Developing Transgenic Tolerance to Environmental Stresses Including Drought in Plant

Kazuko Yamaguchi-Shinozaki[1] and Kazuo Shinozaki[2]

Abstract

Many plant genes have been shown to be induced by drought stress and function in drought tolerance. We isolated more than 40 independent genes that are responsive to drought in *Arabidopsis thaliana* and cowpea and analyzed the structure of their gene products. Sequence analyses of these genes indicate that their gene products may function in protecting cells from dehydration. We also analyzed the expression of these genes under various stress conditions. Some of the drought-responsive genes are induced by plant hormone abscisic acid (ABA), unlike others. There seem to be at least four independent signal transduction pathways between initial drought stress signal and gene expression. Two of the pathways are ABA-dependent and two are ABA-independent. We precisely analyzed the promoter regions of two drought-inducible *Arabidopsis* genes, *rd29A* and *rd29B*, in both transgenic *Arabidopsis* and tobacco, and identified a novel cis-acting element containing 9 bp, TACCGACAT (DRE, Dehydration Responsive Element), that is involved in the ABA-independent response of *rd29A* to conditions of dehydration and high salt. DRE is also involved in the induction by low temperature, but does not function in the ABA-responsive, slow expression of *rd29A*.

Introduction

It is important to breed crops for tolerance to various environmental stresses to solve the food crisis and environmental pollution in the 21st century. Biotechnology has a potential to improve the tolerance of crops to environmental stresses using transgenic plant technology. Most important points for the development of transgenic tolerance to stresses are the isolation of genes that function in plant responses against various environmental stresses and the precise understanding of the molecular process of stress response in plants. Plants respond to conditions of environmental stresses with various physiological and developmental changes to tolerate these stresses. Drought is one of the most severe environmental stresses and affects almost all the plant functions including photosynthesis, growth and development. Abscisic acid(ABA) appears to be involved in the ability of plants to tolerate drought stress (Chandler and Robertson, 1994). ABA is produced under water deficit conditions and plays important roles in the tolerance against drought.

[1] Biological Resources Division, Japan International Research Center for Agricultural Sciences (JIRCAS), Ministry of Agriculture, Forestry and Fisheries, 2-1 Ohwashi, Tsukuba, Ibaraki, 305 Japan

[2] Laboratory of Plant Molecular Biology, The Institute of Physical and Chemical Research (RIKEN), Tsukuba Life Science Center, 3-1-1 Koyadai, Tsukuba, Ibaraki, 305 Japan

Recently, a number of genes have been described that respond to drought at the transcriptional level(Bray, 1993; Bohnert, 1995; Shinozaki and Yamaguchi-Shinozaki, 1996). Most of the genes that have been studied to date are also induced by ABA. It appears that dehydration triggers the production of ABA, which, in turn, induces various genes. Many genes that respond to ABA are also expressed at the late stages of embryogenesis during the development of seeds, and are thought to function in the protection of cells from dehydration. Cis-and trans-acting factors involved in ABA-induced gene expression have been extensively analyzed. A conserved sequence, PyACGTGGC, has been reported to function as an ABA-responsive element (ABRE) in many ABA-responsive genes. cDNAs encoding DNA-binding proteins that specifically bind to the ABRE have been cloned and shown to contain the bZIP structure (Marcotte *et al.*, 1989; Mundy *et al.*,1990). Several reports have described genes that are induced by dehydration but are not responsive to exogenous ABA treatments. These findings suggest the existence of ABA-independent as well as ABA-dependent signal transduction cascades between the initial signal of drought or cold stress and the expression of specific genes (Guerrero *et al.*, 1990; Yamaguchi-Shinozaki *et al.*,1992).

In this article, we summarize recent progress of our research on the characterization of drought-induced genes and the expression of these genes. We identified a novel cis-acting element that is involved in the ABA-independent response to conditions of dehydration, low temperature or high salt.

Function of plant genes induced by drought stress

We used *Arabidopsis thaliana* as a model plant for the analysis of the molecular process of drought tolerance. Plants were grown for four weeks at 22℃and harvested plants were dehydrated on chromatography paper at 22℃ and 60 % relative humidity under dim light. *Arabidopsis* plants dehydrated for 10 hr under such conditions recovered within a few hours after being rewatered, which indicated that they were still alive even when they had lost 90 % of their net weight. To identify the genes induced by dehydration stress, both water content (RWC) and accumulation of ABA were determined in *Arabidopsis* rosette plants during dehydration stress. When plants were subjected to dehydration stress, they lost gradually 90 % of water and a plateau was reached after dehydration stress for 10hr. Accumulation of ABA began to increase after dehydration stress for 2hr, and reached a maximum level at 10 hr. We constructed two cDNA libraries from polyA RNA prepared from *Arabidopsis* plants dehydrated for 10-hr or 1-hr. We isolated 9 and 16 independent cDNA clones from cDNA libraries prepared from plants dehydrated for 10-hr and 1-hr by the differential screening method, respectively, and designated them as RD (Responsive to Desiccation) or ERD (Early Responsive to Dehydration), respectively (Yamaguchi-Shinozaki *et al.*, 1992; Kiyosue *et al.*,1994). All the genes, designated as *rd* and *erd*, corresponding to RD and ERD cDNAs, respectively, were induced by dehydration. Sequence analysis of these cDNAs revealed that the genes induced by dehydration encode proteins that seem to function in the protection of cells from dehydration as shown in Fig. 1. For instance,

they encode putative proteinases that may degrade denatured or unnecessary proteins, water channel proteins involved in altering cellular water potentials, the enzymes required for the biosynthesis of osmoprotectants such as proline and sugars, protective proteins such as late embryogenesis abundant (LEA) proteins and chaperones, detoxification proteins such as glutathione S-transferase and soluble epoxide hydrolase, protein kinases and transcription factors, which are involved in further regulation of signal transduction and gene expression(Yamaguchi-Shinozaki *et al.,* 1992; Kiyosue *et al.,* 1993a; Kiyosue *et al.*,1993b; Koizumi *et al.*, 1993; Yamaguchi-Shinozaki and Shinozaki, 1994). We used cowpea as a crop which has the ability to tolerate severe drought conditions to isolate drought-inducible genes. We isolated 10 independent cDNA clones by the differential screening method, and designated them as CPRD(Cowpea Genes Responsive to Dehydration; Iuchi *et al.*, 1996). Sequence analysis of the cDNA of the CPRD8, CPRD14 and CPRD22 genes which exhibited typical responses to water stress revealed that they encoded several proteins including those related to group 2 LEA proteins which may play a role in the protection of cowpea cells from drought stress.

These genes could be used for the construction of transgenes to transform crop plants and produce drought-tolerant transgenic crops in the future.

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

We characterized the expression patterns of nine RD genes in *Arabidopsis* by Northern blotting. We found at least three RD genes that show ABA-independent expression in *Arabidopsis* (Koizumi *et al.*, 1993; Yamaguchi-Shinozaki *et al.*,1992). Northern blot analysis has revealed broad variations in the timing of induction of RD genes and has shown that six of these genes respond to ABA while three do not, which indicates that there are at least two independent signal transduction pathways from initial dehydration stress signal and gene expression. In the case of cowpea, the CPRD8 and CPRD22 genes were induced significantly by the application of exogenous ABA, unlike the CPRD14 gene. The timing of the accumulation of their mRNAs varies among RD genes, suggesting that there are several signal transduction pathways involved in the induction of these genes(Yamaguchi-Shinozaki *et al.,* 1992). We analyzed the expression of ABA-inducible RD genes and found that RD22 gene requires protein biosynthesis for its induction by ABA. Protein inhibitor, cycloheximide, inhibited the ABA-induced gene expression of RD22 unlike that of RD29(Yamaguchi-Shinozaki and Shinozaki, 1993a; Iwasaki *et al.*, 1995). Therefore, there are at least two independent signal transduction pathways between the production of ABA and gene expression under drought conditions. 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-dependent pathways requires protein biosynthesis (pathway II; Shinozaki and

Yamaguchi-Shinozaki, 1996). The existence of complex signal transduction pathways in drought response gives a molecular basis for the complex physiological responses of plants to drought stress.

Identification of a novel cis-acting element involved in the ABA-independent and drought-responsive expression

The transcription of a gene that corresponds to RD29 cDNA was induced very rapidly 20 min after the start of dehydration, and was followed by a second induction phase which began after about 3 hr of dehydration(Yamaguchi-Shinozaki and Shinozaki, 1993b). The changes in the levels of accumulated RD29 mRNA in response to dehydration, low temperature, salt stress, or exposure to ABA were different. Two genes corresponding to RD29, *rd29A* and *rd29B*, were located in tandem in an 8-kbp region of the *Arabidopsis* genome and encoded hydrophilic proteins. Dehydration induced the *rd29A* gene with two-step kinetics, while the *rd29B* gene was induced within 3 hr of dehydration. The *rd29A* mRNA was induced within 5 hr after exposure to low temperature (4℃) and was detectable for at least 24hr. However, *rd29B* mRNA did not accumulate within 24 hr. The expression of both genes was stimulated about 3 hr after treatment with ABA (Yamaguchi-Shinozaki and Shinozaki, 1993b; Yamaguchi-Shinozaki and Shinozaki,1994). Under the dehydration conditions, endogenous ABA began to accumulate 2hr after dehydration started and reached maximum concentration at 10 hr, which suggests that the first rapid induction of *rd29A* is not mediated by endogenous ABA. Therefore, it appears that *rd29A* has at least two cis-acting elements: one seems to be involved in the ABA-associated slow response to dehydration, and the other may function in the ABA-independent rapid induction.

To analyze 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 *Arabidopsis* by the conditions of dehydration, low temperature, or high salt, or by treatment with ABA (Yamaguchi-Shinozaki, 1993b). We investigated cis-acting elements involved in dehydration-responsive expression in the *rd29A* promoter and identified a novel cis-acting element involved in the first rapid response of *rd29A* to dehydration or high salt stress. A deletion analysis of the promoter regions of *rd29A* and *rd29B* in transgenic tobacco revealed that different cis-acting elements function in the dehydration-responsive expression of the two genes. We precisely analyzed one of the cis-acting elements responsible for the dehydration-induced expression of *rd29A* at the nucleotide sequence level. The deletion and the gain-of-function analysis of the promoter region of *rd29A* fused to the GUS reporter gene in transgenic tobacco and *Arabidopsis* revealed that the 20-bp direct repeat sequence is necessary for the dehydration-responsive expression . The base substitution analysis revealed that the 9-bp conserved core sequence, TACCGACAT (DRE, Dehydration Responsive Element), in the 20-bp direct repeat sequence 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. 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*.

Fig. 3 shows a schematic model of the signal transduction pathways between the expression of *rd29A* and *rd29B* and the initial signal of environmental stresses, such as dehydration, low temperature, or high salt (Yamaguchi-Shinozaki and Shinozaki, 1994). There are at least two independent signal transduction pathways, which are ABA- independent and ABA- responsive, between the environmental stresses and expression for the two *rd29* genes. The *rd29A* promoter contains at least two cis-acting elements that are involved in the induction of *rd29A* by dehydration, high salt, or low temperature. One of these elements is DRE, which functions in the first rapid response of *rd29A* to the environmental signal. ABA is not involved in this process. The other element is located in the 53-bp region containing one ABRE and one *asl*. These elements probably function in the second slow induction of *rd29A*. ABA appears to mediate this slow response of *rd29A* to environmental stresses. The *rd29B* gene exhibited only the slow response to dehydration or salt stress at 24 hr, but did not respond significantly to low temperature within 24 hr. ABA may be involved in part in the slow expression of *rd29B*. This model indicates that different cis-acting elements are responsible for the different expression patterns of *rd29A* and *rd29B* under conditions of dehydration, high salt, or low temperature.

Conclusion

We have isolated a number of drought-induced genes in *Arabidopsis* and cowpea, and analyzed the structure and expression of these genes. Structural analysis of their gene products has revealed that they probably function in drought tolerance. These genes are possible candidates for gene resources to construct transgenes for the production of drought-tolerant transgenic crops. We also analyzed the transcriptional regulatory regions of the drought-induced genes and identified several cis-acting elements that are involved in drought-induced gene expression. These promoters can be used to express genes involved in drought tolerance only when the transgenic plants are exposed to drought conditions. Several transcription factors involved in drought-responsive gene expression will be used for the control of many drought-inducible genes under water deficit conditions. We are planning to use these genes, their promoters and regulatory factors in the production of drought tolerant transgenic crops, which should enable to develop technologies to alleviate the food crisis and environmental disruption in the 21st century.

References

1) Bray, E.A. (1993): Molecular responses to water deficit. Plant Physiol. **103** : 1035-1040.

2) Bohnert, H.J., Nelson D.E. and Jensen, R.G.(1995): Adaptations to environmental stresses. Plant Cell **7** : 1099-1111.
3) Chandler, P.M. and Robertson (1994): Gene expression regulated by abscisic acid and its relation to stress tolerance. Annu. Rev. Plant Physiol.Plant Mol.Biol. **45** : 113-141.
4) Guerrero, F.D., Jones, J.T. and Mullet, J.E. (1990): Turgor-responsive gene transcription and RNA levels increase rapidly when pea shoots are wilted. Sequence and expression of three inducible genes. Plant Mol. Biol. **15** : 11-26.
5) Iuchi, S., Yamaguchi-Shinozaki, K., Urao, T., Terao, T. and Shinozaki, K. (1996): Novel drought-inducible genes in the highly drought-tolerant cowpea: cloning of cDNAs and analysis of their gene expression. Plant Cell Physiol., in press.
6) Iwasaki, T., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1995): Identification of a cis-regulatory region of a gene in *Arabidopsis thaliana* whose induction by dehydration is mediated by abscisic acid and requires protein synthesis. Mol. Gen. Genet. **246** : 331-340.
7) Kiyosue, T., Yamaguchi-Shinozaki,K. and Shinozaki K. (1993a): Characterization of cDNA for a dehydration-inducible gene that encodes a CLP A, B-like protein in *Arabidopsis thaliana* L. Biochem. Biophys. Res. Comm. **196** : 1214-1220.
8) Kiyosue, T., Yamaguchi-Shinozaki,K. and Shinozaki K. (1993b): Characterization of two cDNAs (ERD11 and ERD13) for dehydration-inducible genes that encode putative glutathione S-transferases in *Arabidopsis thaliana* L. FEBS lett. **335** : 189-192.
9) Kiyosue, T., Yamaguchi-Shinozaki,K. and Shinozaki,K. (1994): Cloning of cDNA for genes that are early responsive to dehydration-stress (ERDs) in *Arabidopsis thaliana* L.: Identification of three ERDs as cognate genes. Plant Mol. Biol.
10) Koizumi, M., Yamaguchi-Shinozaki, K., Tuji, H. and Shinozaki, K. (1993): Structure and expression of two genes that encode distinct drought-inducible cysteine proteinases in *Arabidopsis thaliana*. Gene **129** : 175-182.
11) Marcotte, W.R., Jr, Russell, S.H. and Quatrano, R.S. (1989): Abscisic acid-response sequences from the *Em* gene of wheat. Plant Cell **1** : 969-976.
12) Mundy, J., Yamaguchi-Shinozaki, K. and Chua, N.-H. (1990): Nuclear proteins bind conserved elements in the abscisic acid-responsive promoter of rice rab genes. Proc. Natl Acad. Sci. USA **87** : 406-410.
13) Shinozaki, K. and Yamaguchi-Shinozaki , K. (1996): Molecular responses to drought and cold stress. Curr. Opon. Biotech. **7** : 161-167.
14) Yamaguchi-Shinozaki, K., Koizumi, M., Urao, S. and Shinozaki, K. (1992): Molecular cloning and characterization of 9 cDNAs for genes that are responsive to desiccation in *Arabidopsis thaliana*: Sequence analysis of one cDNA clone that encodes a putative transmembrane channel protein. Plant Cell Physiol. 33 : 217-224.
15) Yamaguchi-Shinozaki, K. and Shinozaki, K. (1993a): The plant hormone abscisic acid mediates the drought-induced expression but not the seed-specific expression

of rd22, a gene responsive to dehydration stress in *Arabidopsis thaliana*. Mol. Gen. Genet. **238** : 97-105.

16) Yamaguchi-Shinozaki, K. and Shinozaki, K. (1993b): Characterization of the expression of a desiccation-responsive *rd29* gene of *Arabidopsis thaliana* and analysis of its promoter in transgenic plants. Mol. Gen. Genet. **236** : 331-340.

17) Yamaguchi-Shinozaki, K. and Shinozaki, K. (1994): A novel cis-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell **6** : 251-264.

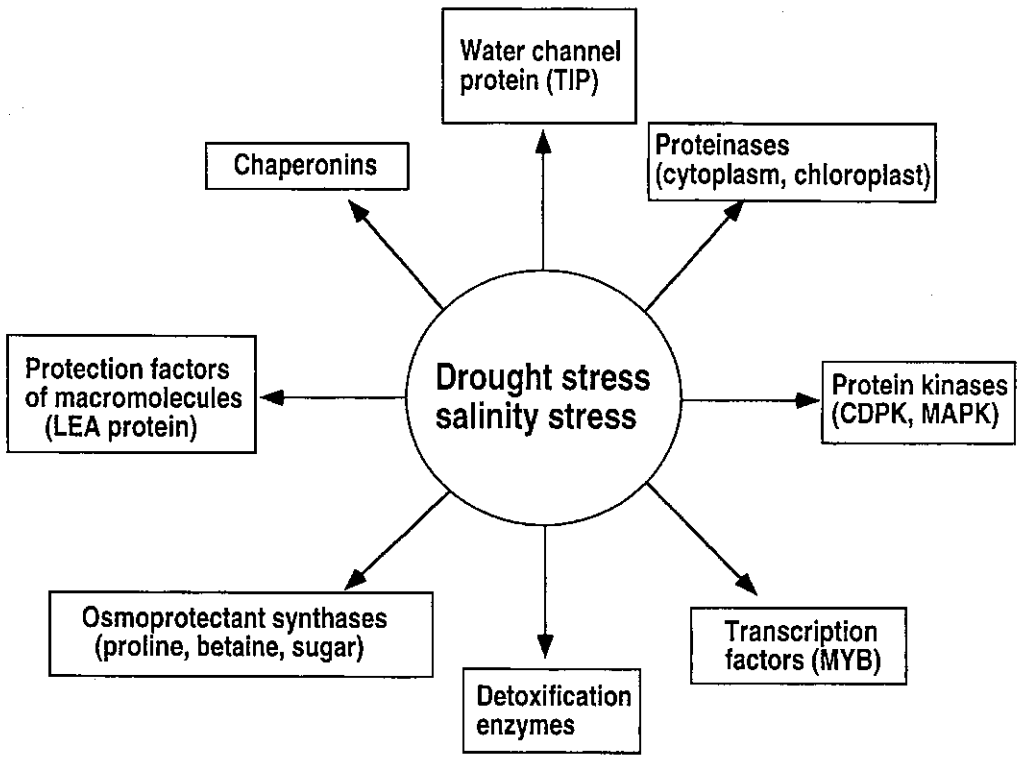

Fig. 1 Drought stress-inducible genes and their possible functions in drought tolerance in *Arabidopsis thaliana*

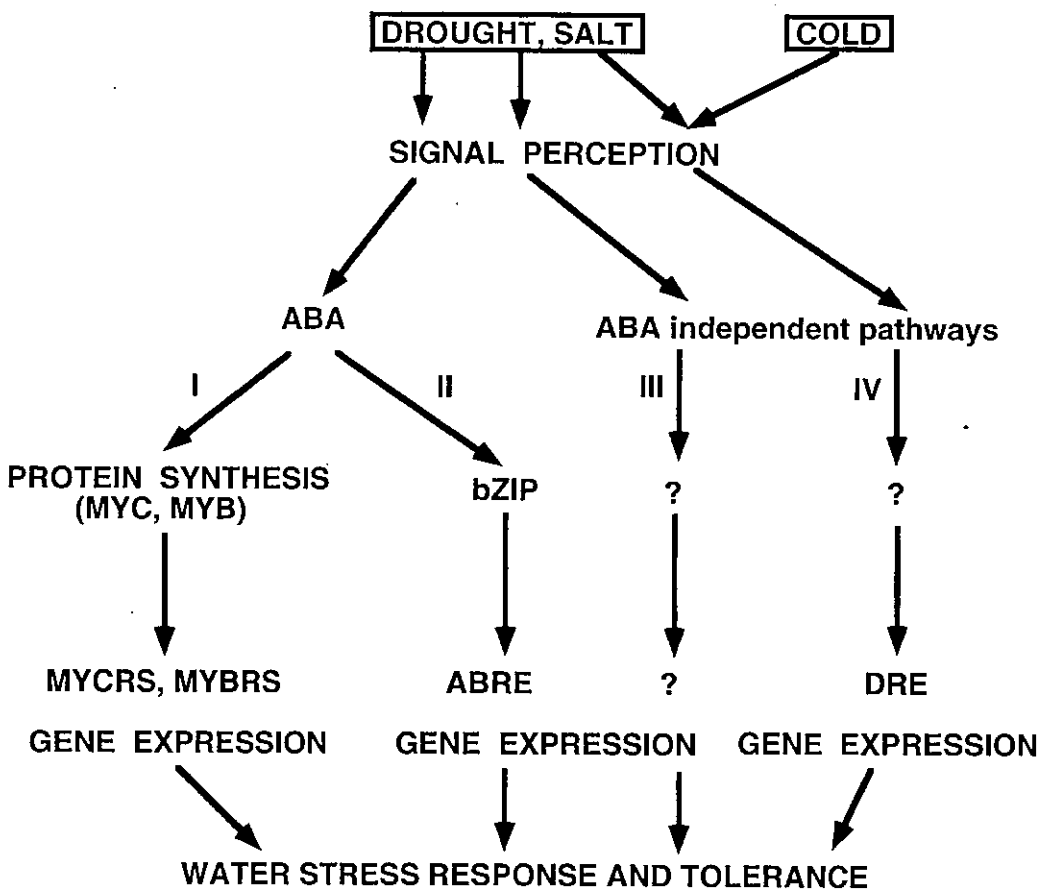

Fig. 2 Signal transduction pathways between initial dehydration stress signal and gene expression

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),and DRE is involved in one of the ABA-independent pathways (IV).

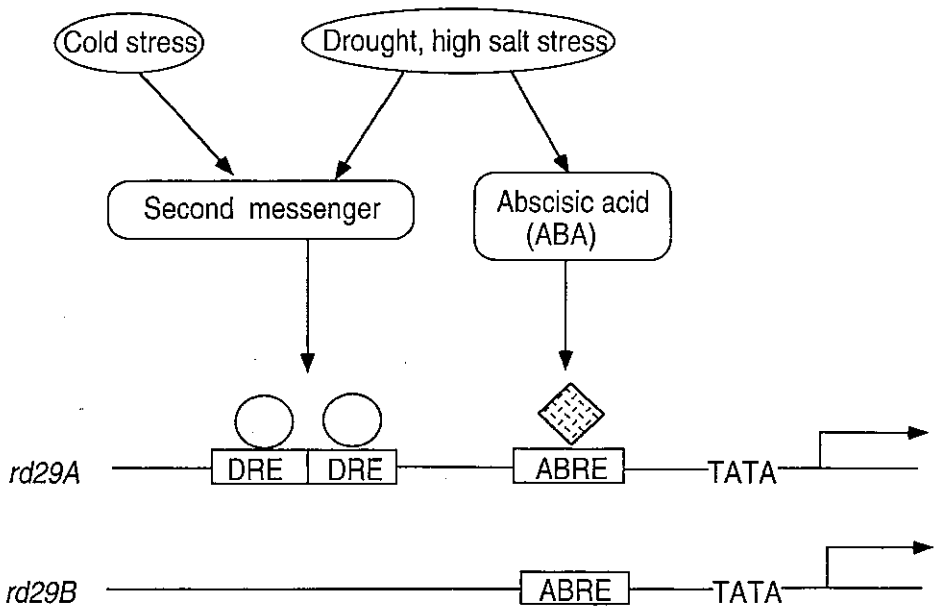

Fig. 3 Schematic representation of the induction of two *rd29* genes and their cis-acting elements involved in stress-responsive expression

Different cis-acting elements, DRE and ABRE, may function in the ABA-independent and ABA-responsive induction of *rd29A*, respectively while ABRE seems to be involved in the ABA-responsive expression of *rd29B*.