Function and Regulation of Genes that are Induced by Dehydration Stress in *Arabidopsis thaliana*.

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Abstract

Plants respond to drought through physiological and developmental changes. Many genes have been shown to be induced by dehydration stress and function in the tolerance of plants to drought. We isolated 25 independent genes that are responsive to dehydration in *Arabidopsis thaliana* and analyzed the structure of their gene products. Sequence analyses of these genes indicate that their products may function in protecting cells from dehydration. We also analyzed the expression of these genes under various stress conditions. Some of the dehydration-responsive genes are induced by the plant hormone abscisic acid (ABA), unlike others. There seem to be at least three independent signal transduction pathways between initial dehydration stress signal and gene expression. We precisely analyzed the promoter of two dehydration-inducible *Arabidopsis genes, rd29A* and *rd29B*, in transgenic plants, 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 dehydration or high salt conditions. DRE is also involved in the induction by low temperature, but does not function in the ABA-responsive, slow expression of *rd29A*. One of the *myb*-related genes, *Atmyb2*, that is responsive to water stress and abscisic acid (ABA) has been cloned from *Arabidopsis thaliana*. *Atmyb2* encodes a transcription factor and binds to conserved MYB recognition sequence. These results suggest that the ABA-mediated induction of dehydration-inducible genes whose expression requires protein synthesis may be regulated by the ATMYB2 protein.

Additional key words : signal transduction, abscisic acid, *cis*-acting element, transcription factor, transgenic plant

Introduction

The genetic improvement of the drought tolerance of plants is an important problem for the future of agriculture in desert and savanna areas of developing countries. Classical breeding methodologies to select drought-tolerant cultivars have already made some progress. Biotechnology has the potential to improve the drought tolerance of crops using transgenic plant technology. The limiting factor for developing this technology is the isolation of genes that can improve drought tolerance and the precise understanding of the molecular process of drought tolerance and plant response to water deficit. Plants respond to conditions of drought through a number of physiological and developmental changes. Several genes that respond to dehydration at the transcriptional level have been described recently. Abscisic acid (ABA)[9] which is produced under water deficit conditions appears to play an important role in the ability of plants to tolerate drought stress. Recently, a number of genes have been described that respond to dehydration at the transcriptional level.[1, 14]. Most of the genes that have been studied to date are also induced by ABA[14]. 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 considered to function in the protection of cells from dehydration[14]. *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[10, 11].

Several reports have described genes that are induced by dehydration but are not responsive to exogenous ABA treatments[3, 16]. These findings suggest the existence of ABA-independent as well as ABA-dependent signal-transduction cascades between the initial signal of dehydration or cold stress and the expression of specific genes. Although the *cis*- and *trans*-acting factors of ABA-responsive genes have been extensively analyzed, our understanding of the molecular mechanism of ABA-independent gene expression under dehydration conditions, and their signal pathways, is still limited. Therefore, it is important to investigate the cis- and trans-acting elements that function in the ABA-independent gene expression induced by dehydration.

In this article, we summarize recent progress of our research on the characterization of dehydration-induced genes and functions of their product proteins. Expression patterns of these genes under various environmental stresses, such as drought, low temperature, high salinity and ABA treatment were characterized and classified into three groups[17, 18]. We also precisely analyzed the promoter of dehydration-inducible *Arabidopsis* genes in transgenic plants, and identified a novel *cis*-acting element that is involved in the ABA-independent response to dehydration, low temperature or high salt conditions[19]. A novel gene for a transcription factor *myb*-homologue, *Atmyb2*, has been found to be induced by dehydration and exogenous ABA treatment[15]. We discuss the role of these genes for *cis*-and *trans*-acting factors in dehydration-regulated gene expression and the application of these genes in the development of drought tolerant transgenic crops.

1. Functional analysis of plant genes induced by dehydration stress.

In order to isolate genes induced by dehydration stress, both water content (RWC) and accumulation of ABA were determined in *Arabidopsis* rosette plants during dehydration stress. *Arabidopsis* plants were grown for four weeks at 22℃ under constant light conditions and harvested plants were dehydrated on chromatography paper at a temperature of 22℃

and 60% relative humidity under dim light. When plants were subjected to dehydration-stress, they lost gradually 90% of water and a plateau was reached after dehydration-stress for 10 hr. Accumulation of ABA which began to increase after dehydration-stress for 2 hr, reached a maximum level at 10 hr. We constructed two cDNA libraries from polyA RNA prepared from 10-hr or 1-hr dehydrated *Arabidopsis* plants. We isolated 9 and 16 cDNA clones from cDNA libraries prepared from 10-hr and 1-hr dehydrated plants by the differential screening method, and designated them as RD (Responsive to Desiccation) and ERD (Early Responsive to Dehydration), respectively[7, 16]. 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 Figure 1. For instance, they encode putative proteinases that may degrade denatured or unnecessary proteins, chaperonins that probably renature proteins, water channel proteins that can function in controlling the osmotic potential of stressed cells, and so on[5, 6, 8, 16]. These genes could be used for the construction of transgenes to transform crop plants and produce drought-tolerant transgenic crops in the near future.

2. Analysis of the expression of dehydration-induced genes in response to environmental stresses and ABA.

We characterized the expression patterns of nine *rd* genes by Northern blotting. We identified at least three *rd* genes that show an ABA-independent expression in *Arabidopsis* (Table 1)[8, 16]. Northern blot analysis revealed wide variations in the timing of induction of *rd* genes and showed that six of these genes respond to ABA while three do not, which indicates that there are at least two independent signal transduction pathways between initial dehydration stress signal and gene expression (Fig. 1). The timing of the accumulation of mRNAs varies among *rd* genes, suggesting that there are several signal transduction pathways involved in the induction of these genes[16]. We analyzed the expression of ABA-inducible RD genes and found that the *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*[18]. Therefore, it is postulated that there are at least two independent signal transduction

Table 1 Characterization of the structure and expression of RD clones

cDNA	Homology	Transcript size (kb)	ABA*
RD28	water channel protein	1.4	-
RD19	thiol protease	1.2	-
RD21	thiol protease	1.5	-
RD22	unidentified seed protein	1.9	+
RD29	*lti*78	2.4	+
RD17	*rab*, dehydrin	1.3	+
RD20		1.1	+
RD26		1.5	+
RD2		1.4	+

*: Genes that are induced by ABA (+) or not induced by ABA (-)

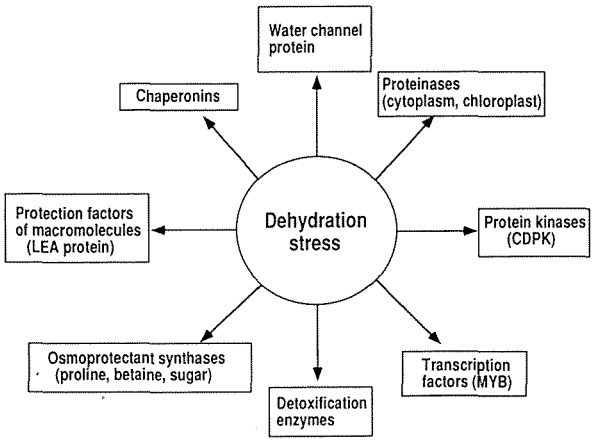

Fig. 1. Dehydration-stress inducible genes and their possible functions in drought tolerance in *Arabidopsis thaliana*

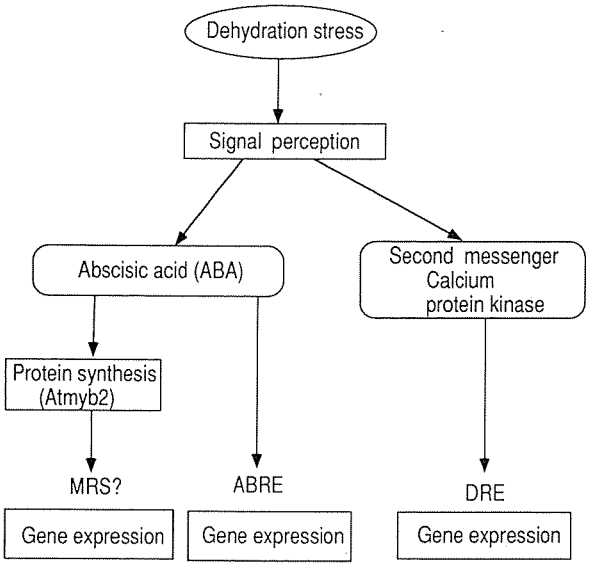

Fig. 2. Signal transduction pathways between initial dehydration stress signal and gene expression. There are at least three signal transduction pathways: one is ABA-independent and two are ABA-dependent. Protein synthesis is necessary for one of the ABA-dependent signal pathways but not for the others

pathways between the production of ABA and gene expression under dehydration conditions (Fig. 2). As shown in Figure 2, we identified at least three independent signal transduction pathways which function under dehydration conditions in *Arabidopsis*. The existence of complex signal transduction pathways in the drought response of plants gives a molecular basis for the complex physiological responses of plants to drought stress.

3. Identification of a novel *cis*-acting element involved in the ABA-independent and dehydration-responsive expression.

The transcription of genes that hybridize to RD29 cDNA is induced very rapidly and at a high rate 20 min after the start of dehydration, and this transcription is followed by a second induction phase which begins after about 3 hr of dehydration.[17] The changes in the levels of RD29 mRNA in response to dehydration, low temperature, salt stress, or exposure to ABA are different[17, 19]. Two genes corresponding to RD29, *rd29A* and *rd29*B, are located in tandem in an 8-kbp region of the *Arabidopsis* genome and encode hydrophilic proteins[17]. Dehydration induces *rd29A* mRNA with two-step kinetics, while *rd29B* is induced within 3 hr of dehydration. The expression of both genes, however, is stimulated about 3 hr after treatment with ABA[17]. Under the dehydration conditions employed in the present study, endogenous ABA began to accumulate 2 hr after dehydration started and reached a maximum level at 10 hr, which suggests that the first rapid induction of *rd29A* is not mediated by endogenous ABA. Therefore, it appears that *rd29A* consists of at least two *cis*-acting elements: one may 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 fusion genes with the *rd29A* promoter fused to β-glucuronidase (*GUS*) reporter gene and transformed *Arabidopsis* and tobacco plants with these constructs (Fig. 3). The *GUS* reporter gene driven by the *rd29A* promoter was induced at significant levels in transgenic *Arabidopsis* under dehydration, low temperature, or high-salt conditions, or by treatment with ABA[17]. We investigated the *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 plants indicated that different *cis*-acting elements function in the dehydration-responsive expression of the two genes[19]. 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 (Fig. 4). The

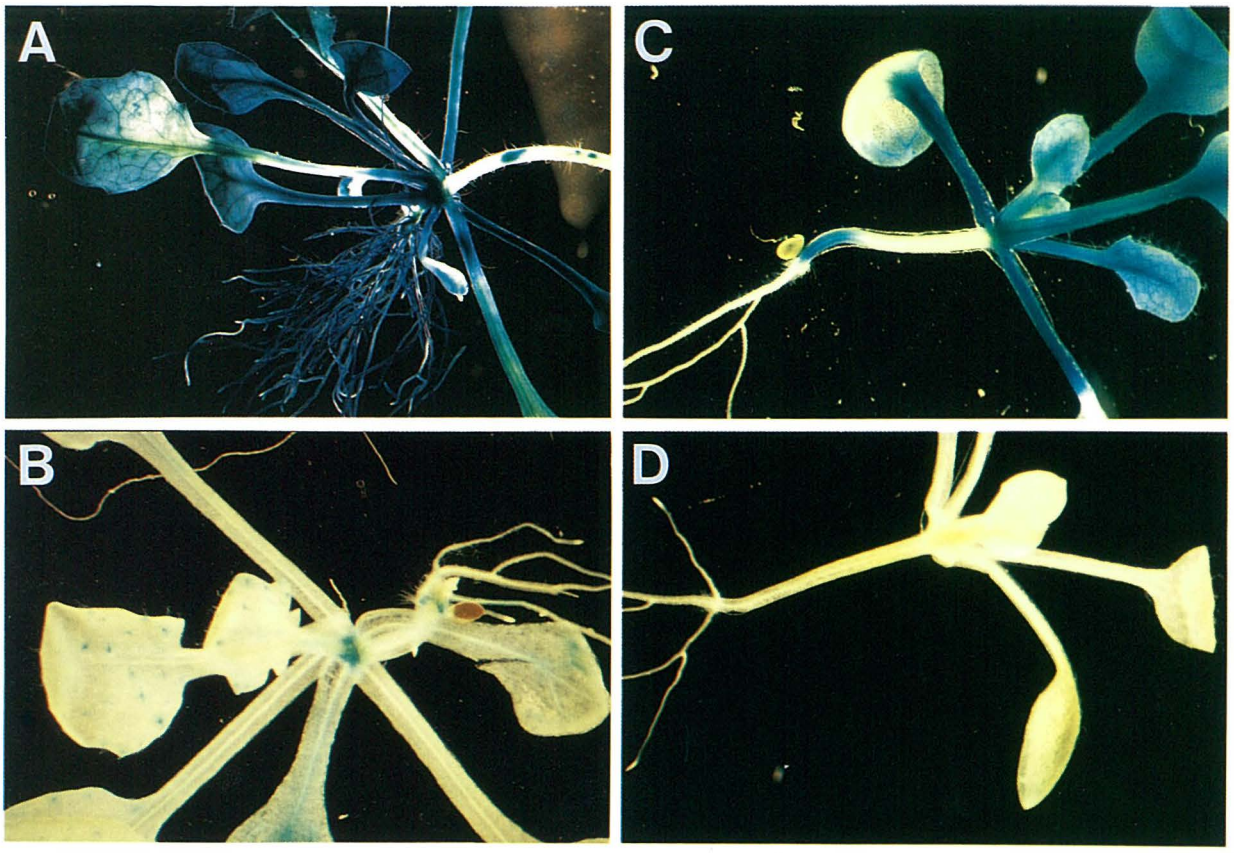

Fig. 3. Histochemical localization of GUS activity (blue color) in transgenic *Arabidopsis*. Transgenic *Arabidopsis* plants containing the -861 *rd29A-GUS* (A, B) or -946 *rd29A-GUS* (C, D) fusion construct were grown on GM agar that contained 10 μg/ml kanamycin and were then exposed to dehydration for 8 hr (A, C) or were grown under normal conditions (B, D)

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 dehydration conditions[19]. Moreover, DRE has been found 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 under dehydration, high-salt, or low temperature conditions, but does not function as an ABA-responsive element in the induction of *rd29A* . Under the stress conditions tested, ABA does not seem to function in the DRE-mediated process in the induction of *rd29A*. RNA gel-blotting analysis indicated that the second slow induction of *rd29A* depends on ABA[19]. The region involved in the ABA-responsive, slow induction of *rd29A* contains one ABRE and *as1*.

Figure 5 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.[11] 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 (TACCGACAT), 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 *as1*. These elements probably function in the second slow induction of *rd29A*. ABA appears to mediate this slow response of

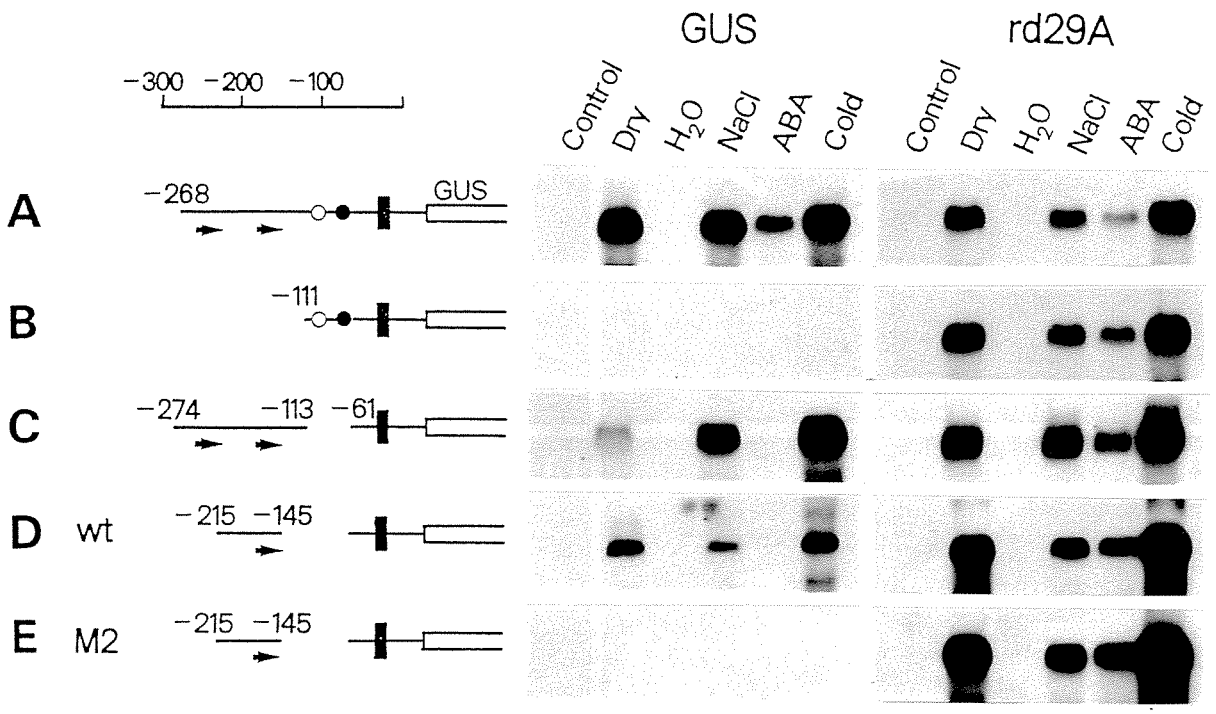

Fig. 4. Analysis of the effect of various treatments on the induction of the *rd29A* promoter-*GUS* fusion genes in transgenic *Arabidopsis*
Schematic representation of the chimeric constructs is indicated on the left side. RNA gel blotting was carried out to measure the amount of *GUS* mRNA or endogenous *rd29A* mRNA in transgenic *Arabidopsis* plants that had been subjected to the following treatments: dehydrated (Dry) for 10 hr (A andB) or 2 hr (C, D, and E), transferred from agar plates for hydroponic culture in water and treated for 10 hr (H_2O), transferred from agar plates to hydroponic culture in 250 mM NaCl (NaCl) and treated for 10 hr (A and B) or 2 hr (C, D, and E), transferred from agar plates to hydroponic culture in 100 μ M ABA for 10 hr (ABA), transferred to and grown at 4℃ for 10 hr (Cold) or untreated (Control). DNA fragments for the coding region of *GUS* or the 3' flanking region of *rd29A* were used as probes. The 71-bp (-215 to -145) DNA fragments that contain base substitutions in DRE (M2) or do not contain the base substitutions (wt) were fused to the -61 *rd29A-GUS*

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. It is thus assumed that 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 dehydration, high-salt, or low temperature conditions.

4. A novel transcription factor, ATMYB2, is involved in dehydration-responsive gene expression.

In higher plants, a number of *myb*-related genes, which encode transcription factors, have been isolated and extensively characterized by genetic analysis. Plant *myb*-related genes are considered to form a large family of genes and may play various roles in the regulation of gene expression[4, 12]. Among these genes, a maize *C1* gene regulates the expression of structural genes that are involved in the biosynthesis of anthocyanin pigments during seed development and the *C1* gene is regulated by the plant hormone abscisic acid (ABA) and *VP1* gene[13]. These studies on plant *myb*-related genes led us to examine whether a *myb*-related gene is involved in the ABA-responsive expression of dehydration-inducible genes in vegetative tissues of a higher plant. We screened a cDNA library prepared from

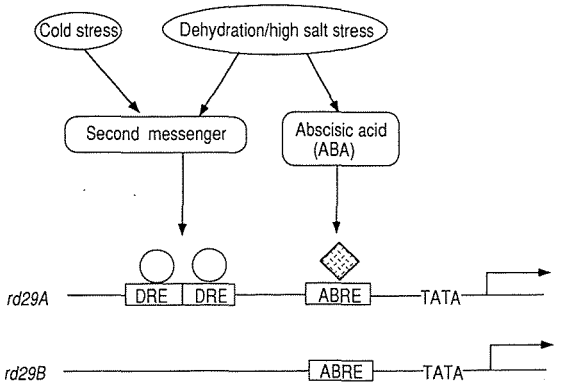

Fig. 5. Schematic representation of the induction of two *rd29* genes and their *cis*-acting elements involved in stress-responsive expression. Different *cis*-acting elements, DRE and ABRE, may function in the ABA-independent and ABA-responsive induction of *rd29A*, while ABRE seems to be involved in the ABA-responsive expression of *rd29B*

Arabidopsis plants that had been dehydrated for 10 hr and cloned a cDNA, designated as *Atmyb2*, that encoded an MYB-related protein[15].

The *Atmyb2* gene was rapidly induced by dehydration stress. Rehydration of dehydrated *Arabidopsis* plants caused a decrease in the level of *Atmyb2* mRNA, which indicates that *Atmyb2* responds specifically to dehydration stress. High-salt conditions and application of exogenous ABA also resulted in the induction of *Atmyb2*, although *Atmyb2* did not respond to cold or heat stresses. Therefore, *Atmyb2* is a gene responsive to dehydration and high-salt stresses, and ABA is involved in the expression of *Atmyb2*[15].

The putative ATMYB2 protein displays several characteristics common to plant homologues of MYB. Based on the comparison of the amino acid sequences of the DNA binding domains of plant MYB-related proteins, *Drosophila* MYB and human c-MYB, ATMYB2 shows two imperfect repeats of 51 to 53 amino acids with conserved tryptophan residues. However, the first tryptophan residue in the second repeat (repeat III) found in animal MYB proteins is replaced by an isoleucine or a phenylalanine residue in plants. The ATMYB2 protein, as well as other plant MYB proteins, lacks repeat I in the DNA binding domain that has been found in human, mouse and *Drosophila* MYB proteins (Fig.6). Products of viral *myb* onco-genes (*v-myb*) also interact with DNA in a sequence-specific manner in spite of the absence of repeat I. These findings imply that only repeats II and III are required for a functional DNA binding domain in plant MYB proteins. The c-MYB protein recognizes the conserved DNA sequence PyAACTG and it has been suggested that AAC was the core sequence of the binding site. The ATMYB2 protein expressed in *E. coli* was found to bind specifically to the MYB-recognition sequence detected in the simian virus 40 enhancer and the maize *Bz-1* promoter[15].

Since *Atmyb2* is induced by water stress, it is likely that the ATMYB2 protein functions as a transcription factor that controls the expression of genes induced under dehydration stress or high-salt conditions. We have isolated nine cDNAs whose corresponding genes (*rd*) are induced by dehydration stress and analyzed their expression[16]. One of the dehydration-responsive genes in *Arabidopsis*, *rd22* , showed a pattern of gene expression similar to that of *Atmyb2*. Both *rd22* and *Atmyb2* are induced by dehydration and high-salt stresses, but not by cold and heat stresses. ABA causes the induction of both *rd22* and *Atmyb2*. Moreover, protein synthesis is necessary for the induction of *rd22* , which suggests that the expression of *rd22* may be regulated by *trans*-acting factors whose synthesis *de novo* is induced by dehydration or ABA[18]. The promoter region of *rd22* that is involved in the dehydration-induced expression contains the sequence of two closely located recognition sites for the transcription factors, MYC and MYB, but not the consensus ABRE sequence[18]. Thus, it appears that the expression of *rd22* is regulated by a novel mechanism. Recently, maize homologues of MYB and MYC, designated as C1 and B, respectively, have been found to act synergistically for the activation of the promoter of the maize *Bz-1* gene, an anthocyanin-biosynthetic gene[2]. As in the case of the *rd22* promoter, an MYC-recognition

Fig. 6. Comparison of amino acid sequences of the DNA-binding domains of MYB-related proteins
The deduced amino acid sequence of ATMYB2 is compared with the DNA-binding domains of MYB-related proteins, namely, maize C1 (ZMC1), barley Hv1 (HV1), *Antirrhinum* 306 (AM306), *Arabidopsis* ATMYB1 and human c-MYB (HUCMYB). Shaded boxes indicate amino acid residues identical with those of ATMYB2 and asterisks represent conserved tryptophan residues. Dashes indicate gaps introduced to maximize alignment. Numbers above the box refer to amino acid positions in ATMYB2

sequence is located near the MYB-recognition site in the *Bz-1* promoter. Maize R and C1 proteins, when constitutively expressed in *Arabidopsis*, have been found to act synergistically for the induction of the production of anthocyanin. We postulated that the ATMYB2 protein induced by ABA may act in concert with the MYC protein to activate the transcription of *rd22*.

Conclusion

In the last five years, we have isolated a number of dehydration-induced genes in a model plant, *Arabidopsis thaliana*, and analyzed the structure and expression of these genes. Structural analysis of their gene products has revealed that they probably function in the drought tolerance of plants. These genes are possible candidates for the construction of transgenes for the production of drought-tolerant transgenic crops. We also analyzed the transcriptional regulatory region of the dehydration-induced genes and identified several *cis*-acting elements that are involved in dehydration-induced gene expression. These promoters can be used to express genes involved in plant drought tolerance only when the trangenic plants are exposed to drought conditions. Several transcription factors involved in the dehydration-responsive gene expression could be used for the control of various dehydration-inducible genes under water deficit conditions. We are planning to use these genes, their promoters and regulatory factors for the production of drought-tolerant transgenic crops, which may contribute to the development of technologies to address the food crisis and environmental degradation in the 21st century.

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モデル実験植物シロイヌナズナの乾燥誘導性遺伝子の機能と発現制御

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摘　要

植物では乾燥ストレスに生理的に応答し，かつ適応して成長，分化を行っている。多くの植物遺伝子が乾燥ストレスによってその発現が制御されていることが明らかになった。乾燥ストレスによって誘導される遺伝子群の機能と遺伝子の発現制御について分子レベルで明らかにすることを目的として，モデル植物のシロイヌナズナ(*Arabidopsis thaliana*)を材料に乾燥で誘導される遺伝子のcDNAをディファレンシャルスクリーニング法によりクローニングした。全部で25個のcDNAクローンを分離し，これらの遺伝子構造を決定した。この結果，乾燥ストレスにより誘導される遺伝子の産物は，いずれも細胞を乾燥から保護するために働いていると推定された。

乾燥ストレスによる遺伝子発現には植物ホルモンのアブシジン酸(ABA)が重要な役割を果していることが明らかになっている。乾燥ストレスによる遺伝子発現の制御機構に関する研究はシグナル伝達系を含めて未知の領域が多い。そこで乾燥誘導性遺伝子の転写レベルでの発現誘導に関してノーザンハイブリダイゼーション法により解析した。その結果，乾燥ストレスによる遺伝子の発現誘導に関しては少なくとも３つのシグナル伝達系があることが明らかになった。乾燥ストレスによって誘導される遺伝子のうち，RD29の遺伝子の転写開始領域の解析を行った。転写開始領域とGUSリポーター遺伝子を連結したキメラ遺伝子を作成し，トランスジェニック植物による発現誘導について解析を行った。RD29の発現パターンの異なる２個の遺伝子*rd29A*と*rd29B*遺伝子の転写開始領域について乾燥ストレス誘導に関与するシスエレメントをトランスジェニック植物実験系を用いて同定した。*rd29A*遺伝子の乾燥，低温，塩によるストレス応答には９塩基の配列(TACCGACAT)が関与していることを明らかにし，この９塩基の配列をDRE(Dehydration Responsive Element)と命名した。DREはABAによる発現誘導には関与しない新しいシスエレメントであることを示した。

乾燥ストレスによって誘導される転写制御因子の*myb*相同遺伝子*Atmyb2*の乾燥ストレス応答に関して，その発現と機能について解析した。*Atmyb2*遺伝子は乾燥ストレス以外に塩ストレスやABAにより誘導されることが明らかになった。さらに大腸菌内で発現したATMYB2タンパク質を用いて，ATMYB2蛋白質はMYB蛋白質の共通の認識配列YAACYRに特異的に結合することをゲルシフト法を用いて明らかにした。*Atmyb2*遺伝子はＡＢＡ処理によって誘導されることから，ATMYB2蛋白質が乾燥ストレス応答における遺伝子発現制御に重要な働きをしていると考えられる。

キーワード：乾燥ストレス，アブシジン酸，遺伝子発現，遺伝子機能，転写制御，シグナル伝達，
トランスジェニック植物