The Plant Cell, Vol. 9, 1859–1868, October 1997

Role of Arabidopsis MYC and MYB Homologs in Drought- and Abscisic Acid–Regulated Gene Expression

Hiroshi Abe,[a,b] Kazuko Yamaguchi-Shinozaki,[a,1] Takeshi Urao,[a] Toshisuke Iwasaki,[c] Daijiro Hosokawa,[b] and Kazuo Shinozaki[c]

[a] Biological Resources Division, Japan International Research Center for Agricultural Sciences (JIRCAS), Ministry of Agriculture, Forestry and Fisheries, 1-2 Ohwashi, Tsukuba, Ibaraki 305, Japan
[b] Department of Applied Biological Sciences, Tokyo University of Agriculture and Technology, Fuchu 183, Japan
[c] Laboratory of Plant Molecular Biology, Tsukuba Life Science Center, Institute of Physical and Chemical Research (RIKEN), 3-1-1 Koyadai, Tsukuba, Ibaraki 305, Japan

In Arabidopsis, the induction of a dehydration-responsive gene, *rd22*, is mediated by abscisic acid (ABA) and requires protein biosynthesis for ABA-dependent gene expression. Previous experiments established that a 67-bp DNA fragment of the *rd22* promoter is sufficient for dehydration- and ABA-induced gene expression and that this DNA fragment contains two closely located putative recognition sites for the basic helix-loop-helix protein MYC and one putative recognition site for MYB. We have carefully analyzed the 67-bp region of the *rd22* promoter in transgenic tobacco plants and found that both the first MYC site and the MYB recognition site function as *cis*-acting elements in the dehydration-induced expression of the *rd22* gene. A cDNA encoding a MYC-related DNA binding protein was isolated by DNA–ligand binding screening, using the 67-bp region as a probe, and designated rd22BP1. The rd22BP1 cDNA encodes a 68-kD protein that has a typical DNA binding domain of a basic region helix-loop-helix leucine zipper motif in MYC-related transcription factors. The rd22BP1 protein binds specifically to the first MYC recognition site in the 67-bp fragment. RNA gel blot analysis revealed that transcription of the *rd22BP1* gene is induced by dehydration stress and ABA treatment, and its induction precedes that of *rd22*. We have reported a drought- and ABA-inducible gene that encodes the MYB-related protein ATMYB2. In a transient transactivation experiment using Arabidopsis leaf protoplasts, we demonstrated that both the rd22BP1 and ATMYB2 proteins activate transcription of the *rd22* promoter fused to the β-glucuronidase reporter gene. These results indicate that both the rd22BP1 (MYC) and ATMYB2 (MYB) proteins function as transcriptional activators in the dehydration- and ABA-inducible expression of the *rd22* gene.

INTRODUCTION

Plants respond to environmental stresses with a number of physiological and developmental changes. Drought stress is one condition that affects almost all plant functions, including growth and development. Plant cells sense the loss of water during drought conditions, and a stress signal is then transduced to the nuclei via as yet unknown pathways. This leads to the expression of many genes that function in drought tolerance or drought response. The plant hormone abscisic acid (ABA) is produced under water-deficit conditions and is instrumental in the development of tolerance against drought.

Recently, a number of genes have been described that respond to drought at the transcriptional level (reviewed in Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996; Bray, 1997). Most of the drought-induced genes studied to date are also induced by ABA. It appears that dehydration triggers the production of ABA, which in turn induces various genes. *cis*- and *trans*-acting factors involved in ABA-induced gene expression have been analyzed (reviewed in Chandler and Robertson, 1994; Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996; Bray, 1997). A conserved sequence, PyACGTGGC, has been reported to function as an ABA-responsive element (ABRE) in many ABA-responsive genes (Guiltinan et al., 1990; Mundy et al., 1990; Yamaguchi-Shinozaki et al., 1990). cDNAs encoding DNA binding proteins that specifically bind to the ABRE have been cloned and are shown to contain the basic region leucine zipper (bZIP) structure (Guiltinan et al., 1990). Recently, coupling elements have been shown to be required to specify the function of ABRE, constituting an ABA-responsive complex (Shen et al., 1996).

[1] To whom correspondence should be addressed. E-mail kazukoys@jircas.affrc.go.jp; fax 81-298-38-6643.

Analyses of drought-induced genes indicate the existence of ABA-independent as well as ABA-dependent signal transduction cascades between the initial signal of water deficit and the expression of specific genes (reviewed in Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996; Bray, 1997). The ABA-independent expression of genes has been analyzed extensively as well. A novel *cis*-acting element, designated a dehydration-responsive element, which contains the sequence TACCGACAT, has been identified as a *cis*-acting element involved in ABA-independent gene expression under drought, high-salt, and low-temperature conditions (Yamaguchi-Shinozaki and Shinozaki, 1994). The core CCGAC sequence has been found in the promoter regions of many cold-inducible genes and designated as the C repeat or low-temperature–responsive element (Baker et al., 1994; Jiang et al., 1996). The existence of multiple signal transduction cascades has been suggested between the perception of the initial signal of water deficit and the expression of genes (reviewed in Shinozaki and Yamaguchi-Shinozaki, 1996).

We have shown that the expression of one of the dehydration-responsive genes, *rd22*, is induced by the application of exogenous ABA in Arabidopsis (Yamaguchi-Shinozaki and Shinozaki, 1993). Because the induction of the *rd22* gene by ABA can be inhibited by the addition of cycloheximide, an inhibitor of protein biosynthesis, the induction of this gene apparently requires de novo protein biosynthesis for its expression under dehydration conditions (Yamaguchi-Shinozaki and Shinozaki, 1993). Although the regulation of many ABA-inducible genes has been postulated to involve the ABRE consensus sequence, the promoter region of *rd22* does not contain a typical consensus sequence of this type. (Yamaguchi-Shinozaki and Shinozaki, 1993). These results suggest the existence of a regulatory system for ABA-responsive gene expression other than the ABRE–bZIP protein system in vegetative tissues under dehydration conditions.

We have analyzed the promoter region of the *rd22* gene in transgenic tobacco plants containing promoter–β-glucuronidase (*GUS*) fusion genes and demonstrated that a 67-bp DNA fragment of the *rd22* promoter contains *cis*-acting elements involved in dehydration- and ABA-responsive gene expression (Iwasaki et al., 1995). In this study, *cis*-acting elements involved in the dehydration-responsive expression of the *rd22* promoter were identified as MYC and MYB recognition sites by using base substitution analysis of the 67-bp region. We cloned a cDNA for the MYC binding site protein by using a DNA–ligand binding screen and determined that it encodes a basic helix-loop-helix–ZIP (bHLH-ZIP) MYC-related protein. The gene encoding this protein is rapidly induced by dehydration and ABA treatment. Cooperation of MYC- and MYB-related proteins was demonstrated for the transcriptional activation of the *rd22* gene. We report here a novel role for MYC- and MYB-related genes in ABA-responsive gene expression in vegetative tissues under dehydration conditions.

RESULTS

Identification of *cis*-Acting Elements Involved in Dehydration-Responsive Expression of *rd22*

Previous experiments established that a 67-bp DNA fragment between positions −207 and −141 of the *rd22* promoter is sufficient for dehydration- and ABA-induced expression (Iwasaki et al., 1995). Two closely located putative recognition sites (CACATG) for MYC-related bHLH DNA binding proteins and one putative recognition site (TGGTTAG, which is complementary to CTAACCA) for MYB-related proteins were found in this 67-bp fragment. To determine whether these recognition sites for MYC and MYB are the *cis* elements involved in the dehydration-induced transcription of the *rd22* gene, we prepared the 67-bp fragment (wt, wild type) and six mutated 67-bp fragments (a to f) with base substitutions in the MYC and MYB recognition sites (Figure 1A). Two tandemly repeated dimer forms of these fragments

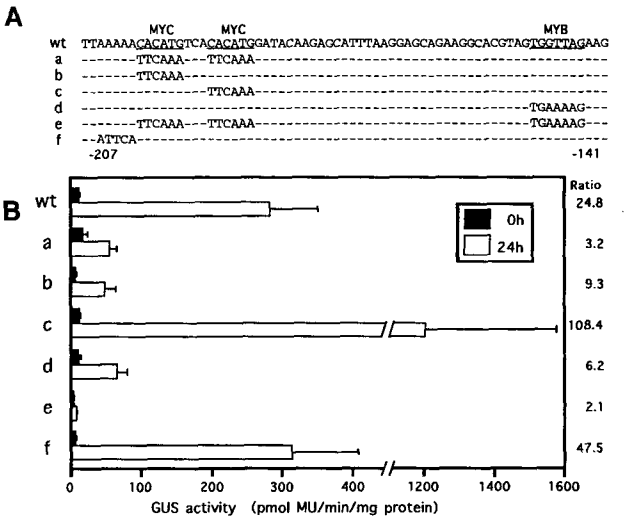

Figure 1. Base Substitution Analysis of the 67-bp Region of the *rd22* Promoter Involved in Dehydration-Responsive Expression in Transgenic Tobacco.

(A) Upper strand sequences of the 67-bp fragment of the *rd22* promoter and its mutated sequences (a to f). The tandemly repeated dimer of each 67-bp fragment containing different mutations was ligated to the −118 *rd22* minimal TATA promoter–*GUS* construct, respectively. Dashes indicate the wild-type sequence, as shown in wt.
(B) Effect of base substitutions in the MYC and MYB recognition sites for dehydration-responsive expression of the *rd22* gene. Half of the leaf was used immediately for the assay of GUS activity, and the other half was dehydrated for 24 hr. GUS activity before and after dehydration was measured in 15 to 32 independent transformants for each construct and is shown as average values. The error bars indicate the standard error of each set of replicates. MU, 4-methylumbelliferone; 0h and 24h, 0 and 24 hr, respectively.

were fused upstream of the −118 *rd22* minimum TATA promoter–*GUS* fusion construct and introduced into tobacco by Agrobacterium-mediated transformation.

We analyzed >15 independent transgenic tobacco plants for the expression of each fusion gene (Figure 1B). The wild-type 67-bp sequence exhibited a 24.8-fold increase in the induction of GUS activity after dehydration (Figure 1B, fragment wt). With base substitutions in both of the two MYC sites, in the first MYC site, or in the MYB site, the level of GUS induction was considerably reduced (3.2-, 9.3-, and 6.2-fold increases, respectively; Figure 1B, fragments a, b, and d). The 67-bp fragment with a base substitution in the second MYC site exhibited four times greater GUS activity (108.4-fold increase) than that of the wild-type fragment (Figure 1B, fragment c). Thus, the second MYC recognition site appears to function as a negative regulator of the expression of the *rd22* promoter–*GUS* fusion gene. The mutant fragment with base substitutions in both MYC sites and the MYB site did not function at all in dehydration-induced expression (2.1-fold increase; Figure 1B, fragment e), whereas the mutant fragment with base substitutions outside of the MYC or MYB recognition sites responded to dehydration stress (47.5-fold increase; Figure 1B, fragment f). These results demonstrate that both the first MYC site and the MYB site function as positive *cis*-acting elements in dehydration-responsive expression of the *rd22* gene.

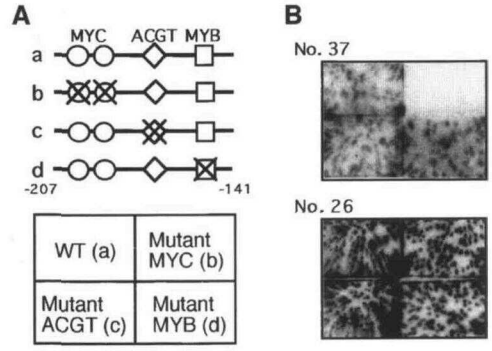

Figure 2. Isolation of a cDNA Encoding a DNA Binding Protein That Interacts with the MYC (CACATG) Motif in the 67-bp Region of the *rd22* Promoter.

(A) A schematic diagram of the probes used in the DNA–ligand binding assay is shown. The wild-type fragment (WT; upper left) or three mutated fragments, mutant in both MYC recognition sequences (upper right), mutant in ACGT core sequence (lower left), or mutant in MYB recognition sequence (lower right), were used. These sequences were described in the legend to Figure 1A. X's indicate the mutated site.

(B) Clone 37 for the MYC motif binding protein and clone 26 for the nonspecific DNA binding protein were plated and blotted on nitrocellulose filters. The filters were sectioned and hybridized individually with each of the DNA probes described in **(A)**.

Isolation of a cDNA Encoding a DNA Binding Protein That Recognizes the MYC Site in the 67-bp DNA Fragment of the *rd22* Promoter

A cDNA expression library was constructed with a λgt11 vector and poly(A)$^+$ RNA from Arabidopsis rosette plants dehydrated for 3 hr. Two million plaques of the cDNA library were screened on the basis of the binding activity of proteins expressed in *Escherichia coli* to the 67-bp fragment probe. Fifteen positive clones were further screened by a DNA binding assay with wild-type and three mutated DNA probes for each recognition site of MYC and MYB and the ACGT motif to examine the DNA binding specificities of proteins produced from isolated clones. The protein derived from a positive phage clone, designated 37, bound to a wild-type probe and probes with mutations at either the MYB recognition site or ACGT motif but not to a probe with mutations at both MYC recognition sites (Figures 2A and 2B). Therefore, this protein had a sequence-specific binding to a MYC recognition site.

Analysis of cDNA clone 37 revealed that the 0.9-kb DNA insert was not full length because it was smaller than predicted by RNA gel blot analysis. A λgt11 library was screened with the clone 37 DNA insert. A cDNA clone containing the entire coding region of this gene was then isolated and designated rd22BP1. The rd22BP1 cDNA contained a single open reading frame of 623 amino acids and encoded a putative protein with a predicted molecular mass of 68 kD (Figure 3A). The putative protein of clone 37 starts at amino acid residue 391 (phenylalanine) of the rd22BP1 cDNA. We searched the DNA and protein databases for sequences homologous to that of the rd22BP1 protein and found its sequence to be homologous with MYC-related DNA binding proteins, such as maize R/S (Perrot and Cone, 1989). The highly conserved C-terminal region of rd22BP1 corresponds to bHLH-ZIP found in MYC-related proteins (Figure 3B). The region near the N terminus of rd22BP1 has a high degree of homology to that of the maize R protein family and bean PG1 (Figure 3C; Kawagoe and Murai, 1996).

Expression of the *rd22BP1* Gene

The expression pattern of the *rd22BP1* gene in Arabidopsis was analyzed using RNA gel blot hybridization to compare it with that of the *rd22* gene. The *rd22BP1* gene was induced within 10 min after dehydration began, as shown in Figure 4A. By contrast, the *rd22* gene was induced 2 hr after dehydration. The accumulation of the *rd22BP1* mRNA was also detected within 10 min after ABA treatment and preceded that of *rd22* mRNA, which was observed 5 hr after treatment and lasted at least 24 hr after treatment. The *rd22BP1* mRNA was detected within 10 min after the initiation of

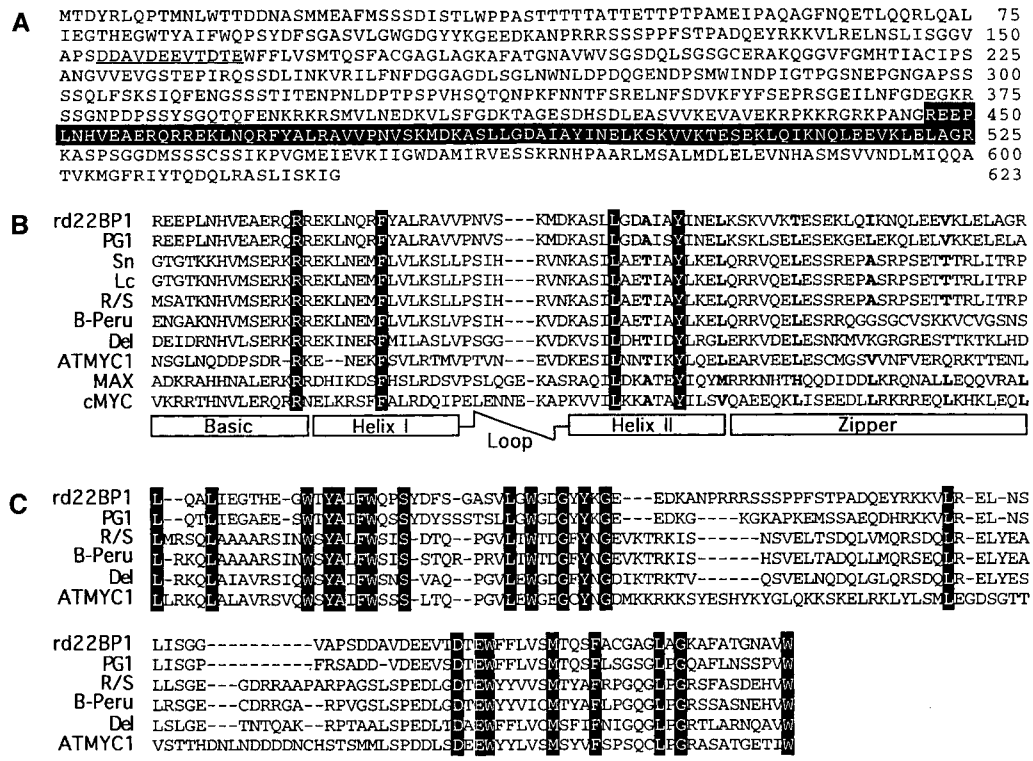

Figure 3. Deduced rd22BP1 Sequence and Comparison with MYC-Related Protein Sequences.

(A) Deduced amino acid sequence of the rd22BP1 protein. The amino acid sequence of the coding region of the putative rd22BP1 protein is shown in single-letter code. The bHLH-ZIP domain is indicated by black boxes. The acidic region is underlined.

(B) Comparison of amino acid sequences of the DNA binding domains of rd22BP1 with MYC-related proteins. The deduced amino acid sequence of rd22BP1 is compared with MYC-related proteins, namely, bean PG1 (Kawagoe and Murai, 1996), maize Sn (Consonni et al., 1992), maize Lc (Ludwig et al., 1989), maize R/S (Perrot and Cone, 1989), maize B-Peru (Radicella et al., 1991), Antirrhinum Del (Goodrich et al., 1992), Arabidopsis ATMYC1 (Urao et al., 1993), human MAX (Blackwood and Eisenman, 1991), and cMYC (Blackwood and Eisenman, 1991). The black background represents perfectly conserved amino acid residues, and dashes indicate gaps introduced to maximize alignment. The rd22BP1 cDNA sequence has been submitted to the GenBank, EMBL, and DDBJ as accession number AB000875. Boldface letters represent the repeat of hydrophobic residues, which extends from helix II into the putative leucine zipper.

(C) Comparison of amino acid sequences of the N-terminal regions of rd22BP1 with MYC-related proteins. The black background represents perfectly conserved amino acid residues, and dashes indicate gaps introduced to maximize alignment.

high-salt treatment, whereas the *rd22* mRNA was not induced until 40 min after high-salt treatment; similar results were observed for the dehydration treatment. When, as a control, the plants were transferred from agar to water, rapid and transient accumulation of the *rd22BP1* mRNA was detected. The *rd22* gene was also induced transiently between 1 and 2 hr. The expression patterns of the *rd22BP1* gene preceded those of the *rd22* gene under stress conditions (Figure 4A). When plants were transferred from normal growth conditions at 22 to 4°C, we did not observe a clear increase in the level of either *rd22BP1* or *rd22* mRNA.

To confirm the induction of the *rd22BP1* gene after ABA treatment, we analyzed RNA isolated from suspension-cultured T87 cells of Arabidopsis (Figure 4B). When the T87-cultured cells were dried on filter paper, the *rd22BP1* gene was rapidly induced within 10 min, reached its maximum at 20 min, and then decreased. The *rd22BP1* mRNA transiently accumulated within 10 min after the addition of the ABA solution to the culture medium. The results indicate that the transcription of *rd22BP1* is regulated by ABA as well as by dehydration stress.

The tissue-specific expression of the *rd22BP1* gene under normal growth conditions was analyzed using RNA gel blot hybridization. As shown in Figure 4C, this gene was expressed in all of the tissues tested, including flowers, siliques, stems, leaves, and roots. The level of its expression

was strong in siliques and stems but weak in leaves and roots. The tissue-specific expression of the *rd22BP1* gene was similar to that of the *rd22* gene (Iwasaki et al., 1995).

The bHLH Region of rd22BP1 Binds Specifically to the First MYC Recognition Site of the rd22 Promoter

To identify the target sequence of the rd22BP1 protein, we constructed a fusion gene with the DNA insert from clone 37 containing the bHLH-ZIP domain and the glutathione *S*-transferase (*GST*) gene by using the pGEX vector. A gel retardation experiment using the recombinant rd22BP1 protein was used to determine its DNA binding ability to the 67-bp fragment probe (Figure 5A). We previously isolated cDNAs for Arabidopsis MYB and MYC homologs Atmyb2 and Atmyc1, respectively (Urao et al., 1993, 1996). The *Atmyb2* gene is induced by dehydration, high-salt conditions, and ABA treatment similar to the *rd22BP1* gene (Urao et al., 1993). By contrast, the *Atmyc1* gene is predominantly expressed in seeds but not induced under stress conditions (Urao et al., 1996). We also examined the DNA binding activities of both GST fusion proteins of ATMYB2 and ATMYC1 to the 67-bp fragment probe (Figure 5A). We detected a shifted band in the gel retardation experiment with the ATMYB2 fusion protein as well as the rd22BP1 protein but not with the ATMYC1 protein. These results indicate that the recombinant rd22BP1 and ATMYB2 proteins bind to the 67-bp region of the *rd22* promoter, but the ATMYC1 protein does not.

The target sequence of the rd22BP1 protein was further analyzed by using base-substituted 67-bp fragments as probes in a gel shift assay (Figure 5B). A shifted DNA band was detected with the wild-type 67-bp DNA fragment (Figure 5C, probe wt) but not with the fragment having mutations in both of the two MYC binding sites (Figure 5C, probe a). With the base-substituted fragment in the first MYC binding site (Figure 5C, probe b), a small amount of shifted band was observed. Base substitutions in the second MYC site (Figure 5C, probe c) made the shifted band stronger than that of the wild-type fragment. In contrast, a similarly shifted band was obtained with fragments having base substitutions in the MYB binding site or the ACGT motif (Figure 5C, probes d and e). These results indicate that the recombinant rd22BP1 protein strongly binds to the first MYC recognition site but does not bind to either MYB or ACGT motifs.

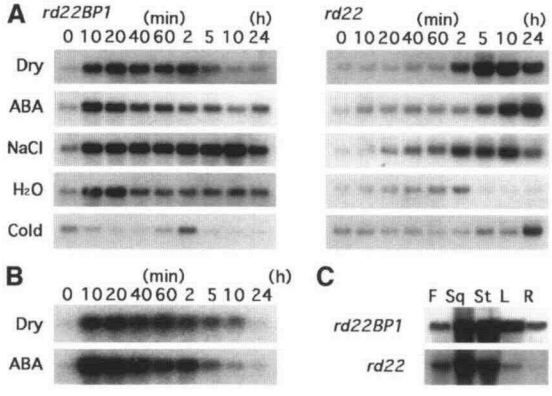

Figure 4. RNA Gel Blot Analysis of the Expression of the *rd22BP1* and *rd22* Genes in Arabidopsis Plants.

(A) Induction of the *rd22BP1* and *rd22* genes by dehydration stress, high-salt conditions, and the application of exogenous ABA. Each lane was loaded with 30 μg of total RNA prepared from unbolted Arabidopsis plants that had been dehydrated (Dry), transferred from agar plates to hydroponic growth conditions in 100 μM ABA (ABA), 250 mM NaCl (NaCl), or water (H_2O), and transferred to and grown at 4°C (Cold). Numbers above each lane indicate the time in minutes or hours (h) after the initiation of treatment.

(B) Expression of the *rd22BP1* gene in Arabidopsis T87 suspension-cultured cells. Each lane was loaded with 30 μg of total RNA prepared from Arabidopsis T87 suspension-cultured cells that had been dehydrated (Dry) or treated with ABA at a final concentration of 100 μM (ABA). Numbers above each lane indicate the time in minutes or hours (h) after the initiation of the treatments before the isolation of RNA.

(C) Expression of the *rd22BP1* and *rd22* genes in a variety of organs of normally grown Arabidopsis. Each lane was loaded with 50 μg of total RNA prepared from flowers (F), siliques (Sq), stems (St), leaves (L), and roots (R).

The rd22BP1 Protein Transactivates the *rd22* Promoter–*GUS* Fusion Gene

To determine whether the rd22BP1 protein is capable of transactivating the transcription driven by the 67-bp DNA fragment of the *rd22* promoter, we performed transactivation experiments using protoplasts prepared from Arabidopsis leaves. Protoplasts were transfected with a *GUS* reporter gene fused to the 67-bp hexamer fragments of the *rd22* promoter and the effector plasmid (Figure 6A). The effector plasmids consisted of the cauliflower mosaic virus 35S promoter fused to the rd22BP1, Atmyb2, or Atmyb1 cDNAs. The tobacco mosaic virus Ω sequence was inserted upstream of these cDNAs to strengthen their translation level.

The *Atmyb2* gene is induced by dehydration, high-salt, and ABA treatment, whereas the *Atmyb1* gene is not (Shinozaki et al., 1992; Urao et al., 1993). Coexpression of the rd22BP1 protein in protoplasts transactivated the expression of the *GUS* reporter gene (Figure 6B). The increase in GUS activity was also achieved by coexpression of the ATMYB2 protein. Coexpression of both the rd22BP1 and ATMYB2 proteins transactivated the expression of the *GUS* reporter gene three times more strongly than when the rd22BP1 protein was used alone. GUS activity obtained by coexpression of the rd22BP1 and ATMYB1 proteins, however, was the same as that obtained when rd22BP1 was used alone. These results suggest that both the rd22BP1 and ATMYB2 proteins may function as interactive transcription activators

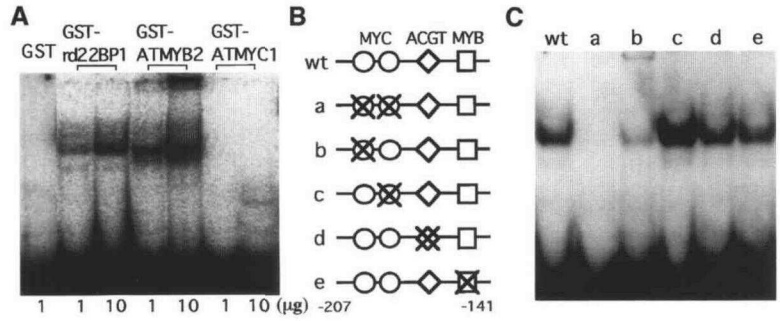

Figure 5. Characterization of DNA Binding Affinity of the Recombinant rd22BP1 Protein to the 67-bp Fragment of the *rd22* Promoter.

(A) Comparison of the binding activity of the rd22BP1, ATMYB2, and ATMYC1 fusion proteins with the 67-bp fragment of the *rd22* promoter. DNA binding reactions were performed with 1 or 10 μg of the GST–rd22BP1, GST–ATMYB2 (Urao et al., 1993), and GST–ATMYC1 proteins (Urao et al., 1996).

(B) The schematic diagram of the probes used in the gel mobility shift assay. Probes a to e contain various mutations in the 67-bp fragment of the *rd22* promoter. X's indicate the mutated site, as described in Figure 1.

(C) Gel retardation assay of the sequence-specific binding of the recombinant rd22BP1 to the 67-bp fragment of the *rd22* promoter. The wt, a, b, c, d, and e probes are diagrammed in **(B)**.

involved in the dehydration- and ABA-responsive expression of the *rd22* gene.

DISCUSSION

To analyze *cis*-acting elements involved in drought- and ABA-responsive gene expression of the *rd22* gene that requires de novo protein biosynthesis for its expression (Yamaguchi-Shinozaki and Shinozaki, 1993), we analyzed the 67-bp region of the *rd22* promoter containing positive regulatory *cis*-acting elements (Iwasaki et al., 1995). The base substitution experiments demonstrated that both the MYB and the first MYC recognition sites in the 67-bp region are involved in dehydration-responsive expression (Figure 1). The second MYC recognition site, however, appears to function as an inhibitory element. These results suggest that the first MYC and the MYB recognition sites function cooperatively in the dehydration-responsive gene expression of the *rd22* gene.

We conducted a DNA–ligand binding screen by using the 67-bp DNA fragment as a probe and isolated a cDNA, rd22BP1, encoding a putative 68-kD protein (Figure 3A). The rd22BP1 protein contains a typical bHLH-ZIP motif at the C terminus (Figure 3B), which is found in human cMYC and MAX, the maize R family (Sn, Lc, and R/S), and bean PG1. Human MyoD, maize B-Peru, and Antirrhinum *del* gene products, on the other hand, have a bHLH but no ZIP motif (Davis et al., 1987; Ludwig et al., 1989; Perrot and Cone, 1989; Blackwood and Eisenman, 1991; Radicella et al., 1991; Consonni et al., 1992; Goodrich et al., 1992; Kawagoe and Murai, 1996). The HLH motif consists of two putative amphipathic α helices that flank an Ω-type loop and mediates formation of homodimers or heterodimers: the basic region is believed to form a DNA contact surface (Ferré-D'Amaré et al., 1993; Ma et al., 1994). Although the CANNTG motif is the recognition sequence of all of the bHLH proteins examined, each bHLH protein has a binding site preference for the central two bases of the CANNTG motif (Blackwell and Weintraub, 1990).

Two MYC recognition sequences in the 67-bp region of the *rd22* promoter are CACATG. The bacterially expressed rd22BP1 fusion protein evidently recognizes and binds only the first CACATG motif (Figure 5C). The fusion protein bound to the wild-type 67-bp DNA fragment but not to the 67-bp DNA fragment with a base substitution in the first CACATG motif. In contrast, the fusion protein bound more strongly to the 67-bp DNA fragment with a base substitution in the second CACATG motif than to the wild-type 67-bp DNA fragment (Figure 5C). These results indicate that not only internal sequences of CANNTG motif but also flanking sequences may affect the binding affinity of the rd22BP1 protein. On the other hand, analysis of the *cis*-acting elements in the 67-bp region, using transgenic tobacco plants, indicates that the first MYC recognition site functions as a *cis*-acting element in the dehydration-induced expression of the *GUS* fusion gene but that the second MYC recognition site functions as an inhibitory element (Figure 1). These results coincide with the DNA binding specificity of the rd22BP1 protein to the two MYC recognition sites (Figure 5C).

The ABA-induced expression of the *rd22* gene requires de novo protein synthesis. Thus, we postulated that regulatory protein factors induced by ABA are involved in transcriptional activation of the *rd22* gene. The *rd22BP1* gene, encoding a transcription factor MYC homolog, was shown to be induced by dehydration, high-salt conditions, and ABA

treatment (Figures 4A and 4B). We found 13 Arabidopsis expressed sequence tags (GenBank, EMBL, and DDBJ accession numbers T46027, T20442, T22106, T46547, H36262, R30455, T20523, T75680, T88024, R65147, T41998, R65140, and R65141) in the DNA database. They all have partial sequence homology with the bHLH region of the rd22BP1 protein. Because three of them, T46027, T20442, and T22106, have high sequence homology with the bHLH region of the rd22BP1 protein, we analyzed the expression of genes corresponding to these three expressed sequence tag clones by RNA gel blot hybridization. However, we could not detect any induced expression of their mRNAs by dehydration stress and ABA treatment (data not shown). The *rd22BP1* gene is the one most likely to be specifically induced by dehydration stress and ABA treatment among the bHLH-related genes in Arabidopsis.

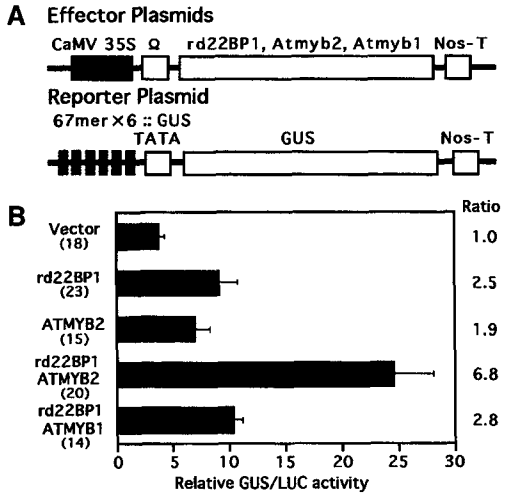

Figure 6. Transactivation of the *rd22* Promoter–*GUS* Fusion Gene by rd22BP1 and ATMYB2 Proteins Using Arabidopsis Protoplasts.

(A) The schematic diagram of the effector and reporter constructs used in cotransfection experiments. The effector constructs contain the cauliflower mosaic virus (CaMV) 35S promoter and tobacco mosaic virus Ω sequence (Gallie et al., 1987) fused to the rd22BP1, Atmyb2, or Atmyb1 cDNAs (35S–Ω–rd22BP1, 35S–Ω–ATMYB2, and 35S–Ω–ATMYB1, respectively). Nos-T indicates the polyadenylation signal of the gene for nopaline synthetase. The reporter construct contained the 67-bp fragment of the *rd22* promoter tandemly repeated six times. The promoter was fused to the −61 *rd29A* minimal TATA promoter–*GUS* construct (67mer × 6::GUS).

(B) Transactivation of the *rd22* promoter–*GUS* fusion gene by the rd22BP1 and ATMYB2 proteins. The 67mer × 6::*GUS* reporter gene was transfected with different sets of effector plasmids: pBI35SΩ vector as a control treatment (Vector), 35S–Ω–rd22BP1 (rd22BP1), 35S–Ω–ATMYB2 (ATMYB2), 35S–Ω–rd22BP1 and 35S–Ω–ATMYB2 (rd22BP1 ATMYB2), and 35S–Ω–rd22BP1 and 35S–Ω–ATMYB1 (rd22BP1 ATMYB1). To normalize for transfection efficiency, the cauliflower mosaic virus 35S promoter–luciferase (LUC) plasmid was cotransfected in each experiment. The error bar indicates the standard error of each set of replicates. Numbers in parentheses indicate the number of independent experiments, and ratios indicate the multiplicities of expression compared with the value obtained with pBI35SΩ vector.

The timing of the induction of the *rd22BP1* gene preceded that of the *rd22* gene under various treatments (Figure 4A). The tissue-specific expression of the *rd22BP1* gene was very similar to that of the *rd22* gene (Figure 4C). Moreover, coexpression of rd22BP1 protein in protoplasts transactivated expression of the *rd22* promoter–*GUS* fusion gene (Figure 6B), and the rd22BP1 protein bound to the first MYC recognition site in the 67-bp region of the *rd22* promoter (Figure 5). These results indicate that the *rd22BP1* gene product most likely functions as one of the transcription factors involved in the induction of the *rd22* gene. The expression of the *rd22BP1* gene was superinduced by cycloheximide and ABA treatment (data not shown), whereas the ABA-responsive expression of the *rd22* gene was inhibited by cycloheximide (Yamaguchi-Shinozaki and Shinozaki, 1993). The *rd22BP1* gene is therefore not likely to require de novo protein biosynthesis for its expression in response to dehydration and ABA. The *rd22BP1* gene product, however, appears to function as one of the protein factors that are involved in the expression of the *rd22* gene (Figure 7).

In the 67-bp region of the *rd22* promoter, one MYB recognition site as well as the first MYC motif were shown to be involved in dehydration-responsive gene expression in tobacco (Figure 1). We have isolated a gene for a MYB homolog, *Atmyb2*, that is induced by dehydration and ABA treatment in Arabidopsis (Urao et al., 1993). Coexpression of the ATMYB2 protein in Arabidopsis protoplasts transactivated expression of the *rd22* promoter–*GUS* fusion gene (Figure 6B). The bacterially expressed ATMYB2 protein also bound the MYB recognition site in the 67-bp region of the *rd22* promoter (Figure 5A). These results indicate that the product of the dehydration-inducible *Atmyb2* gene is also involved in the transactivation of the *rd22* gene. Moreover, coexpression of both of the rd22BP1 and ATMYB2 proteins in Arabidopsis protoplasts further transactivated the *rd22* promoter–*GUS* gene (Figure 6B). The levels of transactivation using both the rd22BP1 and ATMYB2 proteins were three times greater than those when only the rd22BP1 protein or the ATMYB2 protein was used. This observation indicates that rd22BP1, a bHLH-ZIP protein, cooperates with ATMYB2, a MYB protein, to transactivate the *rd22* gene under dehydration conditions (Figure 7).

Cooperation of the MYC and MYB proteins has been reported in plants but not in animals (Goff et al., 1990; Roth et al., 1991; Tuerck and Fromm, 1994). The MYC-related proteins or bHLH proteins function as transcriptional regulators in anthocyanin biosynthesis in maize (*R* gene family) and Antirrhinum (*del*) (Ludwig and Wessler, 1990). In maize, the *C1* and *Pl* genes encoding MYB homologs have been reported to require the *R/B* gene product for MYC homologs to transactivate target genes, such as *Bronze1* and *A1* for anthocyanin

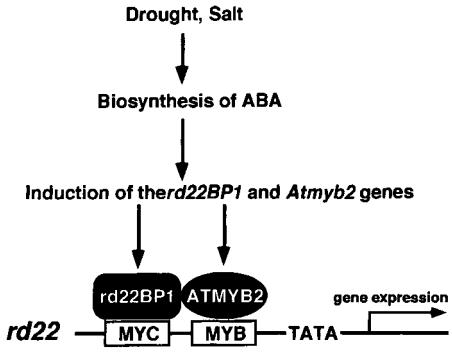

Figure 7. A Model for the Induction of the *rd22* Gene under Water Stress Conditions.

Dehydration or salt stress triggers the production of ABA, which induces the expression of *rd22BP1* and *Atmyb2*. rd22BP1 and ATMYB2 then bind to the MYC and MYB sites of the *rd22* promoter and activate the expression of the *rd22* gene.

biosynthesis (Goff et al., 1990; Roth et al., 1991; Grotewold et al., 1994; Tuerck and Fromm, 1994). Functional analysis of maize *B* and *C1* genes has demonstrated that the N-terminal domain of the B (MYC homolog) protein interacts with the C1 (MYB homolog) protein (Goff et al., 1992). The maize *C1* gene is regulated by ABA and the *VIVIPAROUS1* gene during seed maturation (Hattori et al., 1992). A functional homolog of the maize *R* gene, *TRANSPARENT TESTA GLABRA*, and a *myb* gene, *GLABROUS1*, have been shown to cooperatively regulate trichome development in Arabidopsis (Larkin et al., 1994). This study suggests the interaction of MYC and MYB proteins in ABA-induced gene expression in vegetative tissues under dehydration stress conditions (Figure 7). We are now analyzing the interaction between the rd22BP1 and ATMYB2 proteins.

METHODS

Plant Materials and Stress Treatments

Plants (*Arabidopsis thaliana* ecotype Columbia) were grown on germination medium agar plates for 3 weeks, as described previously (Yamaguchi-Shinozaki and Shinozaki, 1994). Arabidopsis rosette plants were harvested from GM agar plates (Valvekens et al., 1988) and were then dehydrated in plastic culture dishes without covers at 22°C for 60% humidity under dim light. High-salt and cold stress treatments and treatment with abscisic acid (ABA) were performed as described previously (Yamaguchi-Shinozaki and Shinozaki, 1994). The T87 cell line, derived from Arabidopsis, was kindly provided by M. Axelos (CNRS-INRA, Castanet-Tolosan, France). The T87 cells were grown as described previously (Axelos et al., 1992). The T87 cells were harvested by filtration. The cells were then dried on Whatman No. 3MM paper (dehydration treatment), or an ABA solution was added to the culture medium to a final concentration of 100 μM (ABA treatment). The plants or the T87 cells were subjected to the stress treatments for various periods of time and then frozen in liquid nitrogen for further analyses.

Preparation of the 67-bp Fragment of the *rd22* Promoter with or without Base Substitutions

Six 67-bp mutant fragments with base substitutions (a to f) between positions −207 and −141 of the *rd22* promoter with HindIII sites at both ends were prepared by polymerase chain reaction (Figure 1). Forward primers containing mutations used for the amplification of the mutant fragments (underlined nucleotides indicate base substitutions) were 5′-AAGCTTAAAAATTCAATCATTCAAAGATACAAGAGCATTTAAGGA-3′ (mutant fragments a and e), 5′-AAGCTTAAAAATTCAAATCACACATGGATACAAGAGCATTTAAGGA-3′ (mutant fragment b), 5′-AAGCTTAAAAACACATGTCATTCAAAGATACAAGAGCATTTAAGGA-3′ (mutant fragment c), and 5′-AAGCTTATTCACACATGTCACACATGG-3′ (mutant fragment f); a reverse primer containing mutations was 5′-AAGCTTCTTTTCACTACGTGCCTTCTGCTC-3′ (mutant fragments d and e). A wild-type forward primer (5′-AAGCTTAAAAACACATGTCACACA-3′; mutant fragment d) and a wild-type reverse primer (5′-AAGCTTCTAACCACTACGTGCCT-3′; mutant fragments a, b, c, and f) were also used for the amplification of the 67-bp mutant DNA fragments in combination with mutated primers.

The polymerase chain reaction products were cloned into the SmaI site of pBluescript II SK− (Stratagene, La Jolla, CA), and the resulting plasmids were confirmed by sequencing and digested with HindIII. Preparation of the 67-bp fragment of the *rd22* promoter without base substitutions was described previously (Iwasaki et al., 1995). These 67-bp fragments with or without base substitutions were used for the construction of the base-substituted promoter region of the *rd22* gene fused to a β-glucuronidase (*GUS*) gene, the screening of the cDNA expression library, and the gel mobility shift assay.

Construction of the *rd22* Promoter–*GUS* Fusion Gene for Transformation

The tandemly repeated dimeric 67-bp fragments with or without base substitutions were ligated to the HindIII site of the −118 *rd22* minimal TATA promoter–*GUS* fusion construct (Iwasaki et al., 1995). The structures of the fusion constructs were confirmed by sequencing the boundary sites of the fused gene, as described previously (Yamaguchi-Shinozaki and Shinozaki, 1994).

Construction of Effector and Reporter Plasmids Used in a Transient Transactivation Experiment

Effector plasmids used in a transient transactivation experiment were constructed with DNA fragments containing the *rd22BP1*, *Atmyb2*, or *Atmyb1* coding regions that were cloned into polylinker sites of the plant expression vector pBI35SΩ derived from pBI221 (Clontech, Palo Alto, CA). For the construction of the pBI35SΩ vector, pBI221 was digested with SmaI and SacI to delete the *GUS* coding region and ligated with a SmaI-NotI-SacI polylinker. Subsequently, the tobacco mosaic virus Ω sequence (Gallie et al., 1987), which was provided by H. Shinshi (National Institute of Bioscience and Human Technology, Tsukuba, Japan), was ligated with the BamHI site located down-

stream of a cauliflower mosaic virus 35S promoter. To construct 35S–Ω–rd22BP1, the NotI fragment containing the coding region of the rd22BP1 cDNA was cloned into the NotI site of the pBI35SΩ vector. To construct a reporter plasmid, the *rd29A* minimal TATA promoter (Yamaguchi-Shinozaki and Shinozaki, 1994) was replaced with the 35S promoter of pBI221, and then the 67-bp fragments of the *rd22* promoter tandemly repeated six times were ligated into the HindIII site located upstream of the *rd29A* minimal TATA promoter.

Transgenic Plants and Assays of GUS Activity

The *rd22* promoter–*GUS* fusion constructs were introduced into *Agrobacterium tumefaciens* LBA4404 and used to transform *Nicotiana tabacum* cv SR1, as described previously (Yamaguchi-Shinozaki and Shinozaki, 1994). GUS activity was measured as described previously (Jefferson et al., 1986).

Screening of the cDNA Expression Library

The Arabidopsis cDNA expression library was constructed using the λgt11 expression vector with an oligo(dT) primer, and poly(A)$^+$ RNA was prepared from total RNA that had been isolated from rosette plants dehydrated for 3 hr. The expression library was screened using a DNA–ligand binding assay, according to a standard protocol using the 67-bp fragment of the *rd22* promoter with or without base substitutions as probes (Singh et al., 1988). DNA probes were labeled by filling in 5′ overhangs with α-^{32}P-dCTP and the Klenow fragment of DNA polymerase I.

DNA and RNA Gel Blot Analyses

DNA and RNA gel blot hybridizations were performed as described previously (Yamaguchi-Shinozaki and Shinozaki, 1994).

Gel Mobility Shift Assay

A BamHI fragment derived from clone 37 was cloned with pGEX-3X vector (Smith and Johnson, 1988) and then transformed into *Escherichia coli* JM109 cells. Production and purification of the glutathione *S*-transferase (GST) fusion protein were performed as described previously (Urao et al., 1993). The 67-bp fragment of the *rd22* promoter with or without base substitutions was labeled with α-^{32}P-dCTP, as described previously. Gel mobility shift assays were conducted as described previously (Urao et al., 1993).

Transactivation Experiments in Protoplasts

Isolation of Arabidopsis mesophyll protoplasts and polyethylene glycol–mediated DNA transfection was performed as described previously (Abel and Theologis, 1994). GUS activity was assayed as described above. Luciferase assays were performed using the PicaGene luciferase assay kit (Toyo-Ink, Tokyo, Japan), according to the manufacturer's instructions. Protein concentration was determined by the Bradford method (Bio-Rad).

ACKNOWLEDGMENTS

We thank Setsuko Miura, Satomi Yoshida, and Atsuko Yahiro for their excellent technical assistance. This work was supported in part by a grant from the Program for Promotion of Basic Research Activities for Innovative Biosciences to K.Y.-S. This work was also supported in part by a Grant-in-Aid from the Ministry of Education, Science, and Culture of Japan to K.S.

Received May 9, 1997; accepted July 21, 1997.

REFERENCES

Abel, S., and Theologis, A. (1994). Transient transformation of Arabidopsis leaf protoplasts: A versatile experimental system to study gene expression. Plant J. **5,** 421–427.

Axelos, M., Curie, C., Mazzolini, L., Bardet, C., and Lescure, B. (1992). A protocol for transient gene expression in *Arabidopsis thaliana* protoplasts isolated from cell suspension cultures. Plant Physiol. Biochem. **30,** 123–128.

Baker, S.S., Wilhelm, K.S., and Thomashow, M.F. (1994). The 5′-region of *Arabidopsis thaliana cor15a* has *cis*-acting elements that confer cold-, drought- and ABA-regulated gene expression. Plant Mol. Biol. **24,** 701–713.

Blackwell, T.K., and Weintraub, H. (1990). Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. Science **250,** 1104–1110.

Blackwood, E.M., and Eisenman, R.N. (1991). Max: A helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science **251,** 1211–1217.

Bray, E.A. (1997). Plant responses to water deficit. Trends Plant Sci. **2,** 48–54.

Chandler, P.M., and Robertson, M. (1994). Gene expression regulated by absicic acid and its relation to stress tolerance. Annu. Rev. Plant Physiol. Plant Mol. Biol. **45,** 113–141.

Consonni, G., Viotti, A., Dellaporta, S.L., and Tonelli, C. (1992). cDNA nucleotide sequence of Sn, a regulatory gene in maize. Nucleic Acids Res. **20,** 373.

Davis, R.L., Weintraub, H., and Lassar, A.B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell **51,** 987–1000.

Ferré-D'Amaré, A.R., Prendergast, G.C., Ziff, E.B., and Burley, S.K. (1993). Recognition by Max of its cognate DNA through a dimeric b/HLH/Z domain. Nature **363,** 38–45.

Gallie, D.R., Sleat, D.E., Watts, J.W., Turner, P.C., and Wilson, T.M.A. (1987). A comparison of eukaryotic viral 5′-leader sequences as enhancers of mRNA expression in vivo. Nucleic Acids Res. **15,** 8693–8711.

Goff, S.A., Klein, T.M., Roth, B.A., Fromm, M.E., Cone, K.C., Radicella, J.P., and Chandler, V.L. (1990). Transactivation of anthocyanin biosynthetic genes following transfer of *B* regulatory genes into maize tissues. EMBO J. **9,** 2517–2522.

Goff, S.A., Cone, K.C., and Chandler, V.L. (1992). Functional analysis of the transcriptional activator encoded by the maize *B* gene:

Evidence for a direct functional interaction between two classes of regulatory proteins. Genes Dev. **6,** 864–875.

Goodrich, J., Carpenter, R., and Coen, E.S. (1992). A common gene regulates pigmentation pattern in diverse plant species. Cell **68,** 955–964.

Grotewold, E., Drummond, B.J., Bowen, B., and Peterson, T. (1994). The *myb*-homologous *P* gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset. Cell **76,** 543–553.

Guiltinan, M.J., Marcotte, W.R., and Quatrano, R.S. (1990). A plant leucine zipper protein that recognizes an abscisic acid response element. Science **250,** 267–271.

Hattori, T., Vasil, V., Rosenkrans, L., Hannah, L.C., McCarty, D.R., and Vasil, I.K. (1992). The *Viviparous-1* gene and abscisic acid activate the *C1* regulatory gene for anthocyanin biosynthesis during seed maturation in maize. Genes Dev. **6,** 609–618.

Ingram, J., and Bartels D. (1996). The molecular basis of dehydration tolerance in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **47,** 377–403.

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. **247,** 391–398.

Jefferson, R.A., Burgess, S.M., and Hirsh, D. (1986). β-Glucuronidase from *Escherichia coli* as a gene-fusion marker. EMBO J. **83,** 8447–8451.

Jiang, C., Iu, B., and Singh, J. (1996). Requirement of a CCGAC *cis*-acting element for cold induction of the *BN115* gene from winter *Brassica napus.* Plant Mol. Biol. **30,** 679–684.

Kawagoe, Y., and Murai, N. (1996). A novel basic region/helix-loop-helix protein binds to a G-box motif CACGTG of the bean seed storage protein β-*phaseolin* gene. Plant Sci. **116,** 47–57.

Larkin, J.C., Oppenheimer, D.G., Lloyd, A.M., Paparozzi, E.T., and Marks, M.D. (1994). Roles of *GLABROUS1* and *TRANSPARENT TESTA GLABRA* genes in Arabidopsis trichome development. Plant Cell **6,** 1065–1076.

Ludwig, S.R., and Wessler, S.R. (1990). Maize *R* gene family: Tissue-specific helix-loop-helix proteins. Cell **62,** 849–851.

Ludwig, S.R., Habera, L.F., Dellaporta, S.L., and Wessler, S.R. (1989). *Lc*, a member of the maize *R* gene family responsible for tissue-specific anthocyanin production, encodes a protein similar to transcriptional activators and contains the *myc*-homology region. Proc. Natl. Acad. Sci. USA **86,** 7092–7096.

Ma, P.C.M., Rould, M.A., Weintraub, H., and Pabo, C.O. (1994). Crystal structure of MyoD bHLH domain–DNA complex: Perspectives on DNA recognition and implications for transcriptional activation. Cell **77,** 451–459.

Mundy, J., Yamaguchi-Shinozaki, K., and Chua, N.-H. (1990). Nuclear proteins bind conserved elements in the abscisic acid–responsive promoter of a rice *rab* gene. Proc. Natl. Acad. Sci. USA **87,** 406–410.

Perrot, G.H., and Cone, K.C. (1989). Nucleotide sequence of the maize *R-S* gene. Nucleic Acids Res. **17,** 8003.

Radicella, J.P., Turks, D., and Chandler, L.V. (1991). Cloning and nucleotide sequence of a cDNA encoding B-Peru, a regulatory protein of the anthocyanin pathway in maize. Plant Mol. Biol. **17,** 127–130.

Roth, B.A., Goff, S.A., Klein, T.M., and Fromm, M.E. (1991). *C1*- and *R*-dependent expression of the maize *Bz1* gene requires sequences with homology to mammalian *myb* and *myc* binding sites. Plant Cell **3,** 317–325.

Shen, Q., Zhang, P., and Ho, T.-H.D. (1996). Modular nature of abscisic acid (ABA) response complexes: Composite promoter units that are necessary and sufficient for ABA induction of gene expression in barley. Plant Cell **8,** 1107–1119.

Shinozaki, K., and Yamaguchi-Shinozaki, K. (1996). Molecular responses to drought and cold stress. Curr. Opin. Biotechnol. **7,** 161–167.

Shinozaki, K., Yamaguchi-Shinozaki, K., Urao, T., and Koizumi, M. (1992). Nucleotide sequence of a gene from *Arabidopsis thaliana* encoding a *myb* homologue. Plant Mol. Biol. **19,** 493–499.

Singh, H., LeBowits, J.H., Boldwin, A.S., Jr., and Sharp, P.A. (1988). Molecular cloning of an enhancer binding protein: Isolation by screening of an expression library with a recognition site DNA. Cell **52,** 415–423.

Smith, D.B., and Johnson, K.S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. Gene **67,** 31–40.

Tuerck, J.A., and Fromm, M.E. (1994). Elements of the maize *A1* promoter required for transactivation by the anthocyanin *B/C1* or phlobaphene *P* regulatory genes. Plant Cell **6,** 1655–1663.

Urao, T., Yamaguchi-Shinozaki, K., Urao, S., and Shinozaki, K. (1993). An Arabidopsis *myb* homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. Plant Cell **5,** 1529–1539.

Urao, T., Yamaguchi-Shinozaki, K., Mitsukawa, N., Shibata, D., and Shinozaki, K. (1996). Molecular cloning and characterization of a gene that encodes a MYC-related protein in *Arabidopsis.* Plant Mol. Biol. **321,** 571–576.

Valvekens, D., Van Montagu, M., and Van Lijsebettens, M. (1988). *Agrobacterium tumefaciens*–mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc. Natl. Acad. Sci. USA **85,** 5536–5540.

Yamaguchi-Shinozaki, K., and Shinozaki, K. (1993). 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,** 17–25.

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.

Yamaguchi-Shinozaki, K., Mundy, J., and Chua, N.-H. (1990). Four tightly linked *rab* genes are differentially expressed in rice. Plant Mol. Biol. **14,** 29–39.