Gene networks involved in drought stress response and tolerance

Kazuo Shinozaki1,* and Kazuko Yamaguchi-Shinozaki2,3

1 RIKEN Plant Science Center, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, 203-0045 Japan
2 Laboratory of Plant Molecular Physiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1 Yayoi, Bunkyo-ku, Tokyo, 113-8657 Japan
3 Biological Resources Division, Japan International Research Center for Agricultural Sciences (JIRCAS), 1-1 Ohwashi, Tsukuba, Ibaraki, 305-8686 Japan

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Abstract
Plants respond to survive under water-deficit conditions via a series of physiological, cellular, and molecular processes culminating in stress tolerance. Many drought-inducible genes with various functions have been identified by molecular and genomic analyses in Arabidopsis, rice, and other plants, including a number of transcription factors that regulate stress-inducible gene expression. The products of stress-inducible genes function both in the initial stress response and in establishing plant stress tolerance. In this short review, recent progress resulting from analysis of gene expression during the drought-stress response in plants as well as in elucidating the functions of genes implicated in the stress response and/or stress tolerance are summarized. A description is also provided of how various genes involved in stress tolerance were applied in genetic engineering of dehydration stress tolerance in transgenic Arabidopsis plants.

Key words: Drought stress, gene expression, microarray, stress tolerance, molecular breeding.

Introduction
Drought stress induces a range of physiological and biochemical responses in plants. These responses include stomatal closure, repression of cell growth and photosynthesis, and activation of respiration. Plants also respond and adapt to water deficit at both the cellular and molecular levels, for instance by the accumulation of osmolytes and proteins specifically involved in stress tolerance. An assortment of genes with diverse functions are induced or repressed by these stresses (Shinozaki et al., 2003; Bartels and Sunkar, 2005; Yamaguchi-Shinozaki and Shinozaki, 2005). Most of their gene products may function in stress response and tolerance at the cellular level. Significantly, the introduction of many stress-inducible genes via gene transfer resulted in improved plant stress tolerance (Zhang et al., 2004; Umezawa et al., 2006a). Recently, a number of stress-inducible genes have been identified using microarray analysis in various plant species, such as Arabidopsis and rice. Now, analysing the functions of these genes is critical to further our understanding of the molecular mechanisms governing plant stress response and tolerance, ultimately leading to enhancement of stress tolerance in crops through genetic manipulation.

Drought triggers the production of the phytohormone abscisic acid (ABA), which in turn causes stomatal closure and induces expression of stress-related genes. Several drought-inducible genes are induced by exogenous ABA treatment, whereas others are not affected. Indeed, evidence exists demonstrating the presence of both ABA-independent and ABA-dependent regulatory systems governing drought-inducible gene expression (Yamaguchi-Shinozaki and Shinozaki, 2005). Both cis-acting and trans-acting regulatory elements functioning in ABA-independent and/or
ABA-responsive gene expression induced by drought stress have been precisely analysed at the molecular level (Yamaguchi-Shinozaki and Shinozaki, 2005). This short article summarizes recent results obtained through transcriptome analysis of drought-inducible gene expression in Arabidopsis and rice using microarrays, including information supporting potential functions of drought-inducible genes in stress response and tolerance. Examples are also cited of how drought-inducible genes are utilized in improvement of drought-stress tolerance by gene transfer. The specific functions of these genes in stress tolerance are also discussed, mainly in Arabidopsis.

Transcriptome analysis of drought-inducible genes in Arabidopsis and rice using microarrays

Microarray technology employing cDNAs or oligonucleotides is a powerful tool for analysing gene expression profiles of plants exposed to abiotic stresses such as drought, high salinity, or cold, or to ABA treatment (Seki et al., 2001, 2002a, b; Krebs et al., 2002). There are two predominant varieties of microarray technology available, the cDNA microarray (Seki et al., 2001, 2002a, b) and the oligonucleotide microarray, the most prominent being the Affimetrix GeneChip (Kreps et al., 2002). A 7000 full-length cDNA microarray was utilized to identify 299 drought-inducible genes, 54 cold-inducible genes, 213 high salinity-inducible genes, and 245 ABA-inducible genes in Arabidopsis (Seki et al., 2002a, b). More than half of these drought-inducible genes were also induced by high salinity and/or ABA treatments, implicating significant cross-talk between the drought, high salinity, and ABA response pathways. In contrast, only 10% of the drought-inducible genes were also induced by cold stress. Thousands of stress-inducible genes were identified using the Affimetrix GeneChip array containing oligonucleotides representing ~8000 independent Arabidopsis genes (Kreps et al., 2002). The stress-inducible genes identified from the cDNA microarray analysis did not coincide with those recognized through GeneChip analyses. This discrepancy in results is primarily due to differences between the sets of genes arrayed in the two systems (only 1919 genes were arrayed in both systems), as well as disparities in plant growth and stress treatment conditions (Maruyama et al., 2004). Recently, the AtGenExpress project assayed the Arabidopsis transcriptome using the Affymetrix 23 000 ATH1 GeneChip, generating thousands of transcriptome data points identifying genes expressed in various tissues and under defined growth conditions, stress induction, and phytohormone treatment (Schmid et al., 2005).

Rice gene expression in response to high salinity stress was first analysed using cDNA [expressed sequence tag (EST)] microarray technology (Kawasaki et al., 2001). Recently, stress-inducible genes in rice were analysed using a microarray including ~1700 independent rice cDNAs (ESTs). These cDNAs were harvested from rice plants exposed to drought, cold, or high salinity stresses (Rabbani et al., 2003). Stress-inducible expression of the candidate genes identified via microarray analysis was confirmed using RNA gel-blot analysis. These analyses confirmed that 73 of these genes were truly stress inducible. Expression of ~40% of drought- or high salinity-inducible genes was also induced by cold stress. In contrast, expression of >98% of the high salinity- and 100% of ABA-inducible genes were also induced by drought stress. These data implicated the existence of a substantial common regulatory system or very significant cross-talk between drought and high salinity stress signalling and between drought and the ABA-induced pathways. These results also indicate a somewhat weaker relationship between the signalling pathways activated in response to cold versus drought stress or between cold versus ABA treatment (Rabbani et al., 2003). These results in rice are consistent with the overlap of gene expression in response to drought and high salinity observed in Arabidopsis. Recently, microarrays constructed using larger oligonucleotides were produced based on full-length cDNA information obtained by the Rice Genome Program of Japan. This rice oligonucleotide array is available through the Agilent Company Ltd. This 22 000 Agilent oligoarray is now being used for rice transcriptome analysis evaluating abiotic stress responses.

Functions of drought-inducible genes in Arabidopsis and rice

The products of the drought-inducible genes identified through the recent microarray analyses in Arabidopsis can be classified into two groups (Shinozaki et al., 2003; Fig. 1). The first group includes proteins that most probably function in abiotic stress tolerance. These include molecules such as chaperones, late embryogenesis abundant (LEA) proteins, osmotin, antifreeze proteins, and phytohormone treatment (Schmid et al., 2005).

Fig. 1. Functions of drought stress-inducible genes in stress tolerance and response. Gene products are classified into two groups. The first group includes proteins that probably function in stress tolerance (functional proteins), and the second group contains protein factors involved in further regulation of signal transduction and gene expression that probably function in stress response (regulatory proteins).
mRNA-binding proteins, key enzymes for osmolyte biosynthesis, water channel proteins, sugar and proline transporters, detoxification enzymes, and various proteins. The second group is comprised of regulatory proteins, i.e. protein factors involved in further regulation of signal transduction and stress-responsive gene expression. These include various transcription factors, protein kinases, protein phosphatases, enzymes involved in phospholipid metabolism, and other signalling molecules such as calmodulin-binding protein. Many transcription factor genes were stress inducible, suggesting that various transcriptional regulatory mechanisms may function in regulating drought, cold, or high salinity stress signal transduction pathways. These transcription factors could govern expression of stress-inducible genes either cooperatively or independently, and may constitute gene networks in *Arabidopsis*.

Similar to the *Arabidopsis* findings, the products of stress-inducible genes identified in rice can also be classified into functional proteins and regulatory proteins (Rabbani et al., 2003). Comparative analysis of stress-inducible genes in *Arabidopsis* with those in rice revealed a considerable degree of similarity in stress responses between the two genomes at the molecular level. Among the 73 genes identified as stress inducible in rice, 51 have already been reported in *Arabidopsis* to perform a similar function. These results confirm that rice shares common stress-inducible genes with *Arabidopsis*, even though these two plants evolved separately more than a million years ago.

**Gene discovery leading to improved drought stress tolerance in plants via gene transfer**

Introduction by gene transfer of several stress-inducible genes has demonstrably enhanced abiotic stress tolerance in transgenic plants (Zhang et al., 2004; Bartels and Sunkar, 2005; Umezawa et al., 2006a). These particular genes encode key enzymes regulating biosynthesis of compatible solutes such as amino acids (e.g. proline), quaternary and other amines (e.g. glycinebetaine and polyamines), and a variety of sugars and sugar alcohols (e.g. mannitol, trehalose, galactinol, and raffinose).

Genes encoding LEA proteins and heat shock proteins have also been used to improve drought tolerance in transgenic plants. A gene encoding galactinol synthase (GolS), a key enzyme involved in raffinose family oligosaccharide biosynthesis, was introduced to improve drought-stress tolerance in transgenic *Arabidopsis* (Taji et al., 2002). Prior analyses demonstrate that GolS genes are induced by drought, cold, and ABA. Moreover, expression of the gene encoding raffinose synthase is also induced by drought stress. Additionally, recent metabolome analysis indicated significant accumulation of both galactinol and raffinose under drought stress. Not only metabolites, but also some stress-responsive proteins such as LEAs, have also been implicated in detoxification and alleviation of cellular damage during dehydration. Other studies demonstrate that overexpression of some LEA class genes results in enhanced tolerance to dehydration, although the precise mechanism is still unknown. LEA proteins may also function as chaperone-like protective molecules to combat cellular damage (Umezawa et al., 2006a).

Transcription factors have also proven quite useful in improving stress tolerance in transgenic plants, through influencing expression of a number of stress-related target genes (Shinozaki et al., 2003; Yamaguchi-Shinozaki and Shinozaki, 2005). The specific molecular mechanisms governing this improved stress tolerance are discussed in detail in the next section.

Other regulatory factors, such as protein kinases and enzymes involved in ABA biosynthesis, are also useful for improving stress tolerance by regulating many stress-related genes in transgenic plants. ABA is synthesized *de novo* primarily in response to drought and high salinity stress. Recently, genes involved in ABA biosynthesis and catabolism were identified based on genetic and genomics analyses (Nambara and Marion-Poll, 2005). It was demonstrated that overexpression of the gene encoding 9-cis-epoxycarotenoid dioxygenase (NCED), a key enzyme in ABA biosynthesis, improves drought stress tolerance in transgenic *Arabidopsis* plants (Iuchi et al., 2001). Recently, a cytochrome P450 CYP707A family member was identified as ABA 8'-hydroxylase, an enzyme that degrades ABA during seed imbibition and dehydration stress (Kushiro et al., 2004; Saito et al., 2004). A T-DNA insertion mutant of CYP707A3, which is the most abundantly expressed gene amongst the four CYP707A members under stress conditions, exhibited elevated drought tolerance with a concomitant reduction in transpiration rate (Umezawa et al., 2006b).

The ABA-activated SnRK2 protein kinase (OST1/SRK2E) functions in the ABA signal transduction pathway controlling stomatal closure (Mustilli et al., 2002; Yoshida et al., 2002). SnRK2 is a member of the SNF1-related PKase family, which contains 10 members in *Arabidopsis* and rice. SnRK2s are activated by drought, salinity, and ABA (Yoshida et al., 2002). SRK2E/OST1 is involved in stomatal closure, but not seed germination. Another SnRK2, SRK2C, is activated by osmotic stress, salt stress, and ABA treatment (Umezawa et al., 2004). SRK2C is strongly expressed in the root tip, and is involved in the root response to drought stress. SRK2C also functions in transgenic plants to improve stress tolerance, as many of the downstream genes it influences are stress inducible. In addition, SnRK2 protein kinases may activate transcription factors influencing osmotic stress-responsive gene expression.

**Regulation of gene expression: ABA-independent pathways influencing the drought-stress response**

The promoter of a drought-, high salinity-, and cold-inducible gene, *RD29A/COR78/LTI78*, contains two major
cis-acting elements, ABRE (ABA-responsive element) and DRE (dehydration-responsive element)/CRT (C-RepeaT), both of which are involved in stress-inducible gene expression (Yamaguchi-Shinozaki and Shinozaki, 1994, 2005). ABRE and DRE/CRT are cis-acting elements that function in ABA-dependent and ABA-independent gene expression, respectively, in response to abiotic stress (Fig. 2).

Transcription factors belonging to the ERF/AP2 family that bind to these DRE/CRT elements were isolated and termed CBF/DREB1 and DREB2 (Yamaguchi-Shinozaki and Shinozaki, 2005). Their conserved DNA-binding motif is A/GCCGAC. The CBF/DREB1 genes are rapidly and transiently induced by cold stress, the products of which activate the expression of target stress-inducible genes (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999). Overexpression of CBF/DREB1 in transgenic plants increased stress tolerance to freezing, drought, and salt stresses, suggesting that the CBF/DREB1 proteins function in the development of cold-stress tolerance without modification (Liu et al., 1998). Many CBF/DREB1 target genes have been identified using both cDNA and GeneChip microarrays (Seki et al., 2001; Fowler and Thomashow, 2002; Maruyama et al., 2004; Vogel et al., 2005). Most of the CBF/DREB1 target genes contain the DRE motif with a conserved (A/G)CCGACNT sequence in their promoter regions. The target gene products of these proteins are consequently involved in establishing stress tolerance. The DREB2 genes are induced by dehydration stress and may activate other genes involved in drought stress tolerance (Liu et al., 1998). However, overexpression of DREB2 in transgenic plants did not improve stress tolerance, suggesting involvement of post-translational activation of DREB2 proteins (Liu et al., 1998). Recently, an active form of DREB2 was shown to transactivate target stress-inducible genes and improve drought tolerance in transgenic Arabidopsis (Sakuma et al., 2006). The DREB2 protein is expressed under normal growth conditions and activated by osmotic stress through post-translational modification in the early stages of the osmotic stress response.

Rice homologues of CBF/DREB1 and DREB2, 10 OsDREB1s and four OsDREB2s, respectively, have been identified based on rice genome sequence analyses. The function of these genes in stress-inducible gene expression has been demonstrated in rice. Overexpression of OsDREB1A in Arabidopsis revealed a similar function of the rice genes in stress-responsive gene expression and stress tolerance (Dubouzet et al., 2003). Recently, overexpression of OsDREB1 or Arabidopsis DREB1 also improved drought and chilling tolerance in rice (Ito et al., 2006). These data indicate that similar transcription factors function in abiotic stress tolerance between dicotyledonous and monocotyledonous plants.

Several drought-inducible genes do not respond to either cold or ABA treatment, suggesting the existence of another

Fig. 2. Transcriptional regulatory networks of abiotic stress signals and gene expression. At least six signal transduction pathways exist in drought, high salinity, and cold-stress responses: three are ABA dependent and three are ABA independent. In the ABA-dependent pathway, ABRE functions as a major ABA-responsive element. AREB/ABFs are AP2 transcription factors involved in this process. MYB2 and MYC2 function in ABA-inducible gene expression of the RD22 gene. MYC2 also functions in JA-inducible gene expression. The RD26 NAC transcription factor is involved in ABA- and JA-responsive gene expression in stress responses. These MYC2 and NAC transcription factors may function in cross-talk during abiotic-stress and wound-stress responses. In one of the ABA-independent pathways, DRE is mainly involved in the regulation of genes not only by drought and salt but also by cold stress. DREB1/CBFs are involved in cold-responsive gene expression. DREB2s are important transcription factors in dehydration and high salinity stress-responsive gene expression. Another ABA-independent pathway is controlled by drought and salt, but not by cold. The NAC and HD-ZIP transcription factors are involved in ERD1 gene expression.
ABA-independent pathway regulating the dehydration stress response. These genes include ERD1, which encodes a Clp protease regulatory subunit, ClpD. The ERD1 gene is not only induced by dehydration but is also up-regulated during natural senescence and dark-induced senescence (Nakashima et al., 1997). Promoter analysis of the ERD1 gene in transgenic plants indicates that the ERD1 promoter contains cis-acting element(s) involved not only in ABA-independent stress-responsive gene expression but also in senescence-activated gene expression. Analysis of the ERD1 promoter further identified two different novel cis-acting elements involved with dehydration stress induction and in dark-induced senescence (Simpson et al., 2003). Recently, DNA-binding proteins interacting with these cis-elements were identified as NAC transcription factors (Tran et al., 2004).

**Gene expression controlled by endogenous ABA accumulated under drought stress**

ABRE is a major cis-acting element in ABA-responsive gene expression (Fig. 2). Two ABRE motifs are important cis-acting elements controlling ABA-responsive expression of the Arabidopsis RD29B gene (Uno et al., 2000). Two basic leucine zipper (bZIP) transcription factors, AREB/ABF, can bind to ABRE, thereby activating ABA-dependent gene expression (Choi et al., 2000; Uno et al., 2000). The AREB/ABF proteins require an ABA-mediated signal for their activation, as indicated by their reduced activity in the ABA-deficient aba2 and ABA-insensitive abil mutants and their enhanced activity in the ABA-hypersensitive era1 mutant of Arabidopsis (Uno et al., 2002). This phenomenon is very probably due to the ABA-dependent phosphorylation of the AREB/ABF proteins. Overexpression of ABF3 or AREB2/ABF4 caused ABA hypersensitivity, reduced the transpiration rate, and enhanced drought tolerance in transgenic Arabidopsis plants (Kang et al., 2002). Recently, transgenic plants expressing a phosphorylated form of AREB1 with multisite mutations displayed induction of many ABA-responsive genes without exogenous ABA application (Fujita et al., 2005; Furihata et al., 2006). These data suggest that such constitutively active forms of transcription factors rendered by point mutations may contribute to enhancement of drought tolerance in transgenic plants.

Induction of the drought-inducible RD22 gene is mediated by ABA and requires protein biosynthesis for its ABA-dependent expression. A MYC transcription factor, AtMYC2 (RD22BP1), and a MYB transcription factor, AtMYB2, were shown to bind cis-elements in the RD22 promoter and co-operatively activate RD22 (Abe et al., 1997, 2003; Fig. 2). These MYC and MYB proteins are synthesized following accumulation of endogenous ABA, defining their role in later stage stress responses. Microarray analysis revealed target genes of MYC/MYB in overexpressing transgenic plants, such as alcohol dehydrogenase and ABA- or jasmonic acid (JA)-inducible genes (Abe et al., 2003). Overexpression of both AtMYC2 and AtMYB2 not only resulted in an ABA-hypersensitive phenotype but also improved osmotic stress tolerance of the transgenic plants.

Recently, a drought-inducible RD26 gene encoding a NAC transcription factor was identified (Fujita et al., 2004). Expression of this RD26 NAC transcription factor gene is induced by drought, high salinity, ABA, and JA treatments. RD26 protein is localized in the nucleus and has transcriptional activity. An RD26-overexpressing transgenic plant was hypersensitive to ABA, and an RD26 dominant repressor transgenic was insensitive to ABA. It was observed that ABA- and stress-inducible genes were up-regulated in the RD26-overexpressing transgenics and repressed in the RD26 repressor lines. However, it was also discovered that typical ABA-inducible genes such as LEA, RD, ERD, COR, and KIN are not target genes of RD26, whereas many JA-inducible genes are target genes of RD26 (Fujita et al., 2004). This indicates an important role for RD26 in mediating cross-talk between ABA signalling and JA signalling during drought and wounding stress responses. Moreover, RD26 is induced by JA. Thus, RD26 may be involved in wounding-related gene expression based on the microarray analysis.

**Conclusion**

Transcriptome analyses based on microarrays have provided powerful tools for discovery of stress-responsive genes not only in Arabidopsis but also in various crop plants and tree species. Transgenic plants generated to express antisense or RNAi constructs, as well as T-DNA- or transposon-tagged mutants, were used to analyse the function of these stress-responsive genes based upon phenotypes resulting from loss of function. Moreover, transgenic overexpressors were very useful not only for functional analyses of stress-inducible genes but also for demonstrating improved stress tolerance in these plants generated by gene transfer. Introduction of Arabidopsis stress-related genes proved valuable for improving drought-stress tolerance in transgenic crops and trees, as well as serving as key tools for the discovery of stress-related genes in those systems by a comparative genomics approach.

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