Improving plant drought, salt and freezing tolerance by gene transfer of a single stress-inducible transcription factor

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Abstract. Plant productivity is greatly affected by environmental stresses such as drought, salt loading and freezing. We reported that a *cis*-acting promoter element, the dehydration response element (DRE), plays an important role in regulating gene expression in response to these stresses in *Arabidopsis*. The transcription factor DREB1A specifically interacts with the DRE and induces expression of stress tolerance genes. We show here that overexpression of the cDNA encoding DREB1A in transgenic *Arabidopsis* plants activated the expression of many of theses stress tolerance genes under normal growing conditions and resulted in improved tolerance to drought, salt loading and freezing. However, use of the strong constitutive 35S cauliflower mosaic virus (CaMV) promoter to drive expression of DREB1A also resulted in severe growth retardation under normal growing conditions. In contrast, expression of DREB1A from the stress-inducible rd29A promoter gave rise to minimal effects on plant growth while providing an even greater tolerance to stress conditions than did expression of the gene from the CaMV promoter. As the DRE-related regulatory element is not limited to *Arabidopsis* the DREB1A cDNA and the rd29A promoter may be useful for improving the stress tolerance of agriculturally important crops by gene transfer.

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Drought, salt loading and freezing are environmental conditions that cause adverse effects on the growth of plants and the productivity of crops. Plants respond to these stresses at molecular and cellular levels as well as the physiological level. Expression of a variety of genes is induced by these stresses (Shinozaki & Yamaguchi-Shinozaki 1996, Thomashow 1994). The products of these genes are thought to function not only in stress tolerance but also in the

regulation of gene expression and signal transduction in stress response (Shinozaki & Yamaguchi-Shinozaki 1997).

Genetic engineering may be useful for improving plant stress tolerance, and several different approaches have recently been attempted (Holmberg & Bulow 1998). The genes selected for transformation were those involved in encoding enzymes required for the biosynthesis of various osmoprotectants (Tarczynski & Bohnert 1993, Kavi Kishor et al 1995, Hayashi et al 1997). Other genes that have been selected for transformation include those that encoded enzymes for modifying membrane lipids, LEA protein and detoxification enzyme (Kodama et al 1994, Ishizaki-Nishizawa et al 1996, Xu et al 1996, McKersie et al 1996). In all these experiments, a single gene for a protective protein or an enzyme was overexpressed under the control of the 35S cauliflower mosaic virus (CaMV) constitutive promoter in transgenic plants, although a number of genes have been shown to function in environmental stress tolerance and response. The genes encoding protein factors that are involved in regulation of gene expression, signal transduction and function in the stress response are promising candidates for the genetic engineering of stress tolerance as they can regulate a range of stress-inducible genes.

Drought is one of the most severe environmental stresses and affects almost all plant functions. Abscisic acid (ABA) is produced under water deficit conditions and plays important roles in tolerance against drought. Most of the drought-inducible genes that have been studied to date are also induced by ABA (Shinozaki & Yamaguchi-Shinozaki 1997). Several reports have described genes that are induced by dehydration but are not responsive to exogenous ABA treatments (Shinozaki & Yamaguchi-Shinozaki 1997). These findings suggest the existence of ABA-independent as well as ABA-dependent signal transduction cascades between the initial signal of drought stress and the expression of specific genes (Shinozaki & Yamaguchi-Shinozaki 1997). To understand the molecular mechanisms of gene expression in response to drought stress, we have analysed in detail *cis*- and *trans*-acting elements that function in ABA-independent and ABA-responsive gene expression by drought stress (Shinozaki & Yamaguchi-Shinozaki 1997). In this paper we summarize the recent progress of our research on *cis*- and *trans*-acting factors involved in ABA-independent gene expression in drought stress response. We also report stress tolerance of transgenic plants that overexpress a single gene for a stress-inducible transcription factor using *Arabidopsis* as a model.

The function of water stress-inducible genes

A variety of genes are induced by drought-stress, and the functions of their gene products have been predicted from sequence homology with known proteins.

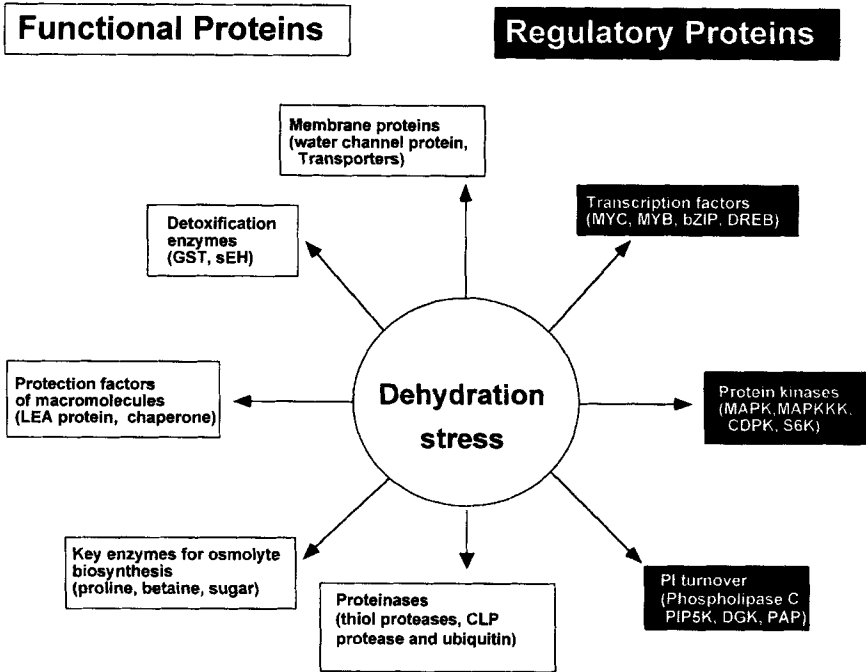| Functional Proteins		Regulatory Proteins

FIG. 1. Drought-stress inducible genes and their possible functions 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); the second group contains protein factors involved in further regulation of signal transduction and gene expression that probably function in stress response (regulatory proteins).

Genes induced during drought stress conditions are thought to function not only in protecting cells from dehydration by the production of important metabolic proteins, but also in the regulation of genes for signal transduction in the drought stress response (Shinozaki & Yamaguchi-Shinozaki 1996, 1997). Thus, these gene products are classified into two groups (Fig. 1).

The first group includes proteins that probably function in stress tolerance (Shinozaki & Yamaguchi-Shinozaki 1997): water channel proteins involved in the movement of water through membranes, the enzymes required for the biosynthesis of various osmoprotectants (sugars, proline and betaine), proteins that may protect macromolecules and membranes (LEA protein, osmotin, antifreeze protein, chaperones and mRNA binding proteins), proteases for protein turnover (thiol proteases, Clp protease, and ubiquitin) and the detoxification enzymes (glutathione S-transferase, soluble epoxide hydrolase, catalase, superoxide dismutase and ascorbate peroxidase). Some of the stress-inducible

genes that encode proteins such as a key enzyme for proline biosynthesis, have been overexpressed in transgenic plants to produce a stress-tolerant phenotype of the plants (Kavi Kishor et al 1995).

The second group contains protein factors involved in the further regulation of signal transduction and gene expression that probably function in the stress response: protein kinases, transcription factors and enzymes involved in phospholipid metabolism (Shinozaki & Yamaguchi-Shinozaki 1997). It is important for us to elucidate the role of these regulatory proteins for understanding plant responses to water stress and for improving the tolerance of plants by gene transfer. The existence of a variety of drought-inducible genes suggests complex responses of plants to drought stress. Their gene products are involved in drought stress tolerance and stress responses.

Expression of dehydration-induced genes in response to environmental stresses and ABA

We analysed the expression patterns of genes induced by drought by RNA gel-blot analysis. The results indicated broad variations in the timing of induction of these genes under drought conditions. Most of the drought-inducible genes respond to treatment with exogenous ABA, but others do not (Shinozaki & Yamaguchi-Shinozaki 1996, 1997). Therefore, there are not only ABA-dependent but also ABA-independent regulatory systems of gene expression under drought stress. Analysis of the expression of ABA-inducible genes revealed that several genes require protein biosynthesis for their induction by ABA, suggesting that at least two independent pathways exist between the production of endogenous ABA and gene expression during stress.

As shown in Fig. 2, we identified at least four independent signal pathways which function under drought conditions: two are ABA-dependent (pathways I and II) and two are ABA-independent (pathways III and IV). One of the ABA-dependent pathways overlaps with that of the cold response (pathway IV). One of the ABA-independent pathways requires protein biosynthesis (pathway II) (Shinozaki & Yamaguchi-Shinozaki 1996, 1997). The existence of complex signal transduction pathways in the drought response gives a molecular basis for the complex physiological responses of plants to drought stress.

Identification of a cis-acting element, DRE, involved in drought-responsive gene expression

A number of genes are induced by drought, salt and cold in *aba* (ABA-deficient) or *abi* (ABA-insensitive) *Arabidopsis* mutants. This suggests that these genes do not require ABA for their expression under cold or drought condition. Among these
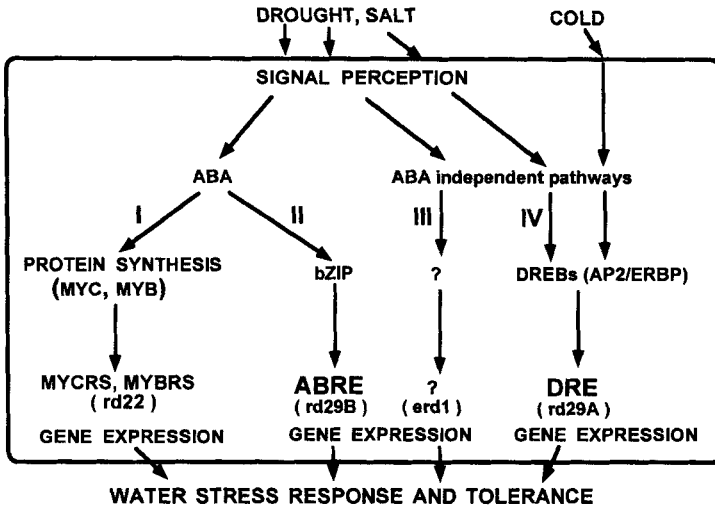
FIG. 2.   Signal transduction pathways between initial dehydration stress signal and gene expression. There are at least four signal transduction pathways: two are ABA-dependent (I and II) and two are ABA-independent (III and IV). Protein synthesis is necessary for one of the ABA-dependent signal pathways (I). ABRE is involved in one of the ABA-dependent pathways (II). In one of the ABA-independent pathways, DRE is involved in the regulation of genes not only by drought and salt but also by cold stress (IV). Another ABA-independent pathway is controlled by drought and salt, but not by cold (III).

genes, the expression of a drought-inducible gene for rd29A/lti78/cor78 was extensively analysed (Yamaguchi-Shinozaki & Shinozaki 1994). At least two separate regulatory systems function in gene expression during drought and cold stress; one is ABA-independent (Fig. 2, pathway IV) and the other is ABA-dependent (pathway II).

To analyse the *cis*-acting elements involved in the ABA-independent gene expression of rd29A, we constructed chimeric genes with the rd29A promoter fused to the β-glucuronidase (*GUS*) reporter gene and transformed *Arabidopsis* and tobacco plants with these constructs. The *GUS* reporter gene driven by the rd29A promoter was induced at significant levels in transgenic plants by dehydration, low-temperature, high-salt or ABA treatment (Yamaguchi-Shinozaki & Shinozaki 1993). The deletion, the gain-of-function and the base substitution analysis of the promoter region of *rd29A* gene revealed that a 9 bp conserved sequence, TACCGACAT (DRE, Dehydration Responsive Element), is essential for the regulation of the expression of *rd29A* under drought conditions. Moreover, DRE has been demonstrated to function as a *cis*-acting element involved in the induction of *rd29A* by either low-temperature or high-salt stress (Yamaguchi-Shinozaki & Shinozaki 1994). Therefore, DRE seems to

be a *cis*-acting element involved in gene induction by dehydration, high-salt, or low temperature, but does not function as an ABA-responsive element in the induction of *rd29A*.

Important roles of the DRE binding proteins during drought and cold stresses

Two cDNA clones that encode DRE binding proteins, DREB1A and DREB2A, were isolated by using the yeast one-hybrid screening technique (Liu et al 1998). The deduced amino acid sequences of DREB1A and DREB2A showed significant sequence similarity with the conserved DNA binding domains found in the EREBP and APETALA2 proteins that function in ethylene-responsive expression and floral morphogenesis, respectively (Okamuro et al 1997, Ohme-Takagi & Shinshi 1995). Each DREB protein contained a basic region in its N-terminal region that might function as a nuclear localization signal and an acidic C-terminal region that might act as an activation domain for transcription. These data suggest that each DREB cDNA encodes a DNA binding protein that might function as a transcriptional activator in plants.

We examined the ability of the DREB1A and DREB2A proteins expressed in *Escherichia coli* to bind the wild-type or mutated DRE sequences using the gel retardation method (Liu et al 1998). The results indicate that the binding of these two proteins to the DRE sequence is highly specific. To determine whether the DREB1A and DREB2A proteins are capable of transactivating DRE-dependent transcription in plant cells, we performed transactivation experiments using protoplasts prepared from *Arabidopsis* leaves. Coexpression of the DREB1A or DREB2A proteins in protoplasts transactivated the expression of the *GUS* reporter gene. These results suggest that DREB1A and DREB2A proteins function as transcription activators involved in the cold- and dehydration-responsive expression, respectively, of the *rd29A* gene (Fig. 3) (Liu et al 1998).

We isolated cDNA clones encoding two DREB1A homologues (named DREB1B and DREB1C). The DREB1B clone was identical to CBF1 (Stockinger et al 1997). We also isolated cDNA clones encoding a DREB2A homologue and named it DREB2B. Expression of the *DREB1A* gene and its two homologues was induced by low-temperature stress, whereas expression of the *DREB2A* gene and its single homologue was induced by dehydration (Liu et al 1998, Shinwari et al 1998). These results indicate that two independent families of DREB proteins, DREB1 and DREB2, function as *trans*-acting factors in two separate signal transduction pathways under low-temperature and dehydration conditions, respectively (Fig. 3) (Liu et al 1998).
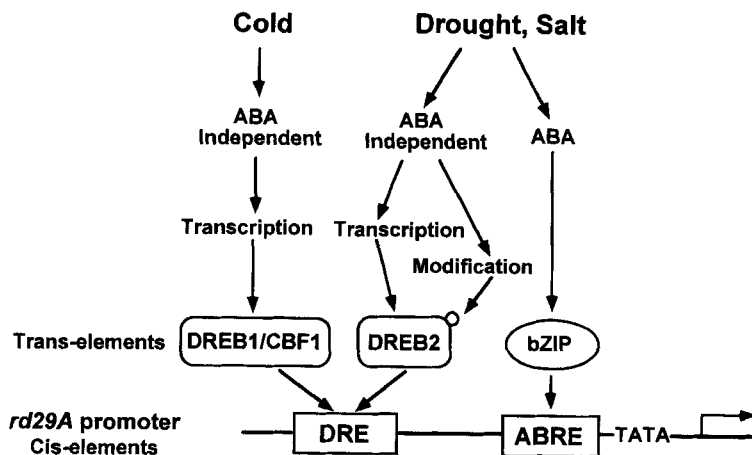
FIG. 3. A model of the induction of the *rd29A* gene and *cis-* and *trans*-acting elements involved in stress-responsive gene expression. Two *cis*-acting elements, DRE and ABRE, are involved in the ABA-independent and ABA-responsive induction of *rd29A*, respectively. Two types of different DRE binding proteins, DREB1 and DREB2, separate two different signal transduction pathways in response to cold and drought stresses, respectively. DREB1s/ CBF1 are transcriptionally regulated whereas DREB2s are controlled post-translationally as well as transcriptionally. ABRE binding proteins encode bZIP transcription factors.

Analysis of the in roles of DREB1a and DREB2a by using transgenic plants

We generated transgenic plants in which *DREB1A* or *DREB2A* cDNAs were introduced to overproduce DREB proteins to analyse the effects of overproduction of DREB1A and DREB2A proteins on the expression of the target *rd29A* gene. *Arabidopsis* plants were transformed with vectors carrying fusions of the enhanced CaMV 35S promoter and the *DREB1A* (*35S:DREB1A*) or *DREB2A* (*35S:DREB2A*) cDNAs in the sense orientation (Liu et al 1998, Mituhara et al 1996). All of the transgenic plants carrying the *35S:DREB1A* transgene (the *35S:DREB1A* plants) showed growth-retardation phenotypes under normal growth conditions. The *35S:DREB1A* plants showed variations in phenotypic changes in growth retardation that may have been due to the different levels of expression of the *DREB1A* transgenes for the position effect (Liu et al 1998).

To analyse whether overproduction of the DREB1A protein caused the expression of the target gene in unstressed plants, we compared the expression of the *rd29A* gene in control plants carrying the pBI121 vector. Transcription of the *rd29A* gene was low in the unstressed wild-type plants but high in the unstressed *35S:DREB1A* plants (Liu et al 1998). The level of the *rd29A* transcripts under the

unstressed control condition was found to depend on the level of the DREB1A transcripts (Liu et al 1998). To analyse whether overproduction of the DREB1A protein caused the expression of other target genes, we evaluated the expression of its target stress-inducible genes. In the *35S:DREB1A* plants the *kin1*, *cor6.6/kin2*, *cor15a*, *cor47/rd17* and *erd10* genes were expressed strongly under unstressed control conditions, as was the *rd29A* gene (Shinozaki & Yamaguchi-Shinozaki 1997, Kiyosue et al 1994).

In contrast, the transgenic plants carrying the *35S:DREB2A* transgene (the *35S:DREB2A* plants) showed little phenotypic change. In *35S:DREB2A* transgenic plants, the *rd29A* mRNA did not accumulate significantly, although the *DREB2A* mRNA accumulated even under unstressed conditions (Liu et al 1998). Expression of the DREB2A protein is not sufficient for the induction of the target stress-inducible gene. Modification, such as phosphorylation of the DREB2A protein, seems to be necessary for its function in response to dehydration (Fig. 3). However, DREB1 proteins can function without modification.

Drought, salt and freezing stress tolerance in transgenic plants

The tolerance to freezing and dehydration of the transgenic plant was analysed using the *35S:DREB1A* plants grown in pots at 2 °C for 3 weeks. When plants were exposed to a temperature of −6 °C for 2 days, returned to 22 °C, and grown for 5 days, all of the wild-type plants died, whereas the *35S:DREB1A* plants survived at high frequency (Liu et al 1998, Kasuga et al 1999). Freezing tolerance was correlated with the level of expression of the stress-inducible genes under unstressed control conditions (Fig. 4; between 80 and 30%, survival) (Liu et al 1998, Kasuga et al 1999).

To test whether the introduction of the *DREB1A* gene enhances tolerance to dehydration stress, we did not water the plants for 2 weeks. Although all of the wild-type plants died within 2 weeks, between 70 and 20% of the *35:SDREB1A* plants survived and continued to grow after re-watering. Drought tolerance was also dependent on the level of expression of the target genes in the *35S:DREB1A* plants under unstressed conditions (Fig. 4) (Liu et al 1998, Kasuga et al 1999).

Overexpression of the *DREB1A* cDNA, driven by the constitutive 35S CaMV promoter in transgenic plants, activated strong expression of the target stress-inducible genes under unstressed conditions, which, in turn, increased tolerance of freezing, salt and drought stresses(Liu et al 1998, Kasuga et al 1999). Jaglo-Ottosen et al (1998) reported that CBF1 overexpression also enhances freezing tolerance. However, the overexpression of stress-inducible genes controlled by the DREB1A protein caused severe growth retardation under normal growth conditions (Liu et al 1998, Kasuga et al 1999).
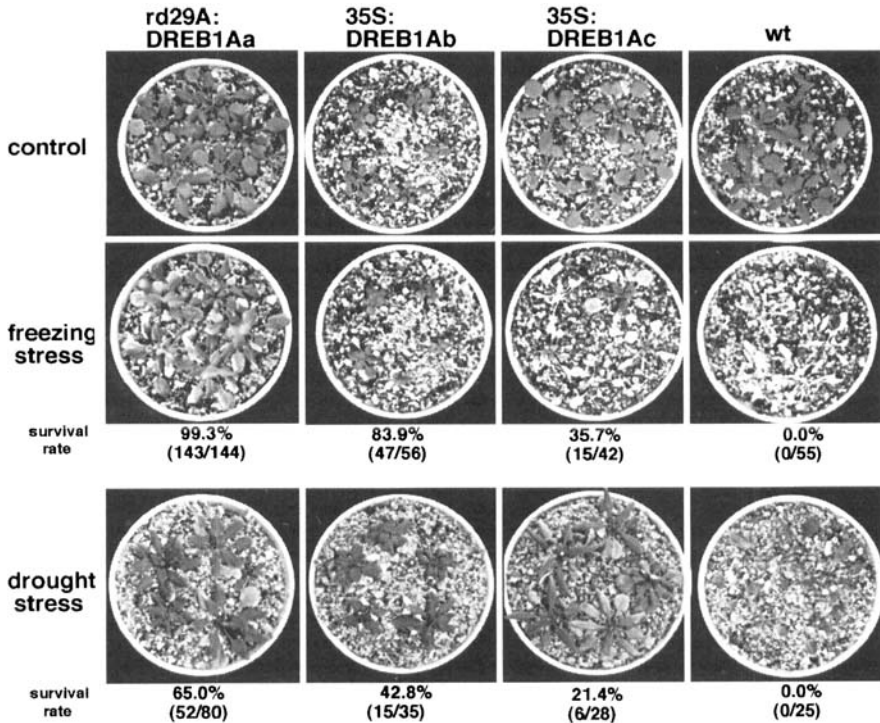
FIG. 4. Freezing and drought stress tolerance of the *35S:DREB1Ab*, *35S:DREB1Ac* and *rd29A:DREB1Aa* transgenic plants. The stress treatments were conducted as described in the text. Control, 3 week old plants growing under normal conditions; freezing, plants exposed to a temperature of −6 °C for 2 days and returned to 22 °C for 5 days; drought, water withheld for 2 weeks. Percentages of surviving plants and numbers of surviving plants per total tested plants are given under the pictures.

To resolve the problem of growth retardation, we used the stress-inducible rd29A promoter to cause overexpression of *DREB1A* in transgenic plants (*rd29A:DREB1A* plants) (Kasuga et al 1999). Because the rd29A promoter was stress-inducible and contained binding sites for the DREB1A protein, it did not cause expression of the *DREB1A* transgene at high levels under unstressed conditions; instead, it rapidly amplified expression of the *DREB1A* transgene only under dehydration, salt and low-temperature stress. The *rd29A:DREB1A* plants revealed strong stress tolerance even though their growth retardation under normal growing conditions was not significant. Moreover, the growth and the productivity of these plants were almost the same as those of the wild-type plants under normal growing conditions (Kasuga et al 1999).

On the contrary, the *rd29A :DREB1A* transgenic plants are more tolerant to the stresses than the *35S:DREB1A* plants that exhibited growth retardation under normal growing conditions (Fig. 4) (Kasuga et al 1999). As the *rd29A* gene is one of the target genes of the DREB1A protein, the rd29A promoter is more suitable for the tissue-specific expression of the *DREB1A* gene in plants than the 35S CaMV promoter. In the *rd29A :DREB1A* plants, the target gene products seem to be strongly accumulated in the same tissues that express the products under stress conditions. These results indicate that combination of the *DREB1A* cDNA with the rd29A promoter would be quite useful for improving drought, salt and freezing-stress tolerance in transgenic plants.

In a previous studies, we showed that DRE also functions in gene expression in response to stress in tobacco plants (Yamaguchi-Shinozaki & Shinozaki 1993, 1994), which suggests the existence of similar regulatory systems in tobacco and other crop plants. DRE-related motifs have been reported in the promoter region of cold-inducible *Brassica napus* and wheat genes (Jiang et al 1996, Ouellet et al 1998). These observations suggest that both the *DREB1A* cDNA and the rd29A promoter can be used to improve the dehydration, salt and freezing tolerance of crops by gene transfer.

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DISCUSSION

Leach: In addition to the non-abiotic stresses, did you test any biological stresses on these plants? Were they protected?

Yamaguchi-Shinozaki: We haven't tried them yet. However, we found that one of the targets for DREB1A is a gene for heat shock protein, so now we are analysing the heat shock tolerance of these *DREB1A* transgenic plants.

Ku: Following on from this, I think it might be worth looking at how these transgenics respond to other stresses, such as pathogen stress. The recent work by Griffith's group at the University of West Ontario (Antikainen & Griffith 1997), found large amounts of cold-induced antifreeze protein in cold-tolerant cereal crops. The twist in the story is that these antifreeze proteins turn out to be pathogenesis-related (PR) proteins that have been recruited by the cereal crops for antifreeze functions. Perhaps your transgenics will also have enhanced pathogen resistance as well as cold and drought tolerance.

Yamaguchi-Shinozaki: We should try this.

Ku: Are you considering a strategy involving the expression of both transcriptional factors in the same plant? If so, would you expect additive effects on stress tolerance?

Yamaguchi-Shinozaki: Perhaps. We have also been working on the gene for proline synthetase. We got good results with the transgenic plants accumulating proline at higher levels. We are now trying to introduce genes for both DREB1A and proline synthetase into plants.

Okita: What do you think is the overall mechanism behind cold and drought tolerance? I am struck by the fact that constitutive expression of the genes involved greatly retards growth in these plants.

Yamaguchi-Shinozaki: Although some of the genes we have targeted affect plant growth, others don't. We have overexpressed rd29A without seeing any growth retardation.

Okita: When you overexpress *DREB2A*, is the plant just maintaining homeostasis of the cells, preventing growth from occurring and thereby allowing the plant to survive the stressful conditions? Or are you building some kind of mechanism into the plant that still allows it to grow under these stress conditions?

Yamaguchi-Shinozaki: We haven't tried this kind of experiment. We are now using the rd29A promoter, which is reversible. After the stress conditions, if the conditions are good, we can get enough seed from this plant.

Dong: Have you tried treating the rd29A-driven *DREB1A* transgenic with drought or cold at different developmental stages?

Yamaguchi-Shinozaki: We treat briefly with stress conditions that are severe enough that the wild-type plant would not survive, so we don't have a comparison.

Bennett: Am I to understand from your paper that the promoter of *DREB1A* is comparatively weak, and that when it is replaced by a stronger promoter with similar induction properties, there is a much higher level of DREB1 protein?

Yamaguchi-Shinozaki: Actually, we use the rd29A promoter to overexpress *DREB1A* in the transgenic plant because this promoter has a binding site for the DREB1 protein. This promoter can self-amplify. This promoter is the strongest one for expressing the DREB1A protein in *Arabidopsis*.

Salmeron: Have you looked for mutations in these transcription factors?

Yamaguchi-Shinozaki: DREB1A has two homologues. When *DREB1A* is disrupted, there is no difference in phenotype because the homologues compensate for its loss. If we wanted to make a mutant with a phenotype we would have to disrupt *DREB1B* and *DREB1C* also.

Mazur: How did you initially identify those stress- and drought-responsive genes?

Yamaguchi-Shinozaki: We used a differential screening method with cDNA probes prepared from dehydrated and non-dehydrated *Arabidopsis*.

Mazur: Did you pre-select the probes at all?

Yamaguchi-Shinozaki: We prepared a library using dehydration-treated *Arabidopsis*. It contained drought-inducible genes, and as a probe we used cDNA prepared from normal or dehydrated plants.

Li: Was the rd29A promoter originally isolated from a resistant genotype, or is it randomly isolated from the induced condition in a susceptible line?

Yamaguchi-Shinozaki: Originally we isolated the cDNAs for drought inducible genes. One of these was *rd29*. We isolated the promoter region of this gene using a genomic library.

Li: Was the cDNA library prepared from a drought-resistant or tolerant genotype?

Yamaguchi-Shinozaki: We used the colombia strain of *Arabidopsis*, which we subjected to dehydration.

Li: Does this line show any drought or salinity tolerance, or any co-tolerance to the stress?

Yamaguchi-Shinozaki: Arabidopsis shows some degree of drought tolerance.

Li: The reason I ask is that I want to know whether the original material shows a stronger tolerance as compared to the transgenic lines: is this strong promoter really from very good germplasm? If this is the case, we might want to screen with this method to find good promoters in rice or other species.

Yamaguchi-Shinozaki: We are also using the cowpea crop grown in a semi-arid region. This shows good drought stress tolerance. In this case we also isolated a similar cDNA.

Li: So what you suggest is that even from a germplasm without a very strong tolerance, we can identify good promoters or good alleles for these tolerant genes or quantitative trait loci (QTLs). This is an important issue. We need to know whether we need to look at the very best germplasm we have, or whether we can just randomly select from diverse sources of germplasm.

Dong: But if you already have such good germplasm you don't need molecular engineering.

Li: That is not the case: drought tolerance in rice is very complex. We will not be able to identify a single gene line in this case. We are very surprised that a single gene promoter can create such a high level of drought or salinity tolerance.

G.-L. Wang: You have shown that the rd29A promoter is much more effective than the 35S promoter. The next step will probably involve field-testing of these plants. Is this promoter also sensitive to temperature, sunlight and other environmental variables? In the greenhouse these can be controlled but it is hard to control them in the fields.

Yamaguchi-Shinozaki: We want to apply this technology to another crop plant. In this case we should try field testing. With *Arabidopsis* the *rd29A* gene is a strong promoter — perhaps even the strongest promoter in *Arabidopsis* under stress conditions. But when we tried this promoter in rice, we got different results. It is also inducible in the rice root, but we couldn't detect induction of the induced genes in the leaves. If we want to apply this technology to another crop plant, we have to isolate the original promoter for that type of crop plant. It may be possible to use DREB1A in rice also, but we need another promoter because of the different tissue specificity.

Reference

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