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# Involvement of Maize Dof Zinc Finger Proteins in Tissue-Specific and Light-Regulated Gene Expression

**Shuichi Yanagisawa[a,b,1] and Jen Sheen[b]**

[a] Department of Life Sciences (Chemistry), Graduate School of Arts and Sciences, University of Tokyo, Meguro, Tokyo 153, Japan

[b] Department of Genetics, Harvard Medical School, and Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114

**Dof is a novel family of plant proteins that share a unique and highly conserved DNA binding domain with one $C_2$–$C_2$ zinc finger motif. Although multiple Dof proteins associated with diverse gene promoters have recently been identified in a variety of plants, their physiological functions and regulation remain elusive. In maize, Dof1 (MNB1a) is constitutively expressed in leaves, stems, and roots, whereas the closely related Dof2 is expressed mainly in stems and roots. Here, by using a maize leaf protoplast transient assay, we show that Dof1 is a transcriptional activator, whereas Dof2 can act as a transcriptional repressor. Thus, differential expression of Dof1 and Dof2 may permit leaf-specific gene expression. Interestingly, in vivo analyses showed that although DNA binding activity of Dof1 is regulated by light-dependent development, its transactivation activity and nuclear localization are not. Moreover, in vivo transcription and in vitro electrophoretic mobility shift assays revealed that Dof1 can interact specifically with the maize C4 phosphoenolpyruvate carboxylase gene promoter and enhance its promoter activity, which displays a light-regulated expression pattern matching Dof1 activity. We propose that the evolutionarily conserved Dof proteins can function as transcriptional activators or repressors of tissue-specific and light-regulated gene expression in plants.**

## INTRODUCTION

Plants and animals respond to a wide range of internal and external stimuli by modulating transcription of diverse genes. To aid in our understanding of the regulatory mechanisms of plant gene expression and signal transduction pathways, numerous *cis* elements and *trans*-acting factors have been identified and characterized; these studies have revealed that many plant DNA binding proteins can be grouped into distinct classes based on their conserved DNA binding domains (Katagiri and Chua, 1992; Menkens et al., 1995; Martin and Paz-Ares, 1997). Each member of these families interacts with closely related DNA sequence motifs that are often found in multiple gene promoters controlled by different regulatory signals. For example, G-box binding factors (GBFs), which belong to a superfamily of basic leucine zipper (bZIP) DNA binding proteins interacting with a CACGTG motif called a G-box, seem to play a role in the responses of plant gene promoters to light, stresses, and phytohormones. It has been proposed that the combination of different *cis* elements and *trans*-acting factors may produce the diversity and specificity required for the regulation of gene expression (Izawa et al., 1993; Menkens et al., 1995; Martin and Paz-Ares, 1997). Although it has been shown that several plant DNA binding proteins containing well-known motifs, such as the leucine zipper and Myb domain, can activate transcription (Katagiri et al., 1990; Lohmer et al., 1991; Oeda et al., 1991; Schindler et al., 1992b; Schmidt et al., 1992; Baranowskij et al., 1994; Izawa et al., 1994; Sablowski et al., 1994; Solano et al., 1995; Ni et al., 1996; Urao et al., 1996), knowledge of the functions and physiological roles of most plant DNA binding proteins is still limited.

Dof (for DNA binding with one finger) is a novel family of DNA binding proteins that have been found in diverse plant species. The proteins comprising this family contain the Dof domain, which is a highly conserved amino acid sequence involved in DNA binding (Yanagisawa, 1995, 1996a). Mutational analysis and chelator experiments have suggested that the Dof domain is a 52–amino acid stretch with a single ($C_2$–$C_2$) zinc finger. The zinc finger of Dof proteins is distinct from other known zinc fingers in terms of its amino acid sequence and arrangement of cysteine residues for the coordination of zinc essential for DNA binding (Yanagisawa, 1995).

Maize Dof1 (MNB1a) is the first member of the Dof protein family to have been identified in plants. It has been shown to bind to the AAAAGG core sequence motif found in many plant promoters (Yanagisawa and Izui, 1993; S. Yanagisawa and J. Sheen, unpublished data). Other cDNA clones encoding Dof2 and Dof3 have also been obtained by screening a

[1] To whom correspondence should be addressed. E-mail csyanag @komaba.ecc.u-tokyo.ac.jp; fax 81-3-3485-2904.

maize cDNA library and using Dof1 as a probe (Yanagisawa, 1995). Recently, a Dof protein OBP1 (for OBF binding protein) was isolated from Arabidopsis. OBP1 can interact with the bZIP protein OBF4 (octopine synthase [*ocs*] element binding factor) and enhance the binding of OBF4 to the *ocs* element in the *ocs* gene promoter. It has also been shown that OBP1 itself has a DNA binding activity similar to that of Dof1. Speculation is that OBP1 might be involved in gene regulation in response to plant hormone and/or stress signals (Zhang et al., 1995; Chen et al., 1996).

Another member of the Dof family of proteins is NtBBF1 (*Nicotiana tabacum* RolB domain B factor 1) from tobacco. NtBBF1 binds to regulatory DNA domain B of the plant oncogene *rolB* promoter that is critical for *rolB* expression in meristems (de Paolis et al., 1996). It has also been reported that a maize endosperm–specific Dof protein PBF binds to the prolamin box, which is a candidate for a positive *cis* element for endosperm-specific gene expression (Vicente-Carbajosa et al., 1997). It is likely that Dof proteins are conserved widely in the plant kingdom and play pivotal roles in the expression of diverse plant genes (Yanagisawa, 1995, 1996a). However, like most plant DNA binding proteins, the physiological functions of Dof proteins have yet to be determined.

Although the Dof domain is highly conserved, the alignment of different Dof domains has revealed less conserved regions, thereby making it possible to classify Dof proteins into subgroups (Yanagisawa, 1996a). Maize Dof1 and Dof2 show the highest amino acid identity (91%) in their Dof DNA binding domains and can interact with each other through Dof domains (Yanagisawa, 1997). These observations suggest that Dof1 and Dof2 might share similar target genes in maize. However, functional analysis in vivo is essential to show the physiological function of each protein. To determine the function of Dof proteins in plants, we investigated the transcriptional activity and regulation of maize Dof1 and Dof2 by using a maize leaf protoplast transient expression assay. Because Dof1 and Dof2 have distinct expression patterns despite their similar DNA binding activities, their distinct transcriptional activities may be responsible for tissue-specific gene expression. We further show that Dof1's ability to differentially activate transcription in green and etiolated leaf cells is regulated through its DNA binding domain. These results suggest that Dof proteins may be involved in novel molecular mechanisms underlying tissue-specific and light-regulated gene expression in plants.

## RESULTS

### A Functional Assay for Transcription Factors in Maize Leaf Protoplasts Is Established

To establish an assay system to analyze the function of plant transcription factors in maize leaf cells, we first performed experiments with the well-characterized yeast transcription factor GAL4 (Ma et al., 1988). A reporter construct (GBS–72–CAT) was created by placing five copies of the GAL4 binding site upstream of a fusion gene carrying the truncated 35S promoter (−72) of cauliflower mosaic virus, the chloramphenicol acetyltransferase (*CAT*) gene, and the nopaline synthase (*nos*) gene terminator (Figure 1A). The effector plasmids (hsp–GAL4) were constructed by fusing a reconstructed GAL4 to the Arabidopsis 18.2 heat shock protein promoter. Use of the heat shock promoter allowed us to control the expression level of the transcription factors in maize mesophyll protoplasts (Sheen et al., 1995). A construct (hsp–none) without any factor and another construct

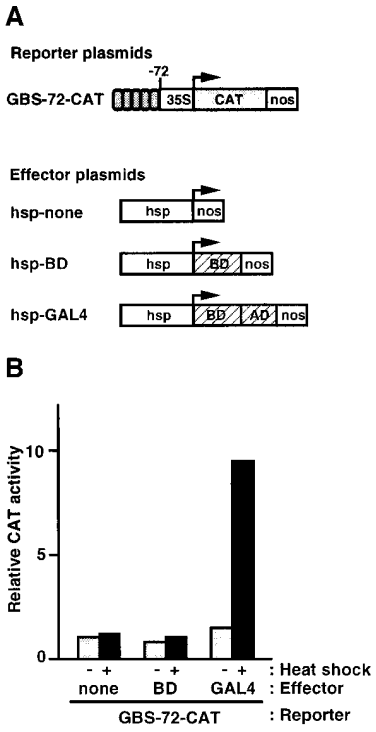


**Figure 1.** The Maize Leaf Protoplast as a System for the Functional Analysis of Transcription Factors.

**(A)** Reporter and effector constructs. GBS–72–CAT contains five copies of the GAL4 binding sites upstream of the 35S promoter truncated at −72. Effector constructs were created with the DNA binding domain (BD) and the transcriptional activation domain (AD) of GAL4, respectively. All effectors are controlled by the Arabidopsis 18.2 heat shock protein (hsp) promoter and the nopaline synthase (nos) terminator. Arrows indicate the transcription initiation sites.

**(B)** Transcriptional activation by GAL4 in maize leaf protoplasts. The GBS–72–CAT construct was cotransfected into greening protoplasts with various effector constructs and the UBI–GUS construct as an internal standard by electroporation. The cotransfected protoplasts were divided into two portions and incubated (16 hr total) at 23°C with (+) or without (−) 2 hr of heat shock at 42°C. CAT activity from protoplasts cotransfected with the construct without a cDNA (hsp–none) and incubated without heat shock was set to 1.

(hsp–BD) carrying only the DNA binding domain of GAL4 were also created as negative controls. The experiment was performed with an internal control (UBI–GUS) with the maize ubiquitin promoter and the β-glucuronidase reporter gene (Sheen, 1996).

Maize mesophyll protoplasts isolated from greening leaves were cotransfected with the reporter plasmid (GBS–72–CAT), the internal control (UBI–GUS), and various effector plasmids by electroporation. The transfected protoplasts were divided into two parts for incubation. CAT activities from protoplasts with and without a heat shock treatment (42°C for 2 hr) were compared. A high level of CAT activity could be detected only in the heat-shocked protoplasts transfected with the hsp–GAL4 effector (Figure 1B). Thus, it is feasible to analyze the activity of transcription factors controlled by a heat shock promoter in maize mesophyll protoplasts.

### Dof1 Is a Transcriptional Activator

Although Dof1 has a specific DNA binding activity in vitro, it remained to be demonstrated whether Dof1 could bind to its target sites and activate transcription in vivo. To test the activity of Dof1 in maize leaf protoplasts, two reporter plasmids (4S–45–CAT and 4S–72–CAT) were constructed by fusing four copies of Dof1 binding sites to the 35S promoter truncated at position −72 or −45 (Figure 2A). In some cases, the −72 basal promoter, containing the TATA box and one TGACG motif, can support the synthetic DNA binding sites for transcription better than the −45 basal promoter, containing only the TATA box (Schäffner and Sheen, 1992). Three reporter constructs, the promoterless construct (PL–CAT) and two constructs including only the truncated 35S promoter (−72–CAT and −45–CAT), were also generated as negative controls (Figure 2A). Two effector plasmids, hsp–Dof1 and hsp–AD–Dof1 (with the activation domain of GAL4), were created in the expression vector with the heat shock promoter. The fusion of a GAL4 activation domain to Dof1 (AD–Dof1) would allow the detection of DNA binding activity even if Dof1 could not activate transcription alone.

Cotransfection of the 4S–72–CAT reporter and various effector constructs showed that Dof1 alone was sufficient to activate transcription after heat shock (Figure 2B). The addition of the GAL4 activation domain to Dof1 enhanced activation further (1.5-fold) after heat shock. The heat shock treatment did not affect CAT activity in the protoplasts cotransfected with the negative control hsp–none (Figure 2B). To confirm that the transcription activation by Dof1 was due to its specific binding to the target sequence in vivo, cotransfection was performed with hsp–Dof1 and various reporter constructs with (4S–45–CAT and 4S–72–CAT) or without the Dof1 binding sites (PL–CAT, −45–CAT, and −72–CAT). The results showed that the Dof1 DNA binding sites are absolutely required for Dof1 to activate the reporter gene in vivo (Figure 2C). These results indicate that Dof1 binds to its target sequence selectively and activates transcription in a sequence-specific manner in vivo.

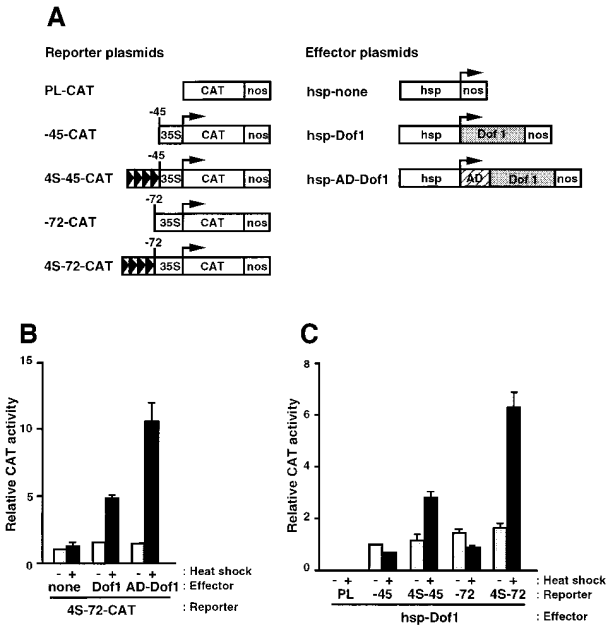


**Figure 2.** Dof1 Is a Transcriptional Activator in Maize Leaf Protoplasts.

**(A)** Dof1 reporter and effector constructs. Reporter constructs were created by placing four copies (arrowheads) of the Dof1 DNA binding sites in front of the 35S promoter truncated at position −45 (4S–45–CAT) or −72 (4S–72–CAT). Arrows indicate the transcription initiation sites. Effector constructs were generated by inserting the Dof1 cDNA between the *hsp* promoter and the *nos* terminator. The construct without a cDNA (hsp–none) was used as a DNA transfection control. In the hsp–AD–Dof1 construct, the activation domain of GAL4 (AD) was fused in frame to Dof1.

**(B)** Dof1 activates transcription in maize leaf protoplasts. The 4S–72–CAT reporter construct was cotransfected with various effector constructs and the UBI–GUS internal control into greening protoplasts. Transfected protoplasts were incubated with (+) or without (−) heat shock. CAT activity from protoplasts cotransfected with hsp–none (none) without heat shock was set to 1.

**(C)** Dof1 transactivation depends on the Dof1 DNA binding sites. The hsp–Dof1 effector construct was cotransfected with various reporter constructs, including −45–CAT (−45), 4S–45–CAT (4S–45), −72–CAT (−72), and 4S–72–CAT (4S–72), into greening protoplasts. PL is the promoterless construct. CAT activity from protoplasts cotransfected with −45–CAT without heat shock was set to 1.

### Dof1 Has Independent Domains for Transcriptional Activation and DNA Binding

We showed previously that the Dof domain in the N-terminal region of Dof1 is sufficient for its DNA binding activity in vitro

(Yanagisawa, 1995). Thus, the C-terminal region may be involved in transcriptional activation. To test this idea, we investigated the transcriptional activation effect of a truncated Dof1 (Dof1ΔC from amino acids 1 to 147) that lacked Dof1's C-terminal region. A cotransfection experiment with 4S–72–CAT showed that Dof1ΔC had lost the ability to activate transcription (Figure 3). However, when the GAL4 activation domain was fused to the Dof1ΔC protein (AD–Dof1ΔC), the ability for sequence-specific transcriptional activation was restored (Figure 3). These results suggest that the N-terminal region of Dof1 is sufficient for DNA binding both in vivo and in vitro, whereas the C-terminal region of Dof1 is essential to activate transcription.

To show that the C-terminal region of Dof1 is sufficient as a transcriptional activation domain, we constructed a plasmid (hsp–BD–Dof1ΔN) that would express a chimeric protein with the GAL4 DNA binding domain and the C-terminal region (amino acids 136 to 238) of Dof1 under heat shock control. When cotransfected with the GBS–72–CAT reporter construct (with the GAL4 binding sites) into greening maize leaf protoplasts, the expression of this chimeric protein activated CAT activity from the reporter construct. The expression of the GAL4 DNA binding domain (hsp–BD) alone could not activate transcription (Figure 3). These results indicate that the C-terminal region (amino acids 136 to 238) of Dof1 can function by itself as a transcriptional activation domain. This domain is a novel transcriptional activation domain because we have not found any homology with known activation domains of diverse transcription factors.

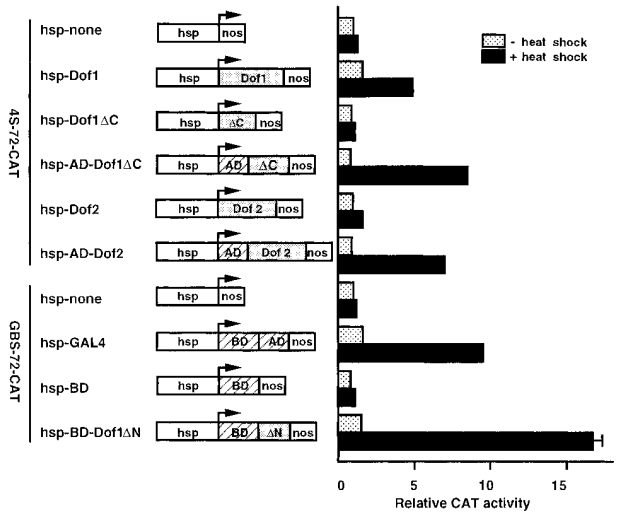


**Figure 3.** Dof1 Has Independent Transcriptional Activation and DNA Binding Domains.

Various effector constructs were cotransfected into greening protoplasts with a reporter construct (4S–72–CAT or GBS–72–CAT) and the UBI–GUS internal control. The hsp–Dof1ΔC construct contains only the N-terminal region of Dof1 (amino acids 1 to 147). The hsp–AD–Dof1ΔC construct contains the GAL4 activation domain (AD) fused to the N-terminal region of Dof1. The hsp–AD–Dof2 construct also carries the activation domain of GAL4. The hsp–BD–Dof1ΔN construct was created by fusing the GAL4 DNA binding domain (BD) to the C-terminal region (amino acids 138 to 238) of Dof1. Arrows indicate the transcription initiation sites. CAT activity from protoplasts cotransfected with the hsp–none construct without heat shock was set to 1.

### Dof2 Can Act as a Transcriptional Repressor

Although Dof1 and Dof2 share high sequence identity in the Dof domain, the C-terminal sequence of Dof2 is completely different from the sequence of Dof1 that is a transcriptional activation domain (Figure 4A; Yanagisawa, 1996a). An electrophoretic mobility shift assay (EMSA) with the recombinant Dof1 and Dof2 proteins revealed that Dof1 and Dof2 can bind similarly to the Dof1 binding sequence in vitro (Figure 4B). By using RNA gel blot analysis with total RNAs from several tissues and a Dof2 gene-specific probe, a single band of 1.1 kb was observed in all lanes (Figure 4C). However, hybridization signals were quite different, showing that Dof2 is highly expressed in stems and roots but weakly expressed in green and etiolated leaves. Analysis with the Dof1-specific probe confirmed the constitutive and ubiquitous expression of Dof1 in maize, as reported previously (Yanagisawa and Izui, 1993; Yanagisawa, 1996b). Thus, expression of Dof2 overlaps with that of Dof1 only in stems and roots. These results suggest that two Dof proteins with similar DNA binding activity might have distinct functions in maize.

To investigate the function of Dof2, a cotransfection experiment was performed with the hsp–Dof2 effector and the 4S–72–CAT reporter constructs. Transcriptional activation by Dof2 was barely detectable (Figure 3). To ensure the heat-induced expression of Dof2 as well as Dof1 in maize protoplasts, Dof1 and Dof2 were fused to a double hemagglutinin (DHA) epitope tag at the C terminus. The expression of Dof1–HA and Dof2–HA proteins in protoplasts was detected by $^{35}$S-methionine labeling and immunoprecipitation (Figure 5). Thus, the inability of Dof2 to transactivate is not due to the lack of expression or instability of the protein. In fact, when the activation domain of GAL4 was fused to Dof2 (AD–Dof2), the heat-induced expression of this protein activated transcription dramatically, as did the AD–Dof1ΔC protein (Figure 3). These results indicate that Dof2 can interact with the Dof1 binding sites in vivo but that Dof2 itself lacks activity for transcriptional activation, at least in maize leaf protoplasts.

Because Dof2 can bind to Dof1 target sites yet cannot activate transcription, Dof2 might act as a competitive inhibitor of Dof1 through its identical or very similar sequence specificity when binding to DNA and repress transactivation by Dof1. To examine this idea, we developed an in vivo competition assay that was designed to express Dof1 under the control of a constitutive promoter (the 35S promoter) and to

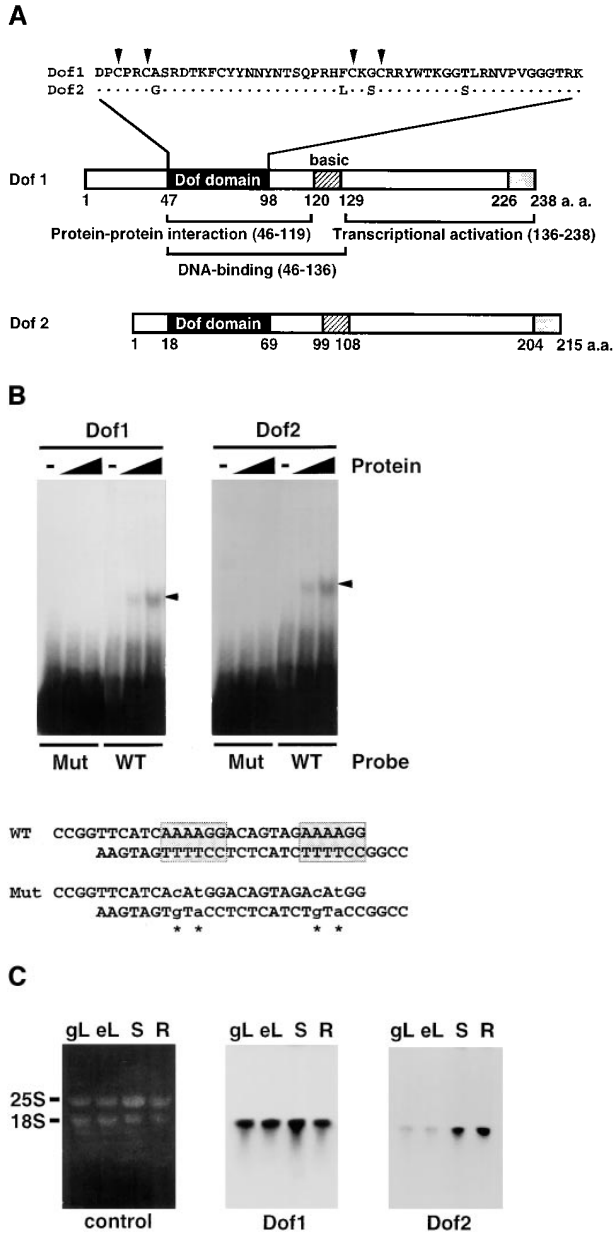


**Figure 4.** Maize Dof1 and Dof2 Share Similar DNA Binding Activity but Show Distinct Expression Patterns.

**(A)** Schematic representation of Dof1 and Dof2. The Dof domains are indicated by the black boxes. The functional domains for DNA binding (Yanagisawa, 1995), self-association (Yanagisawa, 1997), and transcriptional activation (identified in this study) are indicated. The basic boxes are putative nuclear localization signals. The homologous regions at the C-terminal ends that are specific to Dof1 and Dof2 among Dof proteins (Yanagisawa, 1995) are shown as dotted boxes. Cysteine residues that coordinate the binding to zinc are also indicated (arrowheads). a.a., amino acids.

**(B)** EMSA for DNA binding of Dof1 and Dof2. The $^{32}$P-labeled DNA probe with the wild-type (WT, boxed) or mutant (Mut, marked with asterisks) Dof1 binding site AAAAGG was incubated in the absence (−) or increasing (30 and 100 ng, indicated by triangles) amount of recombinant Dof1 or Dof2 proteins. The DNA–protein complex is marked by arrowheads.

**(C)** Differential expression of Dof1 and Dof2. Total RNA (20 μg in each lane) prepared from green leaves (gL), etiolated leaves (eL), stems (S), and roots (R) was used for RNA gel blot analysis by using a $^{32}$P-labeled Dof1- or Dof2-specific probe. rRNAs were used as loading controls.

express a competitive Dof2 factor under the control of an inducible promoter (the heat shock promoter). Cotransfection of the 35S–none effector construct that did not encode any factor had no effect on transcription, whereas cotransfection of the 35S–Dof1 construct activated CAT activity approximately fivefold (Figure 6). This activation was not influenced by cotransfection with the hsp–none construct and the heat shock treatment. However, activation was reduced significantly by heat-induced expression of the Dof1ΔC protein, indicating that this deletion mutant protein without the transcriptional activation domain could function as an inhibitor through competitive binding in vivo. A very similar effect was observed by heat-induced expression of Dof2 (Figure 6). These results suggest that Dof2, lacking transcriptional activation activity, blocked Dof1-mediated transactivation in vivo. Dof2 may act as a repressor in plants. Because the expression patterns of Dof1 and Dof2 can overlap in roots and stems but not in leaves, Dof2 might inhibit Dof1 activity in stems and roots through their competitive binding and allow leaf-specific gene expression controlled by Dof1.

### Dof1 Activity Is Regulated by Light-Dependent Development in Maize Leaves

Many genes that are expressed in a leaf-specific manner are also regulated by light. To investigate the effect of light on the Dof1 activity, we compared the activity of Dof1 in greening and etiolated maize leaf protoplasts. The greening protoplasts were isolated from dark-grown etiolated maize leaves that had been illuminated for 16 hr. The etiolated protoplasts were isolated from dark-grown etiolated leaves that had not been illuminated. It has already been shown that protoplasts freshly prepared from well-differentiated leaves maintain physiological regulation of several maize gene promoters by light, sugars, and hormones (Sheen, 1990, 1991, 1993, 1996; Schäffner and Sheen, 1991, 1992; Jang and Sheen, 1994). As shown in Figure 7, Dof1 did not increase CAT activity significantly in etiolated protoplasts as it did in greening protoplasts. Even the AD–Dof1 protein, which activated transcription by 10-fold in greening protoplasts, was barely able to activate transcription in etiolated protoplasts (Figure 7).

To determine the light-responsive mechanism of Dof1, we compared the activities of GAL4 and two chimeric proteins, AD–Dof1ΔC and BD–Dof1ΔN, in greening and etiolated

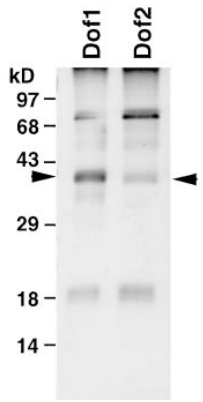


**Figure 5.** Expression of Dof1 and Dof2 in Maize Protoplasts.

The proteins labeled with $^{35}$S-methione and immunoprecipitated by the anti-HA antibody (12CA5) were separated by SDS-PAGE and visualized by fluorography. The positions of Dof1–HA and Dof2–HA (as indicated by arrowheads) were higher than the calculated values, which were also observed with Dof proteins expressed in *Escherichia coli* (Yanagisawa, 1997). The signal of Dof2–HA is weaker than that of Dof1–HA because Dof2–HA has only one methionine residue, whereas Dof1–HA has three methionine residues.

protoplasts. GAL4 had very similar transactivational activity in both types of protoplasts, indicating that cotransfection efficiency and general transcriptional activity are similar in greening and etiolated protoplasts. However, the two chimeric proteins clearly had different activities. Similar to Dof1, the AD–Dof1ΔC protein, with the Dof1 DNA binding domain and the GAL4 activation domain, was strongly activated in greening protoplasts but weakly activated in etiolated protoplasts. However, the BD–Dof1ΔN protein, with the GAL4 DNA binding domain and the Dof1 activation domain, had strong transcriptional activation activity in both types of protoplasts.

### Dof1 Is Localized in the Nucleus of Both Greening and Etiolated Protoplasts

There are at least two possible mechanisms by which the accessibility of Dof1 to its DNA binding sites is controlled in etiolated and greening protoplasts. The Dof1 DNA binding domain may keep Dof1 in the cytoplasm of etiolated protoplasts but allows Dof1 to be translocated into the nucleus to activate transcription in greening protoplasts. Alternatively, Dof1 could be constitutively localized in the nucleus but only able to bind to DNA or/and interact with other transcription factors in greening protoplasts. To investigate these possibilities, we analyzed subcellular localization of Dof1 in greening and etiolated protoplasts by using a green fluorescent protein (GFP) fusion that allowed direct visualization in living cells. Because the N-terminal DNA binding domain of Dof1 is sufficient to mimic the light-responsive behavior of Dof1, a Dof1–sGFP fusion was made with the N-terminal region (amino acids 1 to 147) of Dof1 and a synthetic GFP that gives optimal expression in plants (Chiu et al., 1996). As shown in Figures 8A, 8B, 8E, and 8F, the fluorescence of the sGFP as a control was detected mainly in the cytoplasm of the transfected protoplasts. However, the Dof1–sGFP fusion (Figures 8C, 8D, 8G, and 8H) was expressed and localized in the nucleus of both etiolated and greening protoplasts. Thus, Dof1 seems to accumulate in the nuclei of both greening and etiolated protoplasts. The possibility that the subcellular localization of the Dof1 protein is controlled by light signals can be ruled out. In addition, this result suggests that the N-terminal DNA binding domain is sufficient for the nuclear localization of the Dof1 protein. The N-terminal region of Dof1 contains a region rich in basic amino acids (KKKPASKKRR, amino acids 120 to 129; Figure 4A) that resembles the bipartite nuclear localization sequences in yeast, animals, and plants (Raikhel, 1992). The region could be the nuclear localization signal.

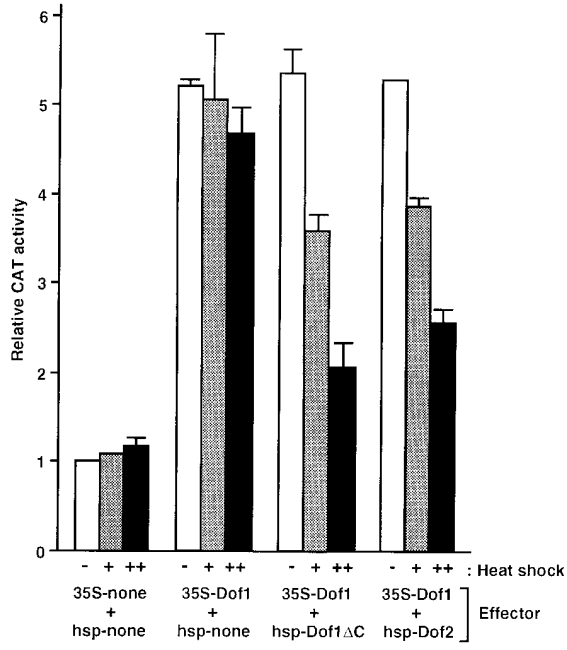


**Figure 6.** Dof2 Represses Transcriptional Activation Mediated by Dof1.

Greening protoplasts were cotransfected with various combinations of reporter and effector constructs and the UBI–GUS construct as an internal control. The transfected protoplasts were divided into three parts and incubated at 42°C for 0 hr (−), 1 hr (+), or 2 hr (++) to control the expression levels of Dof2. CAT activity from protoplasts cotransfected with the 35S–none and hsp–none constructs without heat shock was set to 1.

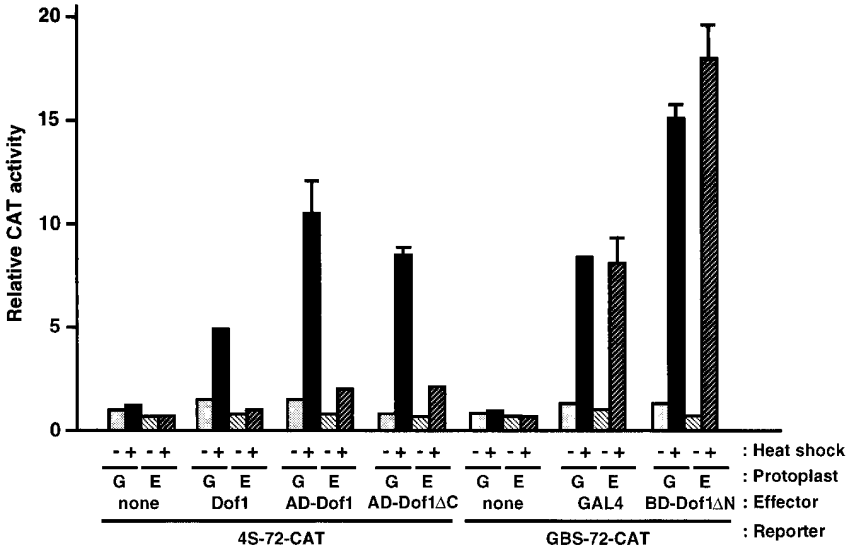


**Figure 7.** Dof1 Activity Is Inhibited in Etiolated Protoplasts.

Greening (G) or etiolated (E) protoplasts were cotransfected with the 4S–72–CAT reporter construct and various effector constructs: hsp–none (none), hsp–Dof1 (Dof1), hsp–AD–Dof1 (AD-Dof1), or hsp–AD–Dof1ΔC (AD-Dof1ΔC). The two types of protoplasts were also cotransfected with the GBS–72–CAT reporter construct and various effector constructs: hsp–none (none), hsp–GAL4 (GAL4), or hsp–BD–Dof1ΔN (BD-Dof1ΔN). UBI–GUS was used as an internal control. The transfected protoplasts were incubated without (−) or with (+) heat shock at 42°C for 2 hr. CAT activity from greening protoplasts cotransfected with GBS–72–CAT, and hsp–none without heat shock was set to 1.

### Dof1 Binds to a Specific Maize Gene Promoter in Vivo and in Vitro

Although we have shown that Dof1 can activate transcription from a synthetic promoter, its target genes remain to be identified. The sequences and important *cis* elements of the promoters of several maize leaf-specific and light-regulated genes have already been analyzed (Sheen, 1990, 1991, 1993; Schäffner and Sheen, 1991, 1992; H. Huang and J. Sheen, unpublished data). The C4-type phosphoenolpyruvate carboxylase (C4PEPC) gene promoter shows multiple Dof1 binding sites that have been demonstrated to have high enhancer activity. The *cis* elements or promoters of other maize genes expressed in leaves do not have potential Dof1 binding sites. To reveal the potential target genes of Dof1, we performed a cotransfection experiment with the AD–Dof1ΔC chimeric protein by using the *CAT* reporter gene controlled by the C4PEPC gene promoter or other maize photosynthetic gene promoters as controls (Figure 9A). Because these photosynthetic gene promoters are regulated by multiple *cis* elements (Sheen, 1990, 1991, 1993; Schäffner and Sheen, 1991, 1992; H. Huang and J. Sheen, unpublished data), the use of AD–Dof1ΔC with a strong activation domain from GAL4 might bypass the requirement of other transcriptional partners in the activation of complex promoters. As shown in Figure 9A, the expression of the AD–Dof1ΔC chimeric protein specifically and significantly activated the promoter of the C4PEPC gene but did not activate or only weakly activated the other four photosynthetic gene promoters. This result indicates that Dof1 may bind specifically to the C4PEPC gene promoter in vivo.

To confirm further that Dof1 might regulate the C4PEPC promoter in vivo, we tested the effect of Dof1 itself. A strong and constitutive (35SC4PPDK) promoter (Sheen, 1993; Chiu et al., 1996) was used to drive the expression of Dof1. As shown in Figure 9B, Dof1 activated light-dependent activity of the C4PEPC gene promoter differentially in greening and etiolated protoplasts. As a control, the expression of Dof2 reduced the activity of the C4PEPC gene promoter. These results suggest that Dof1 and Dof2 not only bind to the C4PEPC promoter but also function in the context of the C4PEPC promoter in vivo.

To identify directly the binding sites of Dof1 and Dof2 in the C4PEPC gene promoter, we performed an in vitro EMSA by using recombinant Dof proteins and synthetic DNA probes. Eight sequence motifs in the C4PEPC promoter showing similarity to the previously identified Dof1 binding sequence AAAAGG were synthesized (Figure 10A) and used as probes. As shown in Figure 10B, both Dof1 and Dof2 bind specifically to five of the probes (a, b, f, g, and h) but not to the other three (c, d, and e). Probes f and g are located in the *cis* elements (A29) identified previously (Schäffner and Sheen, 1992). A cotransfection experiment using a reporter plasmid with a monomer or dimer of the A29 sequence revealed that Dof1 can enhance the light-dependent activity of this *cis* element (data not shown). These results suggest that

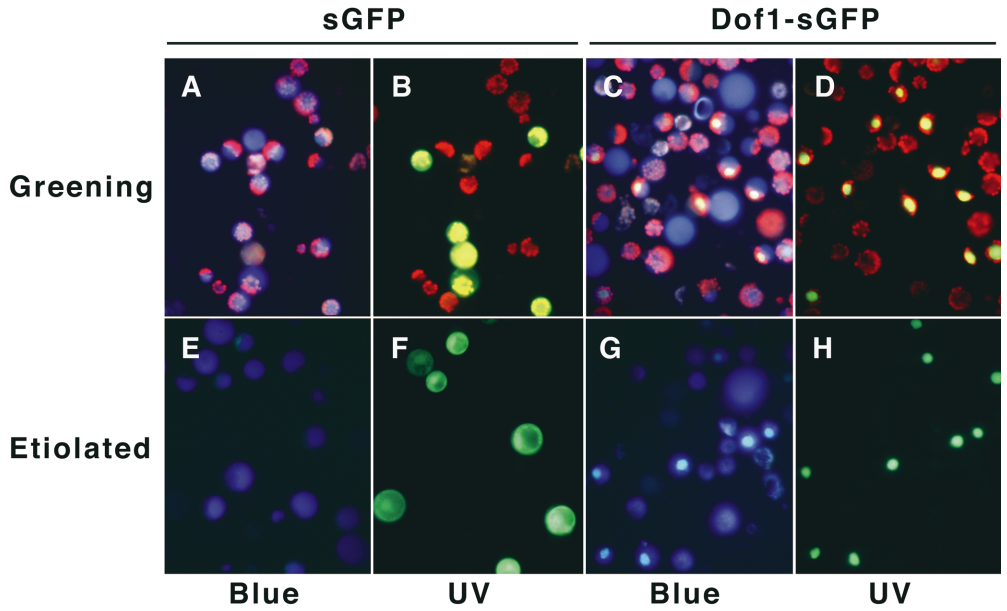


**Figure 8.** Nuclear Localization of Dof1 Is Independent of Light.

**(A)** to **(D)** Greening protoplasts were electroporated with hsp–sGFP (**[A]** and **[B]**) or hsp–Dof1–sGFP (**[C]** and **[D]**) and observed by using blue (**[A]** and **[C]**) or UV (**[B]** and **[D]**) light after heat shock induction (42°C for 2 hr). The green–yellow fluorescence of the Dof1–sGFP fusion in **(C)** and **(D)** is localized in the nuclei; however, that of sGFP **(A)** and **(B)** is not. The red autofluorescence is from chlorophyll. The fluorescence of GFP in greening protoplasts appears yellow because of a mixture of green and red fluorescence.

**(E)** to **(H)** Etiolated protoplasts were electroporated with hsp–sGFP (**[E]** and **[F]**) or hsp–Dof1–sGFP (**[G]** and **[H]**) and observed using blue (**[E]** and **[G]**) or UV (**[F]** and **[H]**) light after heat shock induction (42°C for 2 hr). The green–yellow fluorescence of the Dof1–sGFP fusion **(G)** and **(H)** is localized in the nuclei; however, that of sGFP **(E)** and **(F)** is not. The nuclear localization of the Dof1–sGFP fusion was confirmed by nuclear staining with bisbenzimide Hoechst 33258, as described previously (Sheen, 1993). The experiment was repeated twice with similar results.

the C4PEPC gene promoter might be one of the target promoters of Dof1.

## DISCUSSION

### Structural and Functional Domains of the Dof Proteins

We have shown that Dof1 is a transcriptional activator, with the N-terminal Dof domain for DNA binding (in vivo and in vitro) and with the C-terminal region (amino acids 136 to 238) for transcriptional activation. Based on the analysis of various chimeric proteins constructed with maize Dof1 and yeast GAL4, the DNA binding and the transcriptional activation domains of Dof1 can be considered structurally and functionally independent. Most remarkably, the DNA binding domain can confer light responsiveness on a heterologous transcriptional activation domain. The structural and domain arrangement of maize Dof1 is very similar to that of the well-characterized GAL4 in yeast (Ma and Ptashne, 1987). However, except for the $C_2$–$C_2$ zinc finger, no overt sequence similarity has been found for the two transcription factors. A survey of the yeast genome sequence did not reveal any Dof-like proteins in yeast. Thus, unlike the factors involved in the general transcription machinery, such as the TATA-box binding proteins and CCAAT-box binding proteins, Dof proteins might have evolved to function specifically as transcription factors in multicellular eukaryotes, perhaps for tissue-specific gene regulation not needed in unicellular eukaryotes. Because an extensive sequence search did not reveal any Dof-like proteins in animals, Dof proteins might represent a novel family of zinc finger proteins in higher plants.

The six Dof proteins with established amino acid sequences (Dof1, Dof2, Dof3, OBP1, NtBBF1, and PBF) show extensive homologies only in the Dof domain of their N-terminal regions but not in their C-terminal regions. This observation suggests that each C-terminal region may play a distinct role. With the exception of the Dof1 and Dof2 proteins studied here, there is no direct evidence that Dof proteins play critical roles in transcription. However, the Arabidopsis Dof protein OBP1 interacts selectively with a bZIP protein OBF4 and enhances the binding of OBF4 to DNA (Zhang et al., 1995). In addition, the recently identified maize endosperm–specific Dof protein PBF interacts with a bZIP protein (Opaque2) that activates expression of zein genes (Vicente-Carbajosa et al., 1997). The interaction re-

gions between OBP1 and OBF4 and between PBF and Opaque2 remain to be elucidated. Whether Dof1 interacts with transcription factors other than itself and Dof2 has yet to be determined. Because the C-terminal region of Dof1 acts as a transcriptional activation domain, it is likely that the C-terminal regions of Dof proteins may interact with specific partners, such as bZIP proteins or transcriptional mediators (Bjorklund and Kim, 1996), to activate transcription. The combination of Dof DNA binding domains that determine target sequences and distinct domains that interact with other partners might produce diversity and specificity for Dof protein functions. In addition, there are serine or threonine stretches between the N- and C-terminal regions that might serve as molecular hinges to link the two major domains in Dof proteins.

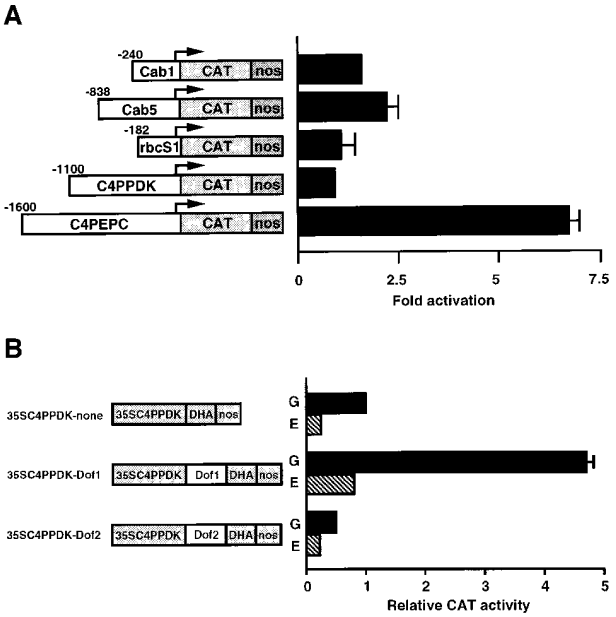


**Figure 9.** The Maize C4PEPC Gene Promoter May Be the Target of Dof1.

**(A)** Dof1 binds to a specific maize photosynthetic gene promoter in vivo. The hsp–AD–Dof1ΔC effector construct was cotransfected with various reporter constructs into greening protoplasts. The promoters tested were two distinct chlorophyll *a*/*b* binding protein gene (Cab1 and Cab5) promoters, a ribulose-1,5-bisphosphate carboxylase small subunit gene (rbcS1) promoter, the C4-type pyruvate orthophosphate dikinase (C4PPDK) gene promoter, and the C4-type phosphoenolpyruvate carboxylase (C4PEPC) gene promoter. The fold induction for each promoter is shown here because each promoter has different activity.

**(B)** Effects of Dof1 and Dof2 on the light-dependent activity of the C4PEPC gene promoter. The C4PEPC–CAT reporter construct was cotransfected with the 35SC4PPDK–none, 35SC4PPDK–Dof1, or 35SC4PPDK–Dof2 effector construct into greening (G) or etiolated (E) protoplasts. CAT activity from greening protoplasts cotransfected with the 35SC4PPDK–none construct was set to 1.

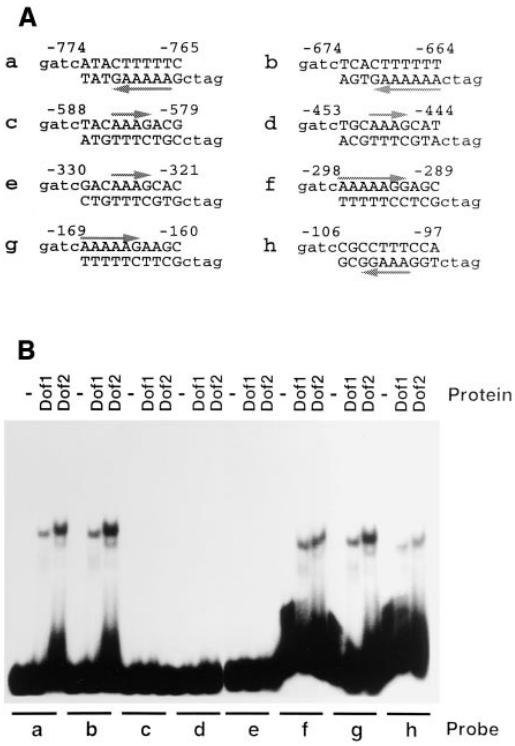


**Figure 10.** Identification of Dof Binding Sites in the Maize C4PEPC Promoter.

**(A)** All sequence motifs (a to h) that resemble AAAAGG (the Dof binding site) in the maize C4PEPC gene promoter (−1015 to +93) (Yanagisawa and Izui, 1989) are shown. Putative Dof binding sites are marked by arrows.

**(B)** EMSA was performed without (−) or with 100 ng of recombinant Dof1 or Dof2 using $^{32}$P-labeled probes carrying putative Dof binding sites (a to h).

Despite the clear domain structure of Dof proteins, Dof1 is an intronless gene (Yanagisawa, 1996b). No homology in the C-terminal region of Dof1 to known transcriptional activation domains or distinctive features in its amino acid sequence has been found. Therefore, the C-terminal region may be a new transcriptional activation domain. Interestingly, the Dof1 activation domain seemed to be much more potent when fused to the GAL4 DNA binding domain than when present in the Dof1 protein (Figure 3). Further analysis is necessary to clarify how this domain contributes to transcriptional activation.

### Dof2 May Act as a Repressor of Dof1-Mediated Transcriptional Activation

The structural similarity between Dof1 and Dof2, the similar binding of these proteins to the DNA motifs in vitro, and the direct protein–protein interaction between Dof1 and Dof2

suggest that Dof1 and Dof2 may participate in the same regulatory mechanism for gene expression (Figures 4B and 10; Yanagisawa, 1995, 1996a, 1997). In this report, we show that Dof2 binds to the Dof1 binding sites in vivo but lacks activity for transcriptional activation. We also demonstrate that Dof2 can block the Dof1-mediated transactivation by an in vivo competition experiment with greening maize protoplasts. These results strongly suggest that Dof2 may be a repressor of transcriptional activation controlled by Dof1. The expression pattern and the inverse functions of Dof1 and Dof2 might be partly responsible for leaf-specific gene expression in maize, although we cannot completely exclude the possibility that Dof2 may also be a transcriptional activator under some conditions. Cases analogous to those of Dof1 and Dof2 have been found in mammals. For example, in a typical Sp1 zinc finger protein family, Sp3 represses Sp1-mediated transcriptional activation, probably through competitive binding (Hagen et al., 1994). The activity of Ikaros proteins, which are essential zinc finger proteins for lymphocyte differentiation, is regulated by protein–protein interactions between DNA binding and non-DNA binding forms (Sun et al., 1996). It is likely that competitive DNA binding of Dof proteins with different activity for transactivation may provide a mechanism for transcriptional regulation. It is also possible that the protein–protein interaction between Dof1 and Dof2 might produce complexes with different activities for DNA binding and/or transcriptional activation. In this scenario, different ratios of Dof1 and Dof2 might modulate activation effects. Based on the ubiquitous expression of Dof1 and predominant expression of Dof2 in stems and roots, we propose that Dof1 and Dof2 may be involved in tissue-specific transcription in maize.

### Light Regulation of Dof1

Light is one of the most important environmental signals influencing plant growth and development. It is well documented that light signals trigger essential plant processes, including leaf and chloroplast development and the activation of photosynthetic genes (Thompson and White, 1991; Nelson and Langdale, 1992; Chory, 1993; Millar et al., 1994; Short and Briggs, 1994; Quail et al., 1995; Terzaghi and Cashmore, 1995; von Arnim and Deng, 1996). Many *cis* elements in light-regulated photosynthetic gene promoters and the factors that bind to these elements have been identified (e.g., Morelli et al., 1985; Green et al., 1987; Giuliano et al., 1988; Staiger et al., 1989; Dehesh et al., 1990; Lam and Chua, 1990; Schindler and Cashmore, 1990; Sarokin and Chua, 1992; Kehoe et al., 1994; Degenhardt and Tobin, 1996). The cDNA clones encoding DNA binding proteins, such as GT-1, GT-2, GBF, and CPRF, which are capable of interacting with these elements, have also been isolated based on their DNA binding activity in vitro (Lam et al., 1990; Weisshaar et al., 1991; Dehesh et al., 1992; Gilmartin et al., 1992; Perisic and Lam, 1992; Schindler et al., 1992a; ). Using a tobacco protoplast transient assay, Ni et al. (1996) have recently shown that the rice GT-2 protein has activities for both DNA binding and transcriptional activation. However, the physiological functions of these factors have not been established, and the precise contribution of most of these factors to light-responsive gene expression remains to be determined.

Using the maize mesophyll protoplast system, we have shown that six maize photosynthetic gene promoters are controlled by two types of light-regulatory mechanisms (Sheen, 1990, 1991, 1993; Schäffner and Sheen, 1991, 1992; H. Huang and J. Sheen, unpublished data). For instance, the C4PPDK gene promoter is activated by light directly after transfection into both etiolated and greening protoplasts (Sheen, 1991, 1993). However, the C4PEPC gene promoter is activated at high levels in greening but not in etiolated protoplasts (Schäffner and Sheen, 1992). The *rbcS* and chlorophyll *alb* binding protein (*Cab*) promoters are activated by both types of light-regulatory mechanisms (Schäffner and Sheen, 1991; H. Huang and J. Sheen, unpublished data). It has been proposed that the light regulation of the C4PEPC gene promoter is coupled to the light-dependent developmental process in illuminated leaves. RNA gel blot analyses have also shown that the endogenous C4PEPC gene is activated in greening maize leaves after 12 hr of illumination but not immediately (Sheen and Bogorad, 1987; Langdale et al., 1991).

Here, we used the maize leaf protoplast system as a powerful tool to investigate the function of plant transcription factors related to leaf specificity and light regulation in etiolated and greening protoplasts. Our results show that Dof1 has a different activity for transactivation in greening and etiolated protoplasts. Further analyses demonstrated that DNA binding activity but not the activity for transcriptional activation or nuclear localization of Dof1 was different in these protoplasts. These results strongly suggest that Dof1 is activated in illuminated leaf cells and may be involved in the light regulation of genes coupled to light-dependent processes, such as chloroplast development (Chory et al., 1996). It remains unclear how the DNA binding activity of Dof1 is inhibited in etiolated leaf cells. Post-translational modification or interaction with some other factor(s) in the nucleus may be important for light-responsive activity of Dof1, because Dof1 is expressed and accumulated in the nucleus of etiolated and greening protoplasts. The study of Dof1 presented here provides an excellent explanation for how constitutively and ubiquitously expressed DNA binding factors, such as GT-1, GT-2, and GBF, may still participate in tissue-specific and light-regulated gene expression in higher plants.

### What Are Target Promoters of Dof1?

Maize is a C4 plant that has acquired many new genes during evolution to photosynthesize more efficiently under the

conditions of an arid environment, high temperature, and high light intensity. The C4PEPC gene encodes one of the key enzymes in the C4 cycle pathway (Nelson and Langdale, 1993; Ku et al., 1996). Although it has been shown that the expression of this gene is leaf specific and light regulated (Sheen and Bogorad, 1987; Langdale et al., 1991), the underlying molecular mechanism remains unclear. Extensive deletion analyses with the maize C4PEPC gene promoter indicated the presence of multiple *cis* elements in maize mesophyll protoplasts. Further analyses of synthetic promoters revealed two distinct direct repeats (A29 and C14) that can account for the major activity of this promoter (Schäffner and Sheen, 1992). Interestingly, similar direct repeated sequences have also been found in the sorghum C4PEPC gene promoter (Cretin et al., 1991), indicating an evolutionary conservation in the function.

Several lines of evidence suggested that the C4PEPC gene promoter could be a strong candidate of the Dof1 target. First, Dof1 binds specifically to the C4PEPC gene promoter in vivo and in vitro. Second, Dof1 itself enhances the light-dependent activity of the C4PEPC gene promoter. Third, two of the Dof1 binding sites (probes f and g in Figures 10A and 10B) are located in the previously identified functional sequences (A29). Fourth, Dof1 also enhances the light-dependent activity of the A29 sequence as a positive *cis* element. Finally, Dof1 activity matches well with the expression pattern of the C4PEPC gene.

Dof1 has also been shown to bind to the AAGG sequence in the 35S promoter in vitro (Yanagisawa and Izui, 1993). However, the activity of the intact 35S promoter was mostly constitutive in maize greening and etiolated protoplasts (Sheen, 1991). The cotransfection of hsp–AD–Dof1 and 35S–CAT did not reveal any effect of Dof1 on the 35S promoter (data not shown). Thus, the results of the in vitro binding assay need to be potentiated by a complementary in vivo assay to determine the function of any DNA binding factors. In the case of the 35S promoter, it is perhaps difficult to evaluate the contribution of Dof1 to the full activity of this promoter, because the 35S promoter consists of multiple combinatorial *cis* elements (Benfey and Chua, 1990). Although the 35S promoter is very active in maize cells, maize is not a host of cauliflower mosaic virus.

Functional cotransfection assays in vivo are very useful and much more reliable than in vitro binding assays for defining the potential targets of transcription factors. In fact, this approach was applied successfully in the identification of numerous animal transcription factors whose cloning was based on sequence homology or mutations. Based on functional assays, the C4PEPC gene promoter seems to be one of the targets of Dof1. However, further analysis, such as a reverse genetic approach using transgenic and mutant plants, is necessary to establish conclusively the real targets of Dof1.

## A Model for Leaf-Specific and Light-Regulated Transcription Mediated by Dof Proteins

In this report, we provide direct evidence that maize Dof proteins can act as transcriptional activators or repressors and may mediate tissue-specific and light-regulated transcription. Based on our findings, it is most likely that Dof1 is in an inactive form for DNA binding and cannot activate transcription in etiolated leaves. When light stimulates the development of etiolated leaves to greening leaves, Dof1 is converted to an active form and can interact with target DNA to activate transcription. Thus, in leaves in which Dof2 is expressed at a very low level, Dof1 may exist in a state ready to respond. In stems and roots in which Dof2 is expressed significantly, Dof2 may interact with DNA to block the effect of Dof1 completely (Figure 11). In summary, Dof1 seems to be a key factor for the response to light in leaves. Dof2 may be important for tissue-specific gene regulation. In this model, the post-translational regulation of Dof1 is most likely a critical step in mediating light signals. Our demonstration that Dof1 can act as a key transcription factor for

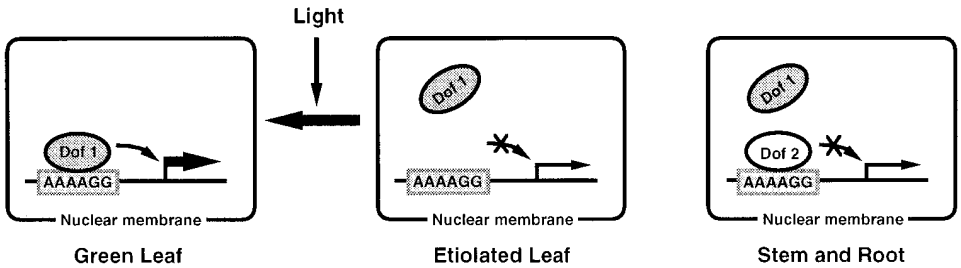


**Figure 11.** Model for Tissue-Specific and Light-Regulated Gene Expression Mediated by Maize Dof Proteins.

Dof1 is expressed ubiquitously and constitutively, whereas Dof2 is expressed mainly in stems and roots. Light stimulates Dof1 binding to its target sites (AAAAGG) in the nucleus of green maize leaves and activates transcription (large arrow). Dof1 binding to its target sites and transcriptional activation are inhibited (small arrows) in the nucleus of etiolated maize leaves. Light-regulated conversion of the DNA binding activity of Dof1 is a nuclear event.

light regulation provides new insights into the molecular mechanism of gene expression that is central to various signal transduction pathways in higher plants.

## METHODS

### Construction of Reporter Plasmids

Reporter plasmids were constructed in pUC8. The −72–CAT and −45–CAT reporter constructs contained the cauliflower mosaic virus 35S promoter truncated at position −72 or −45, the *CAT* gene, and the *nos* terminator (Schäffner and Sheen, 1991, 1992). The construction of a promoterless reporter (PL–CAT) was also described previously (Schäffner and Sheen, 1991). To generate reporter plasmids with Dof1 binding sites, a synthetic DNA with two copies of Dof1 binding sequence (Yanagisawa and Izui, 1993) was cloned into the XmaI (SmaI) site of M13mp18 RF in both orientations. The sequence of this synthetic DNA is shown in Figure 4B. The DNA fragments were recovered from these recombinants by digestion with restriction enzymes and fused to the truncated 35S promoter in −45–CAT or −72–CAT. The resulting plasmids (4S–72–CAT and 4S–45–CAT) contained four copies of Dof1 binding sites in the same and correct orientation. To construct the reporter plasmid for GAL4 (GBS–72–CAT), a synthetic DNA fragment containing five copies of the GAL4 binding sites (17mer) (Ma et al., 1988) was fused to the 35S promoter in the −72–CAT construct. The reporter plasmids with various maize photosynthetic gene promoters fused to the *CAT* gene have been described previously (Sheen, 1990, 1991; Schäffner and Sheen, 1991, 1992). The UBI–GUS construct (Jefferson et al., 1987; Christensen et al., 1992) was used as an internal control and has also been described previously (Sheen, 1996).

### Construction of Effector Plasmids

Effector plasmids were constructed in a plasmid AtHS–CAT (Sheen et al., 1995) containing the Arabidopsis 18.2 heat shock protein promoter, the *CAT* gene, and the *nos* terminator. The *CAT* gene was cut out from this vector by digestion with NcoI and PstI, and various cDNAs were inserted between these sites. The NcoI-PstI DNA fragment of pAX-MNB1a carrying an entire coding region of Dof1 (Yanagisawa, 1995) was used for the hsp–Dof1 construct. To generate the hsp–Dof1ΔC construct, an NcoI-SalI fragment of pCdel529 (Yanagisawa, 1995) was inserted by using two synthetic oligonucleotides—5′-TCGACTGATTGATTGACTGC-3′ and 5′-GTCAATCAATCAG-3′. These oligonucleotides carried a stop codon in frame. The hsp–AD–Dof1 and hsp–AD–Dof1ΔC constructs were created by fusing the GAL4 transcriptional activation domain (AD) and Dof1. For these constructions, the HindIII-EcoRI fragment of pGAD424 for AD (Clontech, Palo Alto, CA) was inserted into the NcoI and SphI sites of the hsp–Dof1 construct or the hsp–Dof1ΔC construct using three synthetic oligonucleotides—5′-CATGGCA-3′, 5′-AGCTTGC-3′, and 5′-AATTCATG-3′. The native translational ATG start codon of Dof1 was in this unique SphI site. To make the hsp–Dof2 construct, we first created pAX-Dof2 by inserting the EcoRI-SalI fragment of Dof2 cDNA into pAX4a vector (Mo Bi Tec GmbH, Gottingen, Germany). An NcoI-PstI fragment of pAX-Dof2 was then inserted between the NcoI and PstI sites of AtHS–CAT. The AD–Dof2 construct was created by replacing the Dof1 cDNA in hsp–AD–Dof1 with an EcoRI-SalI fragment of pAX-Dof2.

Effector plasmids to express GAL4 or its derivative were constructed with two plasmids of the yeast two-hybrid system, pGBT9 and pGAD424 (Clontech). hsp–BD was generated with the NcoI-PstI fragment of AtHS–CAT and the SphI-SalI fragment of pGBT9 that encodes the GAL4 DNA binding domain (BD). The sequences used for ligation are as follows: 5′-TCGACTGATTGATTGACTGC-3′, 5′-GTCAATCAATCAG-3′, 5′-CATGAAGCTACTGTCTTCTATCGAACAAGCATG-3′, and 5′-CTTGTTCGATAGAAGACAGTAGCTT-3′. To generate the hsp–GAL4 construct, the KpnI-BglII fragment of pGAD424 that encodes the activation domain (AD) was inserted between the EcoRI and BamHI sites of the hsp–BD construct by using two synthetic oligonucleotides—5′-AATTCCCGGGTAC-3′ and 5′-GGGCC-3′. The hsp–BD–Dof1ΔN construct was created by ligating the SacI-HindIII fragment of the hsp–Dof1 construct and the EcoRI-HindIII fragment of the hsp–BD construct with two synthetic DNAs—5′-AATTCCCGGAGCT-3′ and 5′-CCGGG-3′. The 35S–Dof1 construct used to express Dof1 constitutively was created by ligating the BamHI-HindIII fragment of pCaMVCN (Pharmacia) and the SphI-HindIII fragment of the hsp–Dof1 construct with a synthetic oligonucleotide—5′-GATCCATG-3′.

The hsp–Dof1–HA, hsp–Dof2–HA, 35SC4PPDK–Dof1, and 35SC4PPDK–Dof2 constructs were generated with cDNAs that were amplified by polymerase chain reaction (PCR) to create StuI sites before the termination codons of Dof1 and Dof2. PCR was performed with the hsp–Dof1 construct using the heat shock primer (5′-GTCTCCCGAAAAGCAACGAACCATGG-3′) and the D1 primer (5′-AAGGCCTCGGGAGGTTGAGGAAGATGCA-3′), which were annealed to the heat shock promoter sequence and the sequence of the 5′ terminus of the Dof1 protein, respectively. PCR was also performed with the hsp–Dof2 construct by using the heat shock primer and the D2 primer (5′-AAGGCCTCGGCAGATTGAGGAACAGCG-3′), which were annealed to the sequence of the 5′ terminus of Dof2. The amplified cDNAs were digested with NcoI and StuI and inserted between NcoI and StuI sites of a plant vector with the 35SC4PPDK promoter and a double hemagglutinin (HA) tag (Sheen, 1996) or another vector with the heat shock promoter and a double HA tag (Sheen, 1996), respectively. Two independent constructs were assayed with the same result, respectively.

### Transient Expression with Maize Leaf Protoplasts and CAT/GUS Assays

Etiolated protoplasts were isolated from the second maize leaves grown in the dark for 11 days. Greening protoplasts were isolated from the second maize leaves grown in the dark for 10.5 days and illuminated for 16 hr, as described previously (Sheen, 1990, 1991). Electroporation was performed with $2 \times 10^5$ protoplasts, 20 μg of a reporter plasmid, 40 μg of an effector plasmid, and 6 μg of a plasmid (UBI–GUS) that was used as an internal control, as described previously (Sheen, 1990, 1996). The electroporated protoplasts were divided into two parts. One part was incubated at 23°C for 18 hr, and the other part was incubated at 23°C for 16 hr after the heat shock treatment (42°C for 2 hr) to induce the expression of various effectors. The greening protoplasts were incubated under illumination. Etiolated protoplasts were incubated in the dark. Preparation of cell lysates and the CAT and GUS assays were performed as previously described (Sheen, 1990, 1991). Relative CAT activities in duplicated samples are shown in Figures 1B, 2B, 2C, 3, 6, 7, 9A, and 9B, with er-

ror bars. Relative activities were determined after the transfection efficiency of each electroplated sample was normalized with GUS activity. All experiments were repeated three times with similar results.

### Electrophoretic Mobility Shift Assay

The plasmid pGST-WT for expression of a fusion protein between glutathione *S*-transferase (GST) and Dof1 was obtained by inserting an NcoI-BglII DNA fragment of pAX-WT (Yanagisawa, 1995), which encodes the DNA binding domain of Dof1 (amino acids 44 to 147), into the EcoRI and SalI sites of pGEX-5X-1 (Pharmacia) by using four oligonucleotides. The sequences of the oligonucleotides are as follows: 5′-AATTCGAGGATCCGGGTAC-3′, 5′-CATGGTACCCGGATCCTCG-3′, 5′-GATCTTGAG-3′, and 5′-TCGACTCAA-3′. For construction of pGST–Dof2ΔC, the EcoRI-DdeI fragments of the Dof2 cDNA were inserted into the EcoRI and SalI sites of pGEX-5X-1 by using two synthetic oligonucleotides (5′-TAAGCGCCGCCGCGTCGCGCCTGCCCCGGAGTGAG-3′ and 5′-TCGACTCACCGGGGCAGGCGCGACGCGGCGGCGC-3′). This plasmid encodes a fusion protein between GST and a region (amino acids 1 to 115) of Dof2.

*Escherichia coli* cells transformed with pGST-WT or pGST–Dof2ΔC were grown in Luria-Bertani medium at 37°C to mid-log phase. The expression of fusion proteins was induced with 1 mM isopropyl β-D-thiogalactopyranoside at 30°C for 2 hr. Cells were harvested and resuspended in PBS supplemented with 1 mM phenylmethylsulfonyl fluoride. The fusion proteins were purified with glutathione–Sepharose 4B (Pharmacia), and the DNA binding domain of Dof1 or Dof2 was obtained by cleavage with factor Xa by the standard method (Ausubel et al., 1989). The purified proteins were incubated in a solution (20 μL) of 17.5 mM Tris–HCl, pH 7.5, 60 mM NaCl, 7% glycerol, 100 ng of poly(dA-dT), 50 ng of poly(dI-dC), and 0.5 ng (10,000 cpm) of probe DNA at room temperature for 20 min. Synthetic DNAs (see Figure 10A) having additional GATC sequences attached to each 5′ end were end labeled with the Klenow fragment of DNA polymerase I and used as DNA probes. Electrophoresis was performed as described previously (Yanagisawa and Izui, 1990), except that a 5% polyacrylamide gel (acrylamide–bisacrylamide, 50:1) was used.

### RNA Gel Blot Analysis

Preparation of total RNAs from tissues of 10-day-old maize seedlings (inbred strain H84) as well as the RNA blot analysis were performed as described previously (Yanagisawa and Izui, 1993; Yanagisawa, 1996b). The gene-specific probes were a 480-bp DNA fragment from the Dof1 cDNA (nucleotides 610 to 1090) and a 436-bp DNA fragment from the Dof2 cDNA (nucleotides 385 to 821). Each of these fragments detected a single band on genomic DNA gel blots (Yanagisawa and Izui, 1993; data not shown).

### Immunoprecipitation of Dof Proteins Tagged with HA

Maize leaf protoplasts were transfected with the hsp–Dof1–HA construct or the hsp–Dof2–HA construct and incubated for 2 hr at 42°C as heat shock treatment. The proteins in the protoplasts were then labeled with $^{35}$S-methionine and immunoprecipitated with anti-HA (12CA5) antibody, as described previously (Sheen, 1996). The proteins were analyzed by PAGE on a 12.5% polyacrylamide gel.

### Construction of the Dof1–sGFP Fusion and Fluorescence Microscopy

The hsp–sGFP construct was created by fusing a DNA fragment encoding engineered GFP[sGFP(S65T)] (Chiu et al., 1996) to the Arabidopsis 18.2 heat shock promoter (Sheen et al., 1995). The hsp–Dof1–sGFP construct was created by fusing sGFP(S65T) at the C terminus of Dof1 (inserting a BamHI-PstI fragment of sGFP(S65T) between the BglII and PstI sites in the hsp–Dof1ΔC). The electroporated etiolated or greening protoplasts were incubated at 23°C for 2.5 hr after heat shock (42°C for 1 hr). Fluorescence photography was as described previously (Sheen et al., 1995; Chiu et al., 1996).

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