a general range of enhancement of about 5-10 kb both sides of the enhancer, thus an insert placed every 10 kb in the genome would be able to activate most genes. We estimated the number of independent insertions required to activate every gene by using the

formula $P = 1 - (1 - d/S)^{nf}$ (Hirochika et al., 2004), where P is the probability of tagging a gene ($P = 0.95$), S is the genome size ($S = 430\ 000$ kb; Arumuganathan and Earle 1991), d is distance between two insertions ($d = 10$ kb), f is the average number of insertion per genome ($f = 1$), the number of insertions (n) can be calculated. A total of 128 815 insertions are required to get inserts near every gene with a 95% probability. This could be made in international collaboration and provided for screening for different phenotypes of interest, e.g. drought and salt stress resistance or for grain quality traits.

ACKNOWLEDGEMENTS

We thank R. Greco and P.B.F. Ouwerkerk for helpful discussions.

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Chapter 7

General Discussion

Drought Resistance

Despite the general awareness of the economic magnitude and social importance of drought in rice, breeding for drought resistance has not been a part of the mainstream of rice improvement programs for decades (O'Toole, 2004). This is partly due to the lack of knowledge about the genetic control of drought resistance mechanisms. The rise of rice as a model plant, the availability of its genome sequence and genomics resources, and the development of advanced analytical tools at the molecular level, however, are believed to provide a basis for understanding the mechanisms of drought resistance. The aim of the research described in this thesis was to use the genomics information and tools from the models Arabidopsis and rice to better understand the genetic mechanisms controlling drought resistance in rice.

Drought resistance is a complex trait. Important mechanisms of drought resistance include at least the following four aspects: (1) drought escape via a short life cycle and developmental plasticity, (2) drought avoidance via enhanced water uptake and reduced water loss, (3) dehydration tolerance via osmotic adjustment and antioxidant capacity, and (4) drought recovery via desiccation tolerance (Fukai and Cooper 1995, Blum 1988). The use of such putative traits to improve grain yield under water-limiting conditions has been demonstrated in maize (Fischer et al., 1989).

Grain yield and its components are important traits for breeders since the ultimate goal in breeding programs is to obtain a high and stable yield. The susceptibility of rice to water stress is more pronounced at the reproductive stage and causes the greatest reduction in grain yield when stress coincides with the irreversible reproductive processes (Matsushima 1966, Cruz and O'Toole 1984).

Heading date (flowering time) has been known as a major determinant of grain yield and its components under drought conditions (Fukai et al., 1999). Any attempt to successfully dissect drought resistance, especially late season drought, needs to minimize the effect of the flowering time. In the strategy followed (Chapter 2), staggered planting date was effective in synchronizing the flowering of test lines during the stress treatment period, as shown by the

absence of any major flowering time-related region associated to grain yield under stress. Elimination of the major effect of variable flowering time, revealed a specific region on chromosome 1 close to the *sd1* locus controlling grain yield under stress and leaf rolling. Comparison with two other populations (Courtois et al., 2000, Price et al., 2002) indicated that this QTL region controlling leaf rolling was conserved across different genetic backgrounds. Leaf rolling has been considered as a good indirect estimate of drought avoidance in rice (O'Toole and Moya 1978). Interestingly, osmotic adjustment capacity was also mapped in the similar region (Robin et al., 2003). Osmotic adjustment capacity allows turgor maintenance as leaf water potential falls under drought. It also helps to delay leaf rolling, yellowing, and death in rice under water stress (Nguyen et al., 1997). The next step is to prove the hypothesis that these QTLs controlling drought avoidance or tolerance are at the *sd1* locus, by conducting fine mapping, a complementation test, or phenotypic analysis of several knock-out mutants in this region.

Drought resistance is a quantitative trait controlled by many genes. Genetic engineering for drought resistance has been seen skeptically, because its limitation to transfer only one or a few genes. The overexpression of a single gene encoding a transcription factor, however, can activate the expression of many target genes encoding proteins that are needed by the plant to survive during drought stress. This has been pioneered by overexpression of the DREB1/CBF AP2/ERF transcription factors conferring drought tolerance in Arabidopsis (Kasuga et al., 1999). Transcription factors are now a powerful tool to engineer stress resistance in plants, as well as to understand the fundamental physiological and molecular basis of drought resistance.

In a similar way rice plants overexpressing the SHINE AP2/ERF transcription factor showed enhancement of drought resistance. In contrast to the production of epicuticular wax in Arabidopsis, rice SHINE overexpressor plants do not produce any visible wax. This essential result helps to dissect the process of epicuticular wax and drought resistance in SHINE overexpressing plants, indicating that epicuticular wax is probably not essential for drought resistance. On the other hand, the chlorophyll leaching experiments indicated an alteration in the cuticle of the rice SHINE overexpressor that also displays a significant reduction in stomatal density. Changes in the cuticle due to SHINE overexpression might influence epidermal cell differentiation that brings about a reduction in stomatal density.

Evapotranspiration studies on rice revealed that from 759 to 1,150 kg of water is required to produce 1 kg of rough rice grain (Shih et al., 1982). Because water is limiting in rainfed environments, a desirable trait for rainfed adapted rice cultivars is to have efficient water use. An increase in water use efficiency indicates that more carbon can be accumulated for growth with the use of less water. The drought resistance of rice SHINE overexpressors might be because of using less water for transpiration due to the reduced stomatal density. The next step should be evaluation whether this reduction of stomatal density limits growth and grain yield. A field test comparing grain yield between rice SHINE overexpressors and the wild type under drought and control conditions would be appropriate. This would help resolve if there would be a yield reduction due to the reduction in stomata and reduced photosynthesis. To ensure that there is minimal yield reduction, the SHINE gene can be introduced into plants with a stress inducible promoter that would reduce stomatal frequency in drought affected environments and not in conditions of normal water availability.

Global expression analysis of drought associated genes

The primary discoveries of stress associated genes were made by identifying genes or proteins that were induced under specific stress conditions. These included genes like *rd, erd, kin, lti, cor* and others, that finally became a signature describing the stress response. The analysis of these genes was initially at the individual gene level using Northern blots and gradually moved towards describing the changes in expression using microarray experiments.

The studies on altered transcription of genes in a stress related process becomes more relevant when analyzing the effect of transcription factors on stress resistance. Transcription factors act through binding to the *cis* elements in the promoter of the target genes and activate or repress the expression of the gene. Transcript profiling studies on drought tolerant genotypes, overexpressing TF genes, can be useful to identify downstream regulated genes of the TFs. Comparison of the target genes of different TFs can unravel the similarity and difference of pathways that the TFs might be involved. Chapter 3 described whole-genome microarray analysis on three Arabidopsis genotypes overexpressing genes encoding different AP2 transcription factors, i.e. DREB1A, SHINE, and HARDY (Kasuga et al., 1999, Aharoni et al., 2004, Dixit et al., unpublished). HARDY showed a similar pattern to DREB1A in the target up-regulated genes comprising a large number of genes that are known to be involved in stress responses. It seems that SHINE affects a different pathway, mainly on wax

biosynthesis even though a limited number of stress regulated genes were also induced. Study on the SHINE dominant repressor (Chapter 5) confirmed the involvement of SHINE proteins on wax biosynthesis. The different pathways affected by these three TFs open a possibility to combine a number of them in one plant in order to provide resistance that is more sustainable.

Microarray analysis on drought-treated wild type Arabidopsis plants showed that some genes encoding enzymes involved in wax biosynthesis were induced in drought stress, i.e. *CER1*, *CUT1/CER6*, *KCS1* (Chapter 3). On this basis it may be deduced that these drought-regulated genes likely function in drought tolerance in Arabidopsis. Activation of the wax biosynthesis pathway by overexpressing SHN in other plants like rice having low wax, would be expected to enhance drought resistance. But overexpression of SHINE in rice did not increase the epicuticular wax to detectable levels, nor did it change the expression level of three putative wax *OsCER1* genes (Chapter 4), indicating that rice probably lacks other functional genes required to activate wax biosynthesis genes like *OsCER1*. The lack of other essential genes might explain the very low level of epicuticular wax in rice compared to other cereals. Probably early in the evolution and selection of rice for water submerged conditions and high humidity, epicuticular waxes might have lost their importance and not be selected for increased crop fitness. However, with the reduced irrigated area due to pressure of population growth and industrialization, rainfed areas will play an important role to increase rice production, and the importance of epicuticular wax will grow. It has been shown that the leaves of upland rice have higher epicuticular wax content than the leaves of lowland rice (O'Toole et al., 1979), indicating that epicuticular wax is an important component of rice adaptation to the upland ecosystem.

Despite the volume of literature available on rice physiology, a substantial gap exists in our understanding of rice responses to water deficit (Lafitte 1999). We do not know whether the basis of rice's sensitivity to drought stress lies in hormonal signals, carbohydrate metabolism, or other processes. Exploitation of the wealth of information available in Arabidopsis can be very useful for comparison, especially from expression profiling studies (Chapter 3). Microarray analysis of drought-treated wild type plants showed the induction of genes encoding proteins involved in the biosynthesis of various osmoprotectants, proteins that may protect macromolecules and membranes, proteases for protein turnover, the detoxification enzymes, protein kinases, and transcription factors.

Comparison of drought-induced genes and target genes induced by the three different AP2 TFs showed co-regulation of some genes. This overlapping set of genes support their association with drought response, signaling and resistance. Comparison of amino acid sequences between these Arabidopsis genes and rice genes showed variable levels of similarity (Table 1). It was relatively easy to find the putative orthologs of some genes encoding transcription factors, protein kinases, and enzymes. Conversely, it was very difficult to identify putative orthologs of most of the genes encoding general stress response proteins, e.g. *rd* and *cor*. The putative orthologs of some drought-related genes in rice are shown in Table 1. Overexpression analysis would be a convenient way to validate their function and conservation between plants.

Gain-of-function approaches

In simple eukaryotic models like Drosophila and Neurospora, suitable tools for genetic dissection of complex processes like development are loss-of-function knockout mutations. These mutants either spontaneous or induced, were also useful to dissect processes dealing with visual phenotypes like those determining color in plant genetic models like maize, tomato, and petunia. For a more complex process like plant development the switch to a genomics and genetics model, Arabidopsis was more appropriate.

However, when the efforts intensified to unravel the function of every gene using a variety of functional genomics and reverse genetics methods, it was evident that not all gene functions could be determined by loss-of-function approaches alone. This was mainly due to the genome redundancy, discovered by sequencing (Arabidopsis Genome Initiative, 2000), as a majority of knock-out mutants did not reveal a mutant phenotype. In addition many phenotypes of complex processes were very subtle and difficult to screen for on a single plant basis.

There have been many attempts to dissect the processes of stress biology with knockout mutants identifying a few genes but a thorough analysis using this approach alone was not satisfactory (Pereira, 2001). The breakthrough came in overexpression analysis of the *DREB1*/*CBF* genes of the AP2/EREBP transcription factor family that revealed stress tolerance (Jaglo-Ottosen et al., 1998., Kasuga et al., 1999). This approach opened the way for large scale systematic overexpression analysis of transcription factors (Mendel Biotechnology, Broun et al., 2004) to reveal their role in complex processes. To date,

knockout mutant analysis of these stress associated AP2 genes has not yet revealed a significant phenotype. Taken together, gain-of-function approaches are valuable to understand stress associated gene functions.

Following this line of reasoning an overexpression strategy was developed using transposon based activation tagging in Arabidopsis (Marsch-Martinez et al., 2002). This led to the screening and identification of Arabidopsis genes conferring stress resistance (Aharoni et al., 2004; Dixit et al., unpublished). The SHINE clade of genes in Arabidopsis, hence, provided a new set of genes in Arabidopsis and rice to study stress resistance mechanisms.

To extend the overexpression approach to other plants like rice, one could overexpress all transcription factors, regulatory and other signaling genes and test the phenotypes, but this can be a very tedious and expensive approach. An overexpression high throughput strategy using transposon-based activation tagging can be an efficient alternative. Chapter 6 describes the development and transformation of *Ac-Ds* and *En-I* based activation tagging constructs into rice and evaluation of their activity in primary transformants. As shown in previous studies (Greco et al., 2003, Greco et al., 2004) the *Ac-Ds* system transposes actively but the transposition of the *En-I* system occurs at a low efficiency. The genotypes produced with the actively transposing *Ac-Ds* system can be used to develop an extensive activation tag population of rice plants. The choice of BAR as positive marker and GFP as negative screenable marker enables the efficient generation for independent transposants.

A parallel approach can be used in gene-by-gene overexpression analysis of candidate genes in rice. These candidates (Table 1) have been identified as closest orthologs of Arabidopsis stress associated genes. Another approach was used to make a dominant loss-of-function mutant of the *SHINE* gene (Chapter 5) and thus to get a mutant phenotype that supports the overexpression phenotype. This dominant loss-of-function approach, by making a protein fusion to a transcriptional repressor domain, can be extended to other transcription factors whose overexpression phenotypes have been described and thus avoid the problems of genome redundancy.

Chapters 4 and 5 describe conservation in the phenotype on overexpression of the SHINE clade members between Arabidopsis and rice. The Arabidopsis SHINE protein retained its function when overexpressed in rice and *vice versa*. Given this, it can be concluded that gain-of-function is also a suitable method to check conservation of gene

function between species. Thus information gained in one species can be translated to the other one.

Conclusions and perspectives

Microarray analysis revealed a variety of genes that were differentially expressed in drought. This affirmed the complex nature of plant responses to drought. Nevertheless not all of these genes might be essential for drought resistance; many of them are probably direct or indirect consequences of stress damage (Zhu et al., 1998). Therefore, the challenge remains to identify the genes and cellular processes crucial for plant drought resistance.

A high throughput overexpression approach using activation tagging has successfully identified Arabidopsis genes conferring drought resistance. Some of these genes are AP2/ERF transcription factor genes. Microarray analysis of the two AP2/ERF overexpressors, together with the well studied stress-related AP2/ERF DREB1A overexpressor, has shown that they are involved partially in different pathways, with some overlap in differential gene expression patterns. Overexpression of one of these genes in rice, in this thesis, has conferred drought avoidance.

To get additional useful genes to improve drought resistance in rice, some parallel approaches can be used. One is the positional candidate gene analysis based on fine mapping of QTLs for drought resistance components. Second, is a high throughput overexpression strategy using transposon-based activation tagging in rice. Finally, a gene-by-gene overexpression approach can be used for rice putative orthologs of Arabidopsis drought stress-related genes identified by microarray analysis.

Engineering a single pathway or mechanism may not be adequate to confer drought resistance in the field because products from several pathways might be required to ensure drought resistance (Ingram and Bartels, 1996). The AP2/ERF transcription factor genes, described here, can be used in combination together and with other genes to induce different pathways. 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 negative effects of the use of a constitutive promoter on growth and yield.

Due to its low transformation efficiency, *indica* rice is infrequently used in transformation research. But *indica* rice is more important economically than *japonica*. Therefore, transformation of these AP2/ERF genes and others into *indica* rice, such as the

high-yielding cultivar IR64 that is planted all over Asia, might have a significant impact on grain yield under drought in the farmer fields.

Table 1: Arabidopsis drought associated genes and their rice orthologs

Arabidopsis	Gene Description	DROUGHT1	DREB1A	HARDY	SHINE	CBF2	cDREB	Rice	P value
At2g34720	CCAAT-binding transcription factor (CBF-B/NF-YA)							Os12g41880	e-57
At3g15210	AtERF4, ethylene-responsive element-binding factor 4							Os01g58420	e-34
At3g23000	CBL-interacting protein kinase 7 (CIPK7)							Os03g43440	e-106
At1g13930	drought-induced protein SDi-6							Os10g18340	e-19
At5g52310	RD29A / COR78							Os04g52960	e-6
At1g20440	dehydrin COR47/RD17							Os02g44870	e-33
At1g20450	dehydrin ERD10 (LTI45)							Os02g44870	e-29
At2g23120	expressed protein (COR8.5)							Os09g30470	e-5
At5g50720	ABA-responsive protein (HVA22e)							Os11g30500	e-44
At1g78070	WD-40 repeat family protein							Os03g26870	e-47
At4g04020	plastid-lipid associated protein PAP / fibrillin							Os09g04790	e-91
At3g57020	strictosidine synthase family							Os03g53950	e-72
At2g42540	cold-regulated protein cor15a: Lea protein							Os03g07180	e-4
At5g15970	stress-responsive protein (KIN2) / COR6.6							Os01g13330	0.097
At2g37760	aldo/keto reductase, similar to chalcone reductase							Os05g38230	e-108
At2g39980	transferase family protein							Os01g63480	e-130
At1g54000	GDSL-like Lipase/Acylhydrolase, myrosinase-associated							Os02g50690	e-31
At5g59320	LTP3, protease inhibitor							Os11g02400	e-25
At5g04340	zinc finger (C2H2 type) family protein							Os12g39400	e-37
At1g01470	LEA14, Late embryogenesis abundant protein							Os01g12580	e-48
At4g24960	ABA-responsive protein (HVA22d)							Os11g30500	e-43
At4g27410	no apical meristem (NAM) family protein (RD26)							Os01g66120	e-6
At2g15970	cold acclimation protein WCOR413-like protein							Os03g55850	e-67
At1g05170	galactosyltransferase family protein							Os03g38050	e-177
At1g58360	amino acid permease I (AAP1)							Os07g04180	e-173
At4g17550	glycerol 3-phosphate permease							Os08g06010	e-156
At3g12580	heat shock protein 70 / HSP70							Os03g60620	0
At3g28270	At14a related protein							Os03g10240	e-37
At5g17460	expressed protein							Os01g03500	e-104
At3g61890	ATHB12, HD-ZIP transcription factor							Os09g35910	e-33
At2g46680	HD-ZIP protein ATHB-7							Os09g35910	e-34
At5g67300	myb family transcription factor							Os09g01960	e-66
At1g01720	ATAF1, no apical meristem (NAM)							Os01g66120	e-101
At2g21620	RD2/ universal stress protein (USP)							Os02g47650	e-74
At3g09390	MT2A, metallothionein protein							Os01g05650	e-25
At1g53580	hydroxyacylglutathione hydrolase / glyoxalase II							Os01g47690	e-114
At4g16760	ACX1, acyl-CoA oxidase							Os06g01390	0
At4g21580	oxidoreductase, zinc-binding dehydrogenase							Os02g56180	e-121
At1g17840	ABC transporter family protein							Os10g35180	0
At2g41190	amino acid/polyamine transporter, family II							Os02g54730	e-175
At1g64780	ATAMT1:2, ammonium transporter 1							Os02g40710	0
At1g19570	dehydroascorbate reductase,							Os05g02530	e-77
At1g19550	dehydroascorbate reductase							Os06g12630	e-42
At4g27560	UDP-glucoronosyl and UDP-glucosyl transferase							Os11g27370	e-77
At4g39730	Polycystin-1, Lipoxygenase, Alpha-Toxin/Lipoxygenase							Os02g51710	e-47
At1g02820	late embryogenesis abundant 3 protein / LEA3							Os01g21250	e-11
At4g27570	UDP-glycosyltransferase							Os11g27370	e-77
At4g36010	pathogenesis-related thaumatin protein, PR5K							Os09g36560	e-97

Microarray results of the drought-treated wild type and three resistant genotypes described in the thesis, are compared to published results on CBF2/DREB1C (Vogel et al., 2005) and on constitutive DREB1A/CBF3 (Maruyama et al., 2004). Dark shade displays induced, light shade repressed genes at more that 2 fold changed in 2 replications. The rice genes similar to the Arabidopsis genes are listed with the probability values showing the extent of identity.

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Summary

Irrigation of rice fields has been a part of Asian culture for many thousands of years. This most productive rice growing system contributes 75% of the world rice production. The combination of human population growth and climate change, however, may soon impact the reliability of the globe's fresh water resources. To meet the increasing demand of rice in the future, therefore, we can not continue to depend solely on irrigated area. We need to improve and exploit the yield potential of the rice crop in rainfed areas.

The major environmental constraint for rice production in rainfed areas is water deficit, to which rice is more susceptible than other cereals. The gap in productivity between irrigated and non-irrigated areas is still huge. Much more effort should be made to narrow the gap through genetic improvement of rice for water-limited environments. Breeding for drought stress resistance is complex due to the large number of genes affecting the trait and the difficulties in evaluation or selection of drought resistant cultivars. However, it is expected that genetic improvement for drought resistance in rice is possible given the recent advances in plant genomics.

This thesis reports the study on the genetic control of drought resistance, using genomics information and tools from the model plants Arabidopsis and rice. The genetic dissection of drought resistance in a rice population, using molecular markers, reveals that a large part of the genetic effect for grain yield under drought condition is determined by a chromosomal region close to the dwarfing gene responsible for the 'Green Revolution'. The parameters associated with the effect on grain yield under stress in this region were percentage of seed-set, grain number per panicle and surprisingly leaf rolling. Comparison with other populations showed that leaf rolling has been mapped in the similar position using different rice genotypes. Leaf rolling is indirect indicator of drought avoidance or tolerance mechanisms.

Drought stress resistance is well studied in Arabidopsis. The experimental information gathered suggests a few principles that enable the information to be used for crop plants like rice. Primarily, although drought resistance is a complex process dealing with hundred of genes, the use of a single transcription factor can regulate a part of this battery of genes to improve drought resistance. Secondly, many genes have conserved functions between plants

even between dicots and monocots and can be used for improvement of a wide variety of crops.

A gene named *SHINE* has been previously identified in Arabidopsis that displayed drought stress resistance when its expression was increased. In this thesis, this gene was introduced into rice by transformation and the plants were analyzed. The rice plants, like in Arabidopsis showed enhanced drought resistance. The mechanism of this conserved function of drought resistance is probably due to the reduced density of stomata in the leaves produced by overexpression of the gene. The reduced density of stomata in rice, without a visible change in the leaf or plant structure, reduces the water loss due to transpiration and thus provides the plant water for a longer period. This is a drought avoidance mechanism.

In rice a gene similar to the Arabidopsis *SHINE* was identified and transformed into Arabidopsis to be expressed at a high level. This rice gene in Arabidopsis also provided the same phenotype, including drought tolerance, as the Arabidopsis gene when over-expressed. These experiments in Arabidopsis and rice showed that this family of genes has conserved functions in providing drought tolerance, although not all aspects of the phenotype are found in both plants.

The most notable phenotype that *SHINE* provided to Arabidopsis plants was shiny leaves due to an increase in epicuticular wax. To confirm that the gene is responsible for wax synthesis regulation, a SHINE protein fusion was made to change it to a repressor of transcription. When this gene was introduced into Arabidopsis there was a reduction in stem wax observed, showing by both overexpression and repression analysis that the *SHINE* family of genes can regulate wax synthesis. However, overexpression of *SHINE* in rice did not produce a visual increase in wax neither did it induce target wax genes in rice. This suggests that the drought stress resistance obtained in rice by overexpression of *SHINE* is independent of wax deposition. However, overexpression of *SHINE* in rice changed the cuticle permeability. The alteration in the cuticle might cause the reduced stomata density contributing to drought stress avoidance.

The effect of drought on a plant was studied by monitoring the changes in expression of all the Arabidopsis genes using whole genome microarrays. A large number of housekeeping genes, required for normal functioning of a plant, were reduced in expression while a set of stress associated genes showed an increase. In addition three drought stress resistant genotypes were analyzed for the changes in expression of the whole set of

Arabidopsis genes. These three genotypes were produced by transforming stress resistance genes, namely *SHINE*, *DREB1A* and *HARDY* into Arabidopsis to be expressed at a high level. An overlap between genes induced in the drought-treated wild type plants and in the drought resistant plants indicated the association of these genes to drought resistance. This analysis also provided a set of differentially regulated transcription factors and other regulatory genes that are candidates to understand drought stress mechanisms and also to engineer drought resistance by overexpressing them in crop plants. Using the complete genome information of Arabidopsis and rice, highly similar rice genes could be identified that probably have the same function as their Arabidopsis counterparts. Thus similar to the *SHINE* example described, overexpression of these Arabidopsis/rice genes would provide information on their possible role in drought resistance.

Analysis of the plant phenotype and expression of genes with altered levels reveals the mechanism involved in the drought stress resistant phenotype. The analysis of the *SHINE* overexpression suggested that the reduction in stomata density was reducing water loss and thus giving a drought avoidance phenotype. However, analysis of the genes differentially expressed in the microarray suggested that stress related genes are also induced. Thus the mechanism of drought stress is also partially a tolerance mechanism in *SHINE*. The two other stress resistant genotypes also show an increased expression of stress related genes that suggest that a drought tolerance mechanism is involved. However the genes induced are not the same, showing that parts of the tolerance mechanism are similar and other parts are unique.

Overexpression analysis of genes provides a convenient way to test for their function between species. One way is to test gene by gene as has been done for the *SHINE* gene or other genes giving stress tolerance. A genome-wide method would be to overexpress all the genes in the plant and select for those lines that are stress tolerant. For this method an activation tag system was developed in rice that would generate genotypes, each having one or a few genes overexpressed that can be screened for drought and other stress resistance parameters. This strategy would provide a systematic analysis of all rice genes by overexpression to select for those that confer a favorable phenotype.

Drought stress resistance in rice can be obtained by increased expression of specific transcription factor genes. Many of such genes can be identified using the available technologies, revealing genes with different mechanisms for stress tolerance. A systematic

analysis of the potential of these different genes and their mechanisms in rice, would contribute to their application towards sustainable grain production and stability under drought stress.

Samenvatting (Summary in Dutch)

Irrigatie van rijstvelden maakt al vele duizenden jaren deel uit van de Aziatische cultuur. Dit productieve systeem van rijst teelt, draagt misschien wel voor 75% bij aan de totale wereld rijstproductie. De combinatie van een groeiende wereldbevolking en klimaatverandering, kan op korte termijn negatieve invloed hebben op de beschikbaarheid van ‘s werelds zoetwaterbronnen. Om te blijven voldoen aan de vraag van rijst in de toekomst, kunnen we niet afhankelijk blijven van alleen maar irrigatie gebieden. We moeten de potentie van de rijstoogst verbeteren en ook benutten in niet-irrigatiegebieden.

De grootste omgevingsbeperking voor rijstproductie in niet-irrigatiegebieden is water tekort op cruciale momenten, waar rijst gevoeliger voor is dan andere granen. Het verschil in productiviteit tussen geïrrigeerde en niet-geïrrigeerde gebieden is nog steeds heel groot. Veel meer inspanning zou gedaan moeten worden om het verschil kleiner te maken door middel van genetische verbetering van rijstcultivars voor gebieden met beperkende waterhoeveelheden. De veredeling op droogtestress resistentie is complex, omdat de hoeveelheid genen die deze eigenschap beïnvloeden groot is en het moeilijk is op droogteresistente cultivars te selecteren. Echter, gezien de recente ontwikkelingen in “plant genomics”, is het te verwachten dat door middel van genetische verbetering het mogelijk moet zijn om meer droogteresistentie aan rijst toe te voegen.

Dit proefschrift gaat over onderzoek naar de genetische controle van droogteresistentie, gebruikmakend van “genomics” informatie en -technieken die in de modelplanten Arabidopsis en rijst voorhanden zijn. De genetische ontrafeling van droogteresistentie in een rijstpopulatie, gebruikmakend van moleculaire merkers, liet een aantal samenhangende parameters zien die de graanopbrengst onder stress bepalen. De belangrijkste waarneming is dat een groot gedeelte van het genetische effect voor droogteresistentie bepaald wordt door genotypisch geconserveerde loci, waar het “dwarfing” (dwerg) gen er één van is dat in het verleden tot de “Green Revolution” (Groene Revolutie) leidde. De eigenschappen gerelateerd aan dit effect op graanopbrengst onder droogtestress waren percentage zaadzetting, korrels per aar en verrassend was bladkrulling. Vergelijking met andere populaties toonde aan dat de eigenschap bladkrulling op dezelfde positie gelegen is in verschillende rijstgenotypen. Bladkrulling is een indirecte indicator van droogtevermijdings of -tolerantie mechanismen.

Resistentie tegen droogtestress is in Arabidopsis goed bestudeerd. De experimentele resultaten samen suggereren een paar principes die het mogelijk maken de informatie te gebruiken voor gewassen zoals rijst. Hoewel droogteresistentie een complex proces is met honderden genen, kan het gebruik van één transcriptiefactor deze verzameling genen zodanig reguleren dat dit tot een verbetering van droogteresistentie leidt. Ten tweede, veel genen hebben geconserveerde functies in planten, zelfs tussen dicotylen en monocotylen, die voor verbetering gebruikt kunnen worden in een scala van gewassen.

Een gen, *SHINE* genaamd, is voorheen in Arabidopsis geïdentificeerd dat tot droogtestress resistentie leidde wanneer de expressie ervan geïnduceerd werd. In dit proefschrift, is dit gen door middel van transformatie in rijst geïntroduceerd en de planten zijn geanalyseerd. De rijstplanten toonden net zoals in Arabidopsis toegenomen resistentie tegen droogte. Het mechanisme van deze geconserveerde functie voor droogteresistentie wordt waarschijnlijk veroorzaakt door het verminderde aantal huidmondjes in de bladeren dat het gevolg is van de geïnduceerde expressie van dit gen. De gereduceerde dichtheid van die huidmondjes in rijst, zonder enig zichtbaar verschil in blad of plantstructuur, veroorzaakt een verminderd waterverlies door verdamping en dus blijft de plant een langere tijd van water voorzien. Dit is dus een resistentiemechanisme van droogtevermijding.

Een soortgelijk gen als het Arabidopsis *SHINE* gen is geïdentificeerd in rijst en is getransformeerd in Arabidopsis om het tot verhoogde expressie te brengen. Het rijst gen in Arabidopsis veroorzaakte hetzelfde fenotype, inclusief droogtetolerantie, zoals het Arabidopsis gen bij overexpressie. Deze experimenten in Arabidopsis en rijst lieten zien dat deze genfamilie geconserveerde functies in droogte tolerantie hebben, hoewel niet alle aspecten van het fenotype in beide planten voorkomen.

Het meest opmerkelijke fenotype dat *SHINE* aan Arabidopsis planten gaf was het fenomeen “glimmende bladeren” als gevolg van een toegenomen epicuticulaire waslaag. Om te bevestigen dat het gen verantwoordelijk is voor wassynthese werd een SHINE eiwitfusie met een remmend effect op transcriptie gemaakt. Toen dit gen geïntroduceerd werd in Arabidopsis werd een reductie in wasafzetting op de stengel waargenomen. Zowel geïnduceerde expressie als verlaagde expressie analyses tonen aan dat de *SHINE* genfamilie wassynthese kan reguleren. Hoewel, geïnduceerde expressie van *SHINE* in rijst gaf niet zichtbaar meer was en ook geen inductie van “target” wassynthese genen in rijst. Dit suggereert dat de droogtestress resistentie verkregen door de geïnduceerde *SHINE* expressie

in rijst onafhankelijk is van wasafzetting. Hoewel, geïnduceerde expressie van *SHINE* in rijst veranderde de doorlaatbaarheid van de "cuticle" (opperhuid). De verandering in de "cuticle" zou de gereduceerde dichtheid van huidmondjes kunnen veroorzaken dat bijdraagt aan de vermijding van droogtestress.

Het effect van droogte op een plant is bestudeerd door het bekijken van veranderingen in expressie van alle Arabidopsis genen met behulp van "volledig genoom" microarrays. Een groot aantal huishoudgenen, nodig voor normale plantengroei, waren gereduceerd in expressie terwijl een groep stress gerelateerde genen verhoogde expressie vertoonden. Hiernaast zijn drie droogtestress resistente genotypen geanalyseerd voor expressieveranderingen van alle Arabidopsis genen. Deze drie genotypen zijn gemaakt door stressresistentie genen, namelijk *SHINE*, *DREB1A* en *HARDY*, te transformeren in Arabidopsis om daarmee een geïnduceerde expressie te bewerkstelligen. Een overlap tussen geïnduceerde genen in droogte behandelde wildtype planten en de drie droogteresistente planten wijzen op een verband van deze genen met droogteresistentie. Deze analyse leverde ook een groep transcriptiefactoren op die verschillend gereguleerd zijn en andere regulatiegenen die kandidaat zijn voor het beter begrijpen van droogteresistentie en die droogteresistentie kunnen geven bij geïnduceerde expressie in gewassen. Gebruikmakend van de beschikbaarheid van de volledige genoominformatie van zowel Arabidopsis als rijst maakte het mogelijk om soortgelijke genen te identificeren in rijst die waarschijnlijk dezelfde functie hebben als hun tegenhangers in Arabidopsis. Dus in parallel aan het beschreven *SHINE* voorbeeld, kan geïnduceerde expressie van andere Arabidopsis/rijst genen informatie geven over hun mogelijke rol in droogteresistentie.

Analyse van het plantenfenotype en genexpressie legt in principe de mechanismen bloot die bij het droogtestress resistente fenotype betrokken zijn. De analyse van *SHINE* geïnduceerde expressie suggereert dat de verminderde dichtheid van huidmondjes een waterbesparing oplevert en dus een verbeterd droogtevermijdingsfenotype tot gevolg heeft. Echter, de analyse van differentiële genexpressie op de microarray suggereert dat gelijktijdig stress gerelateerde genen ook geïnduceerd zijn. Dus het mechanisme van droogtestress is gedeeltelijk ook een tolerantie mechanisme in *SHINE*. De twee andere stress resistente genotypen lieten ook een geïnduceerde expressie van stress gerelateerde genen zien, hetgeen suggereerd dat ook hierbij een droogte tolerantie mechanisme betrokken is. Echter de

geïnduceerde genen zijn niet hetzelfde hetgeen betekent dat een gedeelte van het tolerantie mechanisme overeenkomstig is en andere delen uniek zijn.

Geïnduceerde genexpressieanalyses zijn een handige manier voor het testen en vergelijken van hun functies in verschillende soorten. Eén manier is het testen van gen voor gen dat gedaan is voor *SHINE* of voor andere genen die stresstolerantie geven. Een meer genoom-brede methode zou zijn om alle genen in de plant te induceren en dan de lijnen met stress tolerantie te selecteren. Voor deze methode is een "activation tag" systeem in rijst ontwikkeld dat genotypen kan opleveren met elk één of een paar geïnduceerde genen, die onderzocht kunnen worden op droogte en andere stress resistentie parameters. Deze strategie zal een systematische analyse van alle geïnduceerde rijstgenen opleveren die het mogelijk maakt planten met een gewild fenotype te selecteren.

Droogtestress resistentie in rijst kan verkregen worden door geïnduceerde expressie van één of meer transcriptiefactoren. Veel soortgelijke genen kunnen geïdentificeerd worden op basis van de reeds beschikbare technieken en deze genen zullen verschillende stresstolerantie mechanismen blootleggen. Een systematische waardebepaling van deze verschillende genen en hun bijbehorende mechanismen in rijst, zal in de toekomst bijdragen aan een duurzame graanproductie en de gewenste stabilitiet ervan bij droogtestress.

Ringkasan (Summary in Indonesian)

Irigasi lahan sawah sudah menjadi bagian budaya Asia selama ribuan tahun. Sistem budidaya padi paling produktif ini menyumbang 75% produksi padi dunia. Akan tetapi, kombinasi pertumbuhan penduduk dan perubahan iklim mungkin segera berdampak pada ketersediaan sumberdaya air di bumi. Untuk memenuhi permintaan beras yang meningkat di masa mendatang, kita tidak dapat terus bergantung melulu pada lahan irigasi. Kita perlu mengeksploitasi dan memperbaiki potensi hasil tanaman padi pada lahan tadah hujan.

Kendala utama untuk produksi padi pada lahan tadah hujan adalah kekurangan air, di mana padi lebih peka dari tanaman serealia yang lain. Kesenjangan produktivitas antara lahan irigasi dan non-irigasi masih lebar. Lebih banyak upaya seharusnya dilakukan untuk mempersempit kesenjangan tersebut melalui perbaikan genetik tanaman padi untuk lingkungan terbatas air. Pemuliaan untuk ketahanan kekeringan adalah kompleks karena sejumlah besar gen yang mempengaruhi sifat tersebut dan kesulitan dalam seleksi atau evaluasi kultivar tahan kering. Akan tetapi, diharapkan bahwa perbaikan genetik untuk kekeringan pada padi dimungkinkan oleh kemajuan akhir-akhir ini dalam genomik tanaman.

Thesis ini melaporkan studi pada kontrol genetik dari ketahanan kekeringan, menggunakan informasi dan alat-alat genomik pada tanaman model Arabidopsis dan padi. Analisis genetik sifat ketahanan kekeringan pada sebuah populasi padi, dengan menggunakan marka molekuler, menunjukkan bahwa bagian besar efek genetik untuk hasil gabah di bawah kondisi kekeringan ditentukan oleh bagian kromosom yang posisinya dekat dengan gen semi kate yang bertanggung jawab untuk “Revolusi Hijau”. Parameter-parameter yang terkait dengan efek hasil gabah di bawah cekaman pada posisi ini adalah persentase pembentukan biji, jumlah gabah isi per malai dan secara mengejutkan, penggulungan daun. Perbandingan dengan populasi-populasi yang lain menunjukkan bahwa penggulungan daun sudah dipetakan pada posisi yang mirip menggunakan genotipe-genotipe padi yang berbeda. Penggulungan daun adalah indikator tidak langsung untuk mekanisme pengelakan atau toleransi kekeringan.

Ketahanan terhadap cekaman kekeringan sudah dipelajari dengan baik pada Arabidopsis. Informasi percobaan yang terkumpul menunjukkan beberapa prinsip yang memungkinkan informasi tersebut digunakan untuk tanaman budidaya seperti padi. Pertama, meskipun ketahanan kekeringan adalah sebuah proses kompleks yang melibatkan ratusan gen, penggunaan faktor transkripsi tunggal dapat mengatur ekspresi sebagian dari deretan gen ini

untuk memperbaiki ketahanan kekeringan. Kedua, banyak gen memiliki fungsi yang terpelihara antar tanaman bahkan antara dikotil dan monokotil dan dapat digunakan untuk perbaikan berbagai tanaman budidaya.

Gen yang disebut *SHINE* sebelumnya sudah diidentifikasi pada Arabidopsis yang menunjukkan ketahanan cekaman kekeringan ketika ekspresinya ditingkatkan. Dalam thesis ini, gen ini dimasukkan ke dalam padi melalui transformasi dan kemudian tanamannya dianalisis. Tanaman padi, seperti pada Arabidopsis menunjukkan ketahanan kekeringan yang meningkat. Mekanisme fungsi yang terpelihara untuk ketahanan kekeringan ini mungkin disebabkan oleh densitas stomata yang berkurang pada daun yang dihasilkan oleh ekspresi yang tinggi dari gen tersebut. Densitas stomata yang berkurang pada padi, tanpa perubahan yang kasat mata pada daun atau struktur tanaman, mengurangi kehilangan air yang disebabkan oleh transpirasi dan dengan demikian air dalam tanah tersedia bagi tanaman untuk periode yang lebih lama. Ini adalah mekanisme pengelakan kekeringan.

Pada tanaman padi gen yang mirip dengan *SHINE* Arabidopsis diidentifikasi dan ditransformasi ke Arabidopsis untuk diekpresikan pada level tinggi. Gen padi pada Arabidopsis ini juga memberikan fenotipe yang sama, termasuk ketahanan kekeringan, seperti gen Arabidopsis ketika ekpresinya ditingkatkan. Percobaan pada Arabidopsis dan padi ini menunjukkan bahwa famili gen ini memiliki fungsi yang terpelihara dalam memberikan ketahanan kekeringan, meskipun tidak semua aspek fenotipe ditemukan pada kedua tanaman.

Fenotipe paling menyolok yang diberikan *SHINE* pada tanaman Arabidopsis adalah daun yang mengkilat yang disebabkan oleh peningkatan akumulasi lilin epikutikula. Untuk mengkonfirmasi bahwa gen ini bertanggung jawab terhadap regulasi sintesis lilin, fusi protein SHINE dibuat untuk merubahnya menjadi penekan transkripsi. Ketika gen ini dimasukkan ke dalam Arabidopsis diamati terjadinya reduksi lilin pada batang. Dengan demikian baik analisis peningkatan maupun penekanan ekspresi menunjukkan bahwa famili gen *SHINE* dapat meregulasi sintesis lilin. Akan tetapi, ekspresi *SHINE* pada level tinggi pada padi tidak menghasilkan peningkatan akumulasi lilin yang dapat diamati, tidak juga menginduksi gen-gen target yang terlibat dalam biosintesis lilin pada padi. Ini menunjukkan bahwa ketahanan cekaman kekeringan yang diperoleh pada padi melalui ekspresi *SHINE* pada level tinggi tidak ada hubungannya dengan deposisi lilin. Akan tetapi, ekpresi level tinggi *SHINE* pada padi merubah permeabilitas kutikula. Perubahan pada kutikula ini mungkin menyebabkan densitas stomata yang berkurang yang mendukung mekanisme pengelakan cekaman kekeringan.

Efek kekeringan pada tanaman dipelajari dengan memonitor perubahan pada ekspresi semua gen Arabidopsis menggunakan mikroarai ("*microarray*") genom lengkap. Sejumlah besar gen rumah tangga, yang diperlukan untuk fungsi-fungsi normal dari tanaman, tereduksi ekspresinya sementara sekelompok gen terkait cekaman menunjukkan peningkatan dalam ekspresi. Di samping itu tiga genotipe tahan kekeringan dianalisis untuk perubahan ekspresi dari seluruh gen Arabidopsis. Tiga genotipe ini dihasilkan melalui transformasi gen-gen ketahanan cekaman, yang disebut *SHINE*, *DREB1A*, dan *HARDY* ke dalam Arabidopsis untuk diekspresikan pada level tinggi. Overlap antar gen yang diinduksi pada tanaman tipe liar yang diperlakukan dengan kekeringan dan pada tanaman tahan kering mengindikasikan keterkaitan gen-gen ini dengan ketahanan kekeringan. Analisis ini juga memberikan sekelompok faktor transkripsi dan gen-gen regulator lain yang merupakan kandidat untuk digunakan dalam upaya memahami mekanisme cekaman kekeringan dan juga untuk merekayasa ketahanan kekeringan melalui ekspresi level tinggi pada tanaman budidaya. Menggunakan informasi genom lengkap dari Arabidopsis dan padi, gen-gen padi yang sangat mirip dapat diidentifikasi yang mungkin memiliki fungsi yang sama seperti kerabatnya pada Arabidopsis. Jadi seperti yang sudah digambarkan pada *SHINE*, ekspresi level tinggi gen-gen Arabidopsis atau padi ini akan memberikan informasi mengenai peranan mereka yang mungkin pada ketahanan kekeringan.

Analisis fenotipe tanaman dan ekspresi gen dengan level yang berubah menunjukkan mekanisme yang terlibat dalam fenotipe tahan cekaman kekeringan. Analisis ekspresi level tinggi *SHINE* menunjukkan bahwa reduksi densitas stomata mengurangi kehilangan air dan dengan demikian memberikan fenotipe pengelakan kekeringan. Akan tetapi, analisis gen-gen yang diekspresikan secara diferensial pada mikroarai menunjukkan bahwa gen-gen yang terkait cekaman juga diinduksi. Jadi mekanisme ketahanan cekaman kekeringan sebagian juga merupakan mekanisme toleransi pada *SHINE*. Dua genotipe tahan cekaman yang lain juga menunjukkan ekspresi yang meningkat dari gen-gen terkait cekaman yang menunjukkan bahwa mekanisme toleransi kekeringan terlibat. Akan tetapi, gen-gen yang diinduksi tidak sama, yang menunjukkan bahwa bagian-bagian mekanisme toleransi adalah mirip dan bagian-bagian yang lain unik.

Analisis ekspresi gen level tinggi memberikan cara yang sesuai untuk menguji fungsinya antar spesies. Satu pendekatan adalah menguji satu demi satu gen seperti yang sudah dikerjakan pada gen *SHINE* atau gen-gen lain yang memberikan toleransi kekeringan.

Metode genom lengkap akan berupa ekspresi level tinggi semua gen pada tanaman dan seleksi untuk lini-lini yang toleran cekaman. Untuk metode ini sebuah sistem penanda aktivasi ("*activation tag*") dikembangkan pada padi yang akan menghasilkan genotipe-genotipe, yang masing-masing memiliki satu atau beberapa gen yang diekspresikan pada level tinggi yang dapat disaring untuk parameter-parameter kekeringan atau cekaman yang lain. Strategi ini akan memberikan sebuah analisis sistematik dari semua gen padi melalui ekspresi level tinggi untuk seleksi gen-gen yang memberikan fenotipe yang menguntungkan.

Ketahanan kekeringan pada padi dapat diperoleh dengan ekpresi level tinggi gen-gen faktor transkripsi spesifik. Banyak dari gen-gen tersebut dapat diidentifikasi menggunakan teknologi-teknologi yang tersedia, yang menunjukkan gen-gen dengan mekanisme yang berbeda untuk toleransi terhadap cekaman. Sebuah analisis sistematik mengenai potensi dari gen-gen yang berbeda ini dan mekanismenya pada padi, akan mendukung aplikasi gen-gen tersebut menuju peningkatan dan stabilitas produksi beras di bawah kondisi cekaman kekeringan.

Acknowledgements

In January 2002, when I came to Wageningen to start my sandwich PhD work on molecular markers, I did not imagine that I would work in a wide variety of new techniques that I never knew before. With the completion of this booklet, that is the major outcome of my work, I would like to thank all people for their special contribution in different ways.

First of all I must thank God for everything I have. Health, happiness, and especially, for giving me someone to whom I dedicate this work: You my beloved wife, Nana.

I am very much indebted to my co-promoter, Andy Pereira. Andy, thanks for your guidance during the years, your help and availability whenever needed. Your strategic suggestion to do many things in parallel and then focus on promising results has enabled me to finish my PhD. I enjoyed and learned a lot from the long evening discussions about the research results and plant science world. I would also like to thank for the funds for my 3-months extension period.

I would like give thanks to my second co-promoter, Sugiono Moeljopawiro. Pak Sugiono, thanks for your supervision and help not just during my PhD period but also since I joined your group in 1994. Your efforts to modify my original program enabled me to graduate in the Wageningen University that gives an added value for our institute and for my scientific career.

I would like to thank my promoter, Evert Jacobsen. Evert, thanks for putting me in the "pressure cooker" that enabled me to finish my manuscript on time. I appreciated very much your advice and support especially in the final steps of my thesis.

I would like to say thanks to the head of my institute (RIFCB), Sutrisno. Pak Sutrisno, thanks for your permission for me to leave RIFCB temporarily to do my PhD in Wageningen. Thanks for your help especially for the immigration dealings.

The work presented in this thesis was carried out as part of a BIORIN project involving people from three other partner labs, i.e. Pieter, Sigit, Inez and Joko. Pieter, thanks for your help and discussion, especially for rice transformation. Sigit, thanks for our friendship and discussion during your stay in the Netherlands and also sometime when we met in Indonesia. Bu Inez, thanks for the funds for the development of doubled haploid population in Indonesia. Joko, thanks for your help in taking over my responsibility in our project when I stayed in the Netherlands. I appreciated very much your effort to make all

work on RI population development and field experiment run well, despite many financial and bureaucratic constraints.

I wish to express my thanks to Huub Loffler (BIORIN project leader) for his excellent coordination and leadership in making our BIORIN project to run well. My special thanks are due to my Indonesian friends that are involved in other BIORIN Project: Ence, Tetty, Reni and Vivi. I appreciated very much all of your kindness, especially during the difficult period in the beginning of my stay in Wageningen. I would also like to thank you all for our occasional meetings when Ence was still in Wageningen. I would like to apologize because I could not join in every travel.

Two most important people taught me a wide variety of basic molecular techniques were “Wonderful Gert” and “Super Wim”. Gert, thanks for teaching me making constructs, bacterial transformation, rice transformation, microarray, etc. I would also like to thank you for your availability whenever needed, for your good management in our past lab that enabled me to find easily everything I needed. Wim, thanks for your advice on any lab work, for your help in arranging my AFLP work in other labs, for your help to get advice from people from other groups, and for your help on any “alien police” dealings.

I am indebted to two wonderful couples, Raffa-Asaph and Nayelli-Stefan. Raffa, thanks for your encouragement for me to finish my PhD. I always remember what you said when I came to Wageningen for the second time (October 2003) that I would finish my PhD magically. I would like to thank you for your help whenever needed, even when you were not in Wageningen anymore. Asaph, thanks for teaching me RNA isolation, making cDNA, designing primers, etc. Despite that I made you angry, you still helped me with many kinds of things. Nayelli, thanks for your help and availability whenever needed, especially for answering my questions about Arabidopsis and transposon work. I would also like to thank you for your kind help to critically read one chapter of my thesis. My special thanks for your help on designing the cover of my thesis and the invitation card. Stefan, thank for your suggestions on Arabidopsis work and embedding. I would also like to thank you for your kind help on the samenvatting.

To my past and present colleagues of the former cluster “Genoomanalyse” with whom I shared labs, offices and daily life at PRI: Ellen (thanks for the establishment of rice transformation system at PRI), Doret (thanks for your advice on polyacrylamide gel preparation, silver staining and microarray), Annelies, Marjon, Edwin, Eric, Carolien. And to

the "Greenomics" colleagues: Paul, Tamara, Marleen (who loves my country like I love it … *Terima kasih*…).

I would like to thank also several visiting scientists and students in the group of Andy from abroad. Rajan, thanks for our friendship. I hope we can always keep in touch. Junming and Wang, thanks for our friendship and for long discussions about your country. Aarati, thanks for our fruitful discussion on drought stress physiology. Shital, thanks for your advice on Arabidopsis drought experiments. I would also like to thank you for your kind help to be my paranimph and the organizer of my party. Marco, thanks for your help on mapping work. Good luck with your future. *Ciao*, *pronto*.

To people from the former BU Biodiversity: Hanneke, Wendy, Paul, Danny and Zifu, thanks for your help and advice on microsatellite and silver staining. I would like to thank you Colette for your kind help in microarray work. And to the former Breeding people: Oene, Richard, Christel, Fabrizio and Linda, thanks for your help and advice on AFLP work and analysis.

To people from BU Biointeractions: Odette (thanks for many kinds of help) and Marianna (thanks for our fruitful discussion on microarrays and markers). I would like to thank people from BU Bioscience: Adele and Oscar (thanks for your suggestions on microarray data analysis), Ronny (thanks for your help on operation of FX Pro Plus Multi-imager), John (thanks for your help on microscope photograph and embedding), Xin Ying, Kum Kum and Rada (thanks for always working till late).

I would like give thanks to people from Facility Services and FS-ICT. Yvonne, thanks for your kind help and availability whenever needed. Jan, thanks for the prompt assistance in the many computer emergencies I encountered.

Working on a Ph.D. abroad is not easy. However, when you have friends it becomes nicer and easier. I would like to thank my past and present friends of the Indonesian Student Association Wageningen: Lukas, Pak Hadi and his family, Pak Wiratno, Pak Putu and his family in Indonesia, Hernowo, Ari, Probo and *sedulurku kabeh*. I also wish to thank the Ambassador, Consular Affairs, and Education and Cultural Affairs of Indonesian Embassy in The Hague, The Netherlands for valuable assistance and support.

I would also like to thank my friends in the Student Chaplaincy with whom I enjoyed nice times in my first stay for 6 months in Wageningen. Agnes and Jolande, thanks for our friendship. My wife and I appreciated very much your visit to Yogyakarta in September 2004.

I would like to thank my colleagues from the RIFCB Bogor Indonesia: Mbak Masumah, Pak Iman, Teh Nur, Yeni, Dadang, Harnati, Heri, Tri Joko, Refli, Pak Dedi, Pak Abas, Pak Jarkasih, Nanas, Mitri, Nurul, Pak Herman, El, Mbak Septi, Mike, Pak Yunus, Syarif, Mas Tusran. I would also like to thank my colleagues from Gadjah Mada University Yogyakarta Indonesia: Mas Pri, Rudi HM, Panji and Pak Taryono.

My deepest gratitude goes to my father, Pak Harsoyo, who always supported me to complete my Ph.D. I thank my sister Nunik, my brothers Mas Intoro, Mas Dwi, Sigit and all their family that always prayed for my wife and me. And definitely, I would never have been here without my unforgettable mother Ibu Sutarmi. Unfortunately, you are not here anymore to see another important moment in my life. But your eternal love is my greatest memory that always helps me to cross any kind of constraints.

My special thanks to my mother in law Ibu Lusi, my sister in law Cynthia, my brothers in law Nova and Ancis, and also to Indah, for their care to my wife during the hard times and for their prayers for the success of my Ph.D. My grateful thanks are due to Mbak Tarmi, Mbak Lastri, Mbak Har, Mbah Munah, Mbak Heni, Mas Sigit, Mbak Marta, Teh Riri, Ela, Ulin, Tika, Teh Indah, for their kind help to my wife in different ways during my stay in Wageningen.

Finally, I have no enough words to express my deepest feelings to my beloved wife Sylvia Ratna Wardayanti (Nana), who always amazes me with her love and patience. Despite you had many problems in Indonesia, you still always pushed me to finish my Ph.D. I believe, one day when we recall all our experience during this uneasy period, we will appreciate the power of love unifying us.

Kurniawan Rudi Trijatmiko
Wageningen, September 2005

Curriculum Vitae

Kurniawan Rudi Trijatmiko was born on January 18, 1970 in Surakarta (Solo), a historical town in Central Java, Indonesia. He grew up in this town until he finished high school. He started his studies in Plant Breeding in 1988 at the Gadjah Mada University, Faculty of Agriculture, Yogyakarta, where he obtained his B.Sc. degree in 1994 presenting a thesis work entitled "Screening for regeneration ability through somatic embryogenesis and for responsiveness to *Agrobacterium tumefaciens* infection of Indonesian soybean cultivars". In 1994, he joined the group of Dr. Sugiono Moeljopawiro in the Research Institute for Food Crop Biotechnology (RIFCB) Bogor to work on mapping of QTLs for tolerance to iron toxicity in rice. In 1996 he started his studies in Plant Biotechnology at the Bogor Agricultural University (IPB), where he obtained his M.Sc. degree in 2000 presenting a thesis work entitled "Linkage study between RAPD markers and root penetration ability in rice". In January 2002 he came to Wageningen to start his sandwich Ph.D. program with the supervision of Dr. Andy Pereira and Dr. Sugiono Moeljopawiro, with a research project on molecular breeding for drought resistance in rice. This research is a part of the Biotechnology Research Indonesia-Netherlands (BIORIN) project funded by KNAW, The Netherlands, as a collaboration project between RIFCB and Plant Research International, Wageningen-UR. After obtaining his Ph.D. degree, he will return to Indonesia to continue his work at RIFCB. One of his desires has been to strengthen scientific capacity of his institute through national and international collaboration.

Qui seminant in lacrimis in exultatione metent
(They that sow in tears shall reap in joy, Psalm 125)

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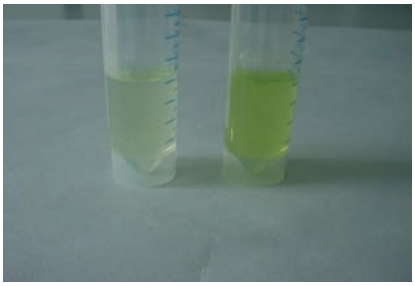

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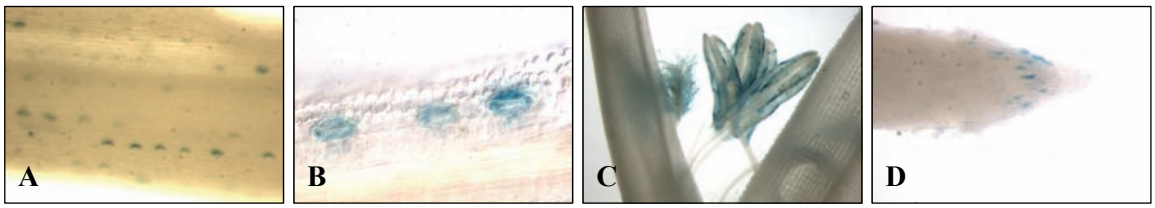

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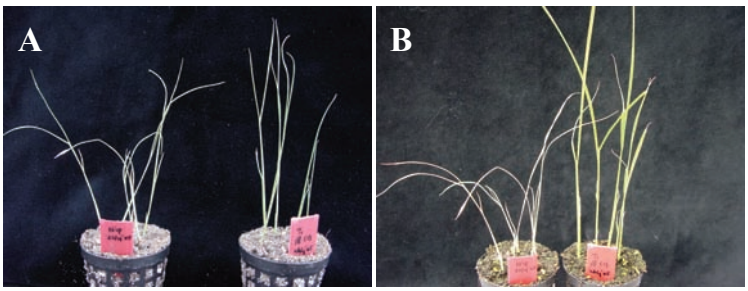

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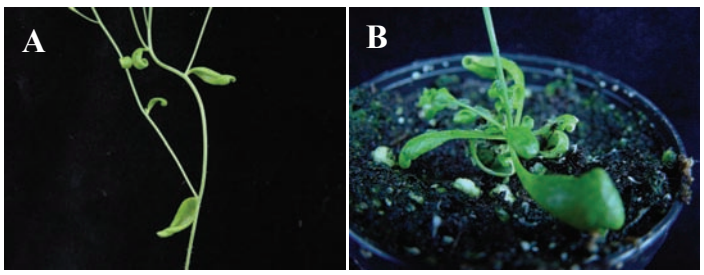

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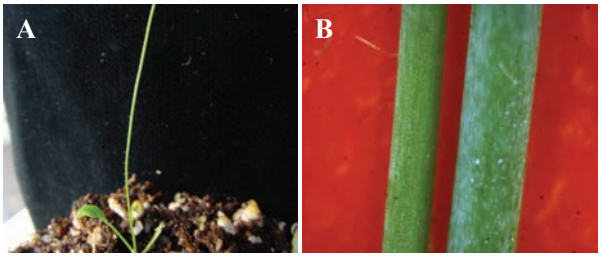
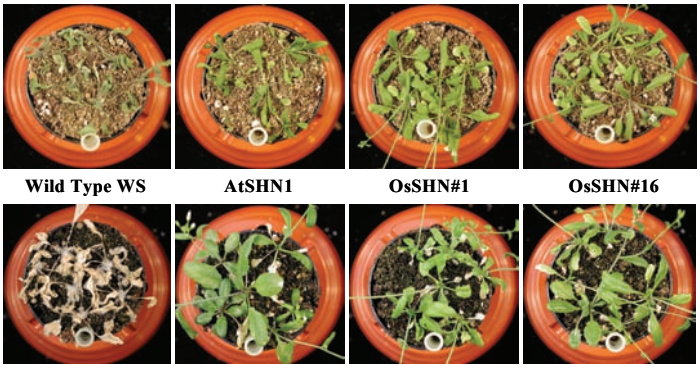


Chapter 5 Fig. 3 page 100



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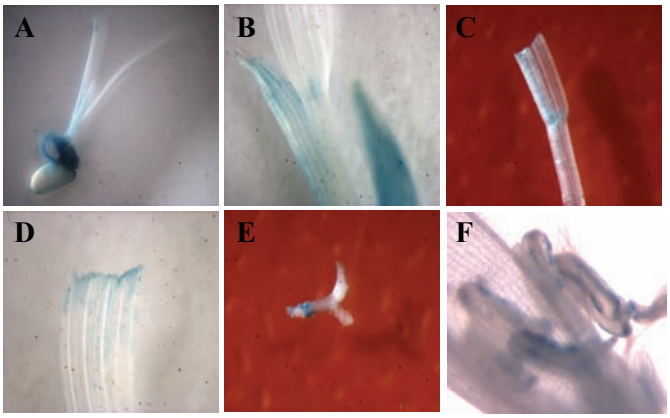

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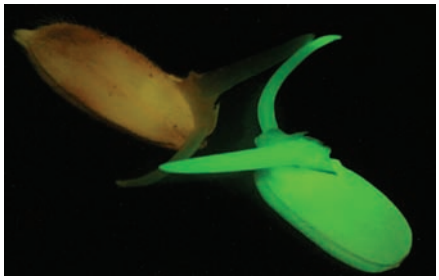

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