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

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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).
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., 1986). 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 (CATACAT) for MYC-related bHLH DNA binding proteins and one putative recognition site (TGTTTAG, which is complementary to CTCAACA) 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.](image-url)
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.

**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 2](image-url)
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 acidic region is underlined. The bHLH-ZIP domain is indicated by black boxes.

(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 PGI (Kawagoe and Murai, 1996), maize Sn (Consonni et al., 1992), maize Lc (Ludwig et al., 1989), maize WS (Perrot and Cone, 1989), maize B-Peru (Radicella et al., 1991), Antirrhinum De1 (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.

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 \textit{rd22BP1} gene was similar to that of the \textit{rd22} gene (Iwasaki et al., 1995).

**The bHLH Region of \textit{rd22BP1} Binds Specifically to the First MYC Recognition Site of the \textit{rd22} Promoter**

To identify the target sequence of the \textit{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 \textit{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 \textit{Atmyb2} gene is induced by dehydration, high-salt conditions, and ABA treatment similar to the \textit{rd22BP1} gene (Urao et al., 1993). By contrast, the \textit{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 \textit{ATMYB2} and \textit{ATMYC1} to the 67-bp fragment probe (Figure 5A). We detected a shifted band in the gel retardation experiment with the \textit{ATMYB2} fusion protein as well as the \textit{rd22BP1} protein but not with the \textit{ATMYC1} protein. These results indicate that the recombinant \textit{rd22BP1} and \textit{ATMYB2} proteins bind to the 67-bp region of the \textit{rd22} promoter, but the \textit{ATMYC1} protein does not.

The target sequence of the \textit{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 \textit{rd22BP1} protein strongly binds to the first MYC recognition site but does not bind to either MYB or ACGT motifs.

**The \textit{rd22BP1} Protein Transactivates the \textit{rd22} Promoter-GUS Fusion Gene**

To determine whether the \textit{rd22BP1} protein is capable of transactivating the transcription driven by the 67-bp DNA fragment of the \textit{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 \textit{rd22} promoter and the effector plasmid (Figure 6A). The effector plasmids consisted of the cauliflower mosaic virus 35S promoter fused to the \textit{rd22BP1}, \textit{ATMYB2}, or \textit{ATMYC1} cDNAs. The tobacco mosaic virus \(\Omega\) sequence was inserted upstream of these cDNAs to strengthen their translation level.

The \textit{Atmyb2} gene is induced by dehydration, high-salt, and ABA treatment, whereas the \textit{Atmyc1} gene is not (Shinozaki et al., 1992; Urao et al., 1993). Coexpression of the \textit{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 \textit{ATMYB2} protein. Coexpression of both the \textit{rd22BP1} and \textit{ATMYB2} proteins transactivated the expression of the GUS reporter gene three times more strongly than when the \textit{rd22BP1} protein was used alone. GUS activity obtained by coexpression of the \textit{rd22BP1} and \textit{ATMYB1} proteins, however, was the same as that obtained when \textit{rd22BP1} was used alone. These results suggest that both the \textit{rd22BP1} and \textit{ATMYB2} proteins may function as interactive transcription activators.
involved in the dehydration- and ABA-responsive expression of the \textit{rd22} gene.

**DISCUSSION**

To analyze \textit{cis}-acting elements involved in drought- and ABA-responsive gene expression of the \textit{rd22} gene that requires de novo protein biosynthesis for its expression (Yamaguchi-Shinozaki and Shinozaki, 1993), we analyzed the 67-bp region of the \textit{rd22} promoter containing positive regulatory \textit{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 \textit{rd22} gene.

We conducted a DNA–ligand binding screen by using the 67-bp DNA fragment as a probe and isolated a cDNA, \textit{rd22BP1}, encoding a putative 68-kD protein (Figure 3A). The \textit{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 \textit{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; Consnoni et al., 1992; Goodrich et al., 1992; Kawagoe and Murai, 1996). The HLH motif consists of two putative amphipathic $\alpha$ helices that flank an $\Omega$-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 \textit{rd22} promoter are CACATG. The bacterially expressed \textit{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 \textit{rd22BP1} protein. On the other hand, analysis of the \textit{cis}-acting elements in the 67-bp region, using transgenic tobacco plants, indicates that the first MYC recognition site functions as a \textit{cis}-acting element in the dehydration-induced expression of the \textit{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 \textit{rd22BP1} protein to the two MYC recognition sites (Figure 5C).

The ABA-induced expression of the \textit{rd22} gene requires de novo protein synthesis. Thus, we postulated that regulatory protein factors induced by ABA are involved in transcriptional activation of the \textit{rd22} gene. The \textit{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.

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 PI 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

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**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 repeatedly six times. The promoter was fused to the –61 rd29A minimal TATA promoter–GUS construct (67mer X 6:GUS).

(B) Transactivation of the rd22 promoter–GUS fusion gene by the rd22BP1 and ATMYB2 proteins. The 67mer X 6:GUS reporter gene was transfected with different sets of effector plasmids: pBl35SCl 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 pBl35SCl vector.
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.

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

**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). 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 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 pBiSS50 derived from pBI221 (Clontech, Palo Alto, CA). For the construction of the pBiSS50 vector, pBi221 was digested with Smal and SacI to delete the GUS coding region and ligated with a Smal–NotI–SacI polylinker. Subsequently, the tobacco mosaic virus (TMV) 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 the GUS coding region.
stream of a cauliflower mosaic virus 35S promoter. To construct 35S–rd22BP1, the NotI fragment containing the coding region of the rd22BP1 cDNA was cloned into the NotI site of the pBl3SSSQ vector. To construct a reporter plasmid, the rd29A minimal TATA promoter (Yamaguchi-Shinozaki and Shinozaki, 1994) was replaced with the 35S promoter of pBl322, 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-32P-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-32P-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.

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Role of Arabidopsis MYC and MYB homologs in drought- and abscisic acid-regulated gene expression.

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*Plant Cell* 1997;9:1859-1868

DOI 10.1105/tpc.9.10.1859

This information is current as of December 22, 2017