Maize Dof1, one of the plant-specific Dof transcription factors, is involved in light-regulated gene expression. To elucidate the molecular mechanism underlying the activity of Dof1, in vivo functional analyses were carried out using transient expression assays with maize mesophyll protoplasts. The results suggest that the Dof domain alone, the region conserved among Dof factors, can mediate interaction with DNA in vivo and distinct Dof1 activities in greening and etiolated protoplasts. A region rich in basic amino acids was identified as a nuclear localization signal. Deletion analysis defined the transcriptional activation domain of 48 amino acids located in the C-terminus of Dof1. This activation domain was also found to be functional in both human cells and yeast, implying that Dof1 may stimulate transcription through a mechanism evolutionarily conserved among eukaryotes. A computer homology search with known transcription factors revealed that the activation domain of Dof1 displayed only a limited similarity to animal transcription factor GATA-4. Mutational analysis revealed the critical role of a tryptophan residue within the activation domain of Dof1, as had been shown in Activation domain II of GATA-4.

Key words: DNA-binding domain — Dof transcription factor — GATA family — Light regulation — Nuclear localization signal — Transcriptional activation domain.

Abbreviations: BGH, bovine growth hormone polyadenylation signal; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus immediate-early promoter; GAL4-AD, transcriptional activation domain of GAL4; GAL4-BD, DNA binding domain of GAL4; GAL4-BD-AD, protein with GAL4-BD fused to GAL4-AD; β-gal, β-galactosidase; GUS, β-glucuronidase; HSP, heat shock protein; NOS, nopaline synthase; NLS, nuclear localization signal; PCR, polymerase chain reaction; sGFP, synthetic green fluorescent protein; SV40, simian virus 40.

Introduction

Transcriptional control in plants as well as in other organisms is of particular importance in regulating many biological processes such as growth, development, differentiation, and metabolism (e.g. Kuhlemeier et al. 1987, Verma 1992, Yanagisawa 1998). To understand the molecular mechanisms of transcriptional control in plants, a number of plant transcription factors have been identified in the past decade, with some showing sequence homology to animal factors and others appearing to be unique to plants (Meshi and Iwabuchi 1995, Schwechheimer et al. 1998). Plant-specific transcription factors include the Dof family, the WAKY family, the AP2/EREBP family, and so forth (Eulgem et al. 2000, Meshi and Iwabuchi 1995, Schwechheimer et al. 1998, Yanagisawa 1996). In general, transcription factors regulate transcription through a two-step process, binding to a specific DNA sequence and subsequent transcriptional activation or repression via interaction with a partner protein such as another activator, a mediator, or a component of the general transcription machinery (Björklund and Kim 1996, Goodrich et al. 1996). It is, therefore, important to define functional domains in vivo to better understand the mechanisms of transcriptional control. Such analyses of animal transcription factors have successfully revealed that the above two steps are usually operated by separable domains. They have also identified not only conserved DNA-binding domains but also similarities among transcriptional activation domains. On the basis of the similarities, transcriptional activation domains are classified mainly into acidic, proline-rich, and glutamine-rich domains (Mitchell and Tjian 1989, Triezenberg 1995). However, examples of in vivo analyses of the functional domains of plant factors are very limited and there are only a few reports on transcriptional activation domains (Döring et al. 2000, Goff et al. 1991, McCarty et al. 1991, Schindler et al. 1992, Schmitz et al. 1997, Urano et al. 1996). Therefore, molecular mechanisms underlying the transcriptional regulation by individual plant transcription factors are still poorly understood.


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The Transcriptional Activation Domain of the Plant-Specific Dof1 Factor Functions in Plant, Animal, and Yeast Cells

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Maize Dof1, the first Dof factor (Yanagisawa 1995, Yanagisawa and Izui 1993), activates transcription from maize promoters responsive to the light signal including the C4 photosynthetic phosphoenolpyruvate carboxylase gene promoter in maize protoplasts, while the activities of these promoters are repressed by the antisense RNA of Dof1. In addition, Dof1 enhances transcription from both native and synthetic promoters differently in greening and etiolated protoplasts. These data suggest that Dof1 is associated with the light-regulated expression of multiple plant-specific genes (Yanagisawa 2000, Yanagisawa and Sheen 1998). Although we have shown that the N-terminal and C-terminal halves of Dof1 function independently as DNA-binding and transactivation domains (Yanagisawa and Sheen 1998), detailed mapping of the functional domains is necessary to unveil the molecular mechanism underlying transcriptional control by Dof1. Here, I present the results of in vivo analyses with maize protoplasts to identify the minimal DNA-binding and transcriptional activation domains and the nuclear localization signal (NLS) of Dof1. I also show that the transcriptional activation domain is functional in human cells and yeast as well as in maize cells.
The region responsible for interaction with DNA in vivo

We have reported previously that the N-terminal half (aa 1–147) of Dof1 can function as a DNA-binding domain in vivo and mediate different Dof1 activity in greening and etiolated protoplasts (Yanagisawa and Sheen 1998). However, questions have remained as to whether the Dof domain alone in the N-terminal half is sufficient for DNA binding in vivo and which amino acid residue(s), ones within the Dof domain or those surrounding the Dof domain, are involved in the modulation of Dof activity. In an attempt to answer these questions, several effector plasmids were constructed to express different portions of Dof1 fused to the transcriptional activation domain of GAL4 (GAL4-AD) under the control of the heat shock protein (HSP) promoter in maize protoplasts (Fig. 1). Since two characteristic regions, the serine-stretch region and a region rich in basic amino acids, were found, effector plasmids were designed to express fusion proteins with or without these regions. These effector plasmids were transfected into maize greening protoplasts together with a reporter plasmid containing Dof1-(119–135)-sGFP gene (E) under the control of the HSP promoter, and were incubated at 23°C for 16 h after heat-shock treatment (40°C, 1 h). Micrographs of protoplasts were taken under visible (B and E) or excitation (D and G) light, or both lights (C and F). The nuclear localization of the Dof1-(119–135)-sGFP protein was confirmed by nuclear staining with DAPI.

Results

Table 1 Different activation by fusion proteins in greening and etiolated protoplasts

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Fold activation (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Greening</td>
</tr>
<tr>
<td>AD-Dof1(1–147)</td>
<td>21.4±0.4</td>
</tr>
<tr>
<td>AD-Dof1(1–119)</td>
<td>19.8±0.6</td>
</tr>
<tr>
<td>AD-Dof1(1–100)</td>
<td>13.8±2.0</td>
</tr>
<tr>
<td>Dof1(1–147)-AD</td>
<td>23.4±0.4</td>
</tr>
<tr>
<td>Dof1(44–147)-AD</td>
<td>21.3±0.8</td>
</tr>
</tbody>
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\(a\) Fold activation is the CAT activity from protoplasts treated with heat shock relative to that from protoplasts that were not treated with heat shock. Values are means of duplicate samples.

Functional domains of maize Dof1 factor

Fig. 2 A basic region of Dof1 is an NLS. (A) Structure of the Dof1 (aa 119–135)-sGFP protein. The amino acid sequence of aa 119–135 of Dof1 is underlined. Basic amino acid residues are indicated by red letters. (B–G) Micrographs of maize protoplasts. Etiolated protoplasts were transfected with plasmids carrying the sGFP gene (B–D) or the Dof1(119–135)-sGFP gene (E–G) under the control of the HSP promoter, and were incubated at 23°C for 16 h after heat-shock treatment (40°C, 1 h). Micrographs of protoplasts were taken under visible (B and E) or excitation (D and G) light, or both lights (C and F). The nuclear localization of the Dof1(119–135)-sGFP protein was confirmed by nuclear staining with DAPI.

two fusion proteins [Dof1(1–147)-AD and Dof1(44–147)-AD] that had the distinct regions of Dof1 fused to the N-terminus of GAL4-AD. Both proteins retained the activity that was similar to that of the AD-Dof1(1–147) protein. These data suggest that the amino acid sequence around the Dof domain is not important for interaction with target DNA in vivo.

To investigate which region mediates different activities of Dof1 in greening and etiolated protoplasts, the activities of several fusion proteins were compared in greening and etiolated protoplasts (Table 1). All fusion proteins examined displayed stronger activity for transcriptional activation in greening protoplasts (3- to 5-fold), while the protein with the DNA binding domain of GAL4 (GAL4-BD) fused to GAL4-AD (GAL4-BD-AD) showed similar activity in greening and etiolated protoplasts (Yanagisawa and Sheen 1998, data not shown). Since deletion of the region surrounding the Dof domain did not affect different activities in greening and etiolated protoplasts,
Functional domains of maize Dof1 factor

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the Dof1 domain itself appears to be responsible for different Dof1 activities in two types of protoplasts.

A 17-amino acid region of Dof1 directs nuclear localization

Three types of NLSs have been identified in eukaryotic nuclear proteins, namely SV40-like NLSs, yeast mating type factor Matα2-like NLSs, and bipartite NLSs (Raikhel 1992, Schwechheimer et al. 1998). Since the fusion protein between synthetic green fluorescent protein (sGFP) and Dof1 has been found in nuclei of maize protoplasts (Yanagisawa and Sheen 1998), some NLS-like sequences were searched for in the amino acid sequence of Dof1. Only a basic region (aa 120–129) resembling to both SV40-type and bipartite type NLSs was found in the middle of Dof1 (Fig. 2A). To examine the abilities of this basic region as an NLS, the subcellular localization of sGFP tagged by this region was analyzed. The tagged sGFP protein displayed quite different subcellular localization from that of sGFP itself and was found to accumulate specifically in the nuclei of maize protoplasts (Fig. 2B–G), suggesting that a 17-amino acid region functions as an NLS.

Identification of the transcriptional activation domain

We have shown that the C-terminal half of Dof1 (aa 136–238) functions as a transcription activation domain when this region is fused to GAL4-BD (Yanagisawa and Sheen 1998). However, no feature of known transcriptional activation domains was found in this region. Thus, the minimal region for transactivation was defined to characterize the activation domain of Dof1. Various effector plasmids were produced to express fusion proteins between GAL-BD and different regions of Dof1 under the control of the HSP promoter. These plasmids were transfected into maize etiolated protoplasts together

Fig. 3 Identification of the transcriptional activation domain. Expression vectors for fusion proteins between GAL4-BD and the distinct regions of Dof1 were transfected into etiolated protoplasts together with the GBS-72-CAT reporter plasmid. Protoplasts were then divided into two parts and incubated with or without heat-shock treatment. The numbers refer to the positions of residues at the ends of the Dof1 regions fused to GAL-BD. Relative CAT activities in duplicate samples are shown after normalization with GUS activity from the reference plasmid. The CAT activity from protoplasts that were co-transfected with the BD-Dof1(136–238) construct and treated with heat shock was set to 1.
Functional domains of maize Dof1 factor

To evaluate this similarity, mutational analysis was carried out using only the amino acid sequence of the transcriptional activation domain of Dof1. The transcriptional activation domain of Dof1 was functional even in animal cells and yeast. However, in a previous survey using the whole amino acid sequence of Dof1, no protein with significant homology to the Dof1 activation domain was found. Therefore, I carried out another survey using only the amino acid sequence of the transcriptional activation domain identified in this study. I could find only a similarity between the activation domain of Dof1 and one of two transcriptional activation domains, referred to as Activation domains I and II, of animal GATA-4 proteins. As shown in Fig. 5A, the similarity between Dof1 and Activation domain II is not high. However, the boundaries of the homologous regions are near the boundaries of each activation domain. I also found that a rice EST encodes a protein with striking homology to the activation domain of Dof1 (Fig. 5A).

A tryptophan residue plays a critical role in transactivation

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Activation domain II has been regarded as unique to the GATA4/5/6 family, and three conserved motifs have been identified in Activation domain II of GATA-4 proteins of the human, mouse, chicken, and frog (Morrisey et al. 1997). Mutational analyses of Activation domain II have revealed that a point mutation on a tryptophan residue in the third motif almost eliminates the transactivation ability, whereas the mutations of other residues in these conserved motifs only slightly affect its ability, if at all (Morrisey et al. 1997). Since such a tryptophan residue is also conserved in the amino acid sequence of Dof1, the effect of the mutation of the tryptophan residue to alanine residue (W220A) was investigated. Two other amino acid residues were also found to be conserved among GATA-4, Dof1, and a rice protein (Fig. 5A), and these amino acid residues were also mutated as controls. As shown in Fig. 5B, the protein carrying the W220A mutation hardly increased CAT activity above the basal level, whereas the protein with the point mutation on a proline residue that is located outside of the activation domain in the GATA-4 sequence had no significant effect. The D215A mutation also appeared to decrease the ability, but this effect was not as strong as that of the W220A mutation.

To confirm that the apparent loss of the transactivation activity of the protein with the W220A mutation was not due to...
a change in accumulation of the protein in protoplasts, an in vivo labeling experiment was carried out. The plasmids for the expression of fusion proteins under the control of a strong and constitutive promoter (35SC4PPDK promoter; Sheen 1990, Sheen 1996) were transfected into maize protoplasts together with a control plasmid allowing the expression of sGFP under the control of the same promoter. Accumulation of the control sGFP protein was clearly observed by SDS-PAGE analysis using whole-cell lysates from labeled protoplasts, whereas bands of fusion proteins were not observed in this analysis (Fig. 5C). Thus, the levels of accumulation of these fusion proteins appeared not to be similar to that of the control protein, and these fusion proteins appeared to be unstable in protoplasts. However, detection of these fusion proteins was achieved by analysis of proteins immunoprecipitated with antibodies against GAL-BD. In correspondence with the observations in whole cell lysates, several degraded forms of each fusion protein were observed. Their patterns were basically similar, and the accumulation of the protein carrying the W220A mutation was relatively larger than those of other proteins. I concluded, therefore, that the observed effect of the W220A mutation reflected a reduced level of activity rather than a change in accumulation of the proteins. These results suggest an important role of the tryptophan residue in the activity of the Dof1 transcriptional activation domain.

Discussion

Dof factors are plant-specific transcription factors associated with a variety of important plant-specific biological processes. Transcription factors basically contain three functional domains that are responsible for DNA binding, nuclear localization, and transcriptional activation or repression, and the activities of transcription factors are often modulated through one of these domains (Calkhoven and Geert 1996). To better understand the gene regulation mechanism involving Dof1, I mapped three functional domains within maize Dof1 in the present study.

The data presented here suggest that the Dof domain alone can mediate DNA binding in vivo. In the case of a plant basic leucine zipper protein, a basic region is involved in both DNA binding and nuclear localization (van der Krol and Chua 1991). Likewise, a dramatic reduction in the DNA-binding activity of Dof1 has previously been observed through an in vitro assay in response to deletion of a basic region (Yanagisawa 1995), which is located outside of the Dof domain and functions as an NLS (see Fig. 1, 2A). However, deletion of this basic region did not affect the apparent DNA-binding activity in vivo. Therefore, an artificial effect through the in vitro assay might be observed previously, and the basic region may be specifically involved in nuclear localization. The results of the experiments regarding the DNA binding domain also suggest that the Dof domain itself mediates different DNA binding activities in greening and etiolated protoplasts. Studies of animal factors have demonstrated that post-translational modification of transcription factors often activates or represses their activity in response to extracellular and/or intracellular signals (Calkhoven and Geert 1996). It is possible, therefore, that the post-translational modification within the Dof domain modulates its DNA binding activity in greening and etiolated protoplasts. However, in vitro analyses have also suggested that the Dof domain is a multifunctional domain that mediates not only DNA binding but also protein–protein interactions, including interactions with other Dof proteins, HMG1 protein, and basic leucine zipper proteins (Kang and Singh 2000, Yanagisawa 1997). Therefore, it is also possible that the activity of Dof1 is regulated through light-dependent protein–protein interactions. Examples of regulation through protein–protein interactions can also be found in animal transcription factors (Calkhoven and Geert 1996).

I have shown in the present study that the basic region (aa 119–135) in the middle of Dof1 functions as an NLS. This NLS resembles both SV40-type NLS and bipartite NLS. Although bipartite-type NLSs usually possess a spacer of more than four residues between two regions of basic amino acids (Raikhel 1992), this region has only a three amino acid spacer (Fig. 2A).

The transcriptional activation domain of Dof1 was also identified in this study. This activation domain of Dof1 functioned not only in plant cells but also in animal cells and yeast. It is likely, therefore, that Dof1 activates transcription through a mechanism evolutionarily conserved among lower and higher eukaryotes. To examine the conservation of transcriptional machinery in eukaryotes, the ability of some mammalian activation domains have been investigated in yeast. The results of these studies suggested both similarities in and differences between transcriptional machinery of lower and higher eukaryotes (Remacle et al. 1997, Escher et al. 2000). The finding of a novel transcriptional activation domain that functions widely in eukaryotes will increase understanding of the machinery controlling transcriptional regulation in eukaryotes.

Despite the high level of activity of the Dof1 activation domain in human cells and yeast (see Fig. 4), no transcription factor, except GATA4/5/6, with an amino acid sequence similar to the Dof1 activation domain could be found. Although the DDED amino acid sequence, a component of GAL4-AD, was found in the activation domain of Dof1 (Fig. 5A), the overall homology between activation domains of Dof1 and GAL4 is not significant and this sequence is not conserved in the related rice protein (Fig. 5A). In addition, the GAL4-AD lacking this sequence has been shown to be still active (Melcher and Johnston 1995). Therefore, the sequence homology between activation domains of Dof1 and GATA4/5/6 may provide a clue to elucidate how Dof1 activates transcription. A tryptophan residue in the Dof1 activation domain is found to be important for transcriptional activation, as has the corresponding residue in mouse GATA-4 (Morrisey et al. 1997). It is interesting to speculate that the activation domain of Dof1 might form a novel class of transcriptional activation domains together with ani-
nal GATA4/5/6 transcription factors involved in cell lineage development and terminal cell differentiation. However, this interesting possibility should be evaluated in detail in future studies, especially with regard to identification of partner proteins of each factor, since the homology between activation domains of Dof1 and GATA4/5/6 is not high. In addition, Activation domain II of GATA-4 does not include the sequence corresponding to aa 226–238 of Dof1 that is also conserved in the related rice protein (Fig. 5A).

The present deletion analysis suggested that the 13-amino acid sequence (aa 226–238) could be a component of the activation domain. However, a point mutation within this sequence did not affect the activity. In addition, this amino acid sequence is conserved in another maize Dof protein, Dof2. Dof1 and Dof2 have completely different amino acid sequences outside the Dof domain, except this 13-amino acid sequence and a basic region corresponding to the Dof1 NLS (Yanagisawa 1995). The action of Dof2 is complex; Dof2 displayed opposite actions on promoters within different contexts (Yanagisawa 2000, Yanagisawa and Sheen 1998). Thus, functional role of this 13-amino acid sequence remains obscure.

The W220A mutation almost eliminated the ability for transactivation, suggesting a critical role of a tryptophan residue in function of the Dof1 activation domain. The importance of aromatic amino acids has been shown in several transcriptional activation domains. For example, the presence of aromatic and bulky hydrophobic residues rather than a net negative charge is critical for the transcriptional activation ability of VP16, a component of the herpes simplex virus 1 virion, although VP16 is a prototypical acidic activator (Cress and Triezenberg 1991, Regier et al. 1993). The D215A mutation also significantly reduced the ability of the Dof1 activation domain. This reduction also might reflect a functional role of the VP16 activation domain revealed that only mutations on specific amino acid residues can affect the ability for transcriptional activation (Sullivan et al. 1998). However, at this stage, I cannot exclude the possibility that this effect might be caused by drastic structural alteration. Although mutational analysis suggests critical amino acid residues for transactivation, it is apparent that the exact role of these amino acid residues in the mechanism for transcriptional activation will be elucidated through analysis of the interactions with functional partner proteins.

Materials and Methods

**Reporter and effector plasmids for the maize transient assay system**

The construction of reporter plasmids has been described previously (Yanagisawa and Sheen 1998). The 4S-72-CAT reporter plasmid contained four Dof1-binding sites immediately upstream of a minimal promoter (the cauliflower mosaic virus 35S promoter truncated at −72), and the GBS-72-CAT plasmid contained five binding sites of the yeast transcription factor GAL4 upstream of the same minimal promoter.

To construct vectors for the expression of distinct regions of Dof1 fused to the N- or C-termini of GAL4-AD, the DNA fragments encoding amino acids (aa) 1−147, 1−119, 1−100, 1−147, or 44−147 of Dof1 were amplified by polymerase chain reactions (PCRs). The PCR products were used to replace the Dof1AC fragment between the coding region for GAL4-AD and the NOS terminator of the plasmid pHSF-AD-Dof1AC (Yanagisawa and Sheen 1998), or for insertion between the HSP promoter and the GAL4-AD coding region of the plasmid pHSP-AD. The plasmid pHSF-AD was created by replacing the Dof1AC fragment of the plasmid pHSF-AD-Dof1AC with a pair of synthetic oligonucleotides (5’-AATCTTAGATGCA-3’ and 5’-TCTAG-3’) encoding a stop codon. These effector plasmids contained the HindIII–EcoRI DNA fragment of pGAD424 (Clontech, USA) encoding GAL4-AD tagged by the NLS from the large T-antigen of simian virus 40 (SV40). Similarly, plant vectors for the expression of diverse regions of Dof1 fused to the C-terminus of the GAL4-BD were constructed through PCR. The Dof1AN fragment between the GAL4-BD coding region and the NOS terminator in plasmid pHSF-BD-Dof1AN (Yanagisawa and Sheen 1998) was replaced with the PCR products encoding aa 136–238, 210–238, 136–174, 175–238, 175–210, 211–238, 191–210, or 211–238.

The expression vectors for fusion proteins containing mutated activation domains were also created by PCR. To produce the plasmid pHSF-BD-Dof1(175−238)D215A, PCR was carried out using primers C2 (5’-CATGAATTCCGCCCCAGACAGAGACGCG-3’) and DA1 (5’-ATTCATGGCAACGAGGGCGGGCCACA-3’), or primers C5 (5’-TATTGTGAATGTTGAGGAGAAT-3’) and DA2 (5’-TATTGATGCGGTGTTGAAAATT-3’). Each primer contained a sequence for a restriction site (indicated by italic letters) and a proper sequence of Dof1 gene (underlined). The DA1 and DA2 primers also contained a mutated nucleotide indicated by bold letters. The PCR products were digested with appropriate restriction enzymes, assembled and then inserted between the GAL-BD coding region and the NOS terminator. Similarly, the plasmid pHSF-BD-Dof1(175−238)W220A was produced with PCR products using primers C2 and WA1 (5’-TATGATGCGGTGTTGAAAATT-3’) and DA1 (5’-ATTCATGGCAACGAGGGCGGGCCACA-3’), or primers C5 and WA2 (5’-TATTGTGAATGTTGAGGAGAAT-3’). The plasmid pHSF-BD-Dof1(175−238)P230A was also created with the PCR product using primers C2 and PA1 (5’-ATGCATGGCGGCTCAGGGGTTGAAACCTT-3’) and two synthetic oligonucleotides (5’-CATCTTTTCTCAACCTCCCTCAGGCTGACTGCA-3’ and 5’-GTCACGGGAGGTTGAGGAGAATGCA-3’). All constructs were verified by DNA sequencing.

**Co-transfection into maize protoplasts and reporter enzyme assays**

Etiolated and greening mesophyll protoplasts were prepared from the second leaves of a maize hybrid line FR992 × FR967 (Illinois Foundation Seed, Champaign, IL, U.S.A.) according to a published method (Sheen 1990). For preparation of etiolated protoplasts, the plants were grown in the dark for 11 d at 25°C. For preparation of greening protoplasts, the plants were grown in the dark for 10.5 d and subsequently illuminated (3,000 lux) for 14 h at 25°C. Electroporation for co-transfection was performed with 3×10⁶ protoplasts using 20 µg of a reporter plasmid, 40 µg of an effector plasmid and 6 µg of an internal control plasmid, as described previously (Yanagisawa 2000). The UBIGUS plasmid that contained the GUS gene under the control of the maize ubiquitin promoter (Christensen et al. 1992, Sheen 1996) was used as an internal control plasmid to normalize transfection efficiency. CAT and GUS assays were also performed as described previously (Sheen 1990, Yanagisawa and Sheen 1998). Each experiment was repeated three times with similar results.
Fluorescence microscopy of sGFP tagged by a basic region of Dof1
The Dof1(119–135)-sGFP construct was produced by the insertion of the BamHI-PstI DNA fragment encoding engineered GFP [sGFP(S65T)] (Chiu et al. 1996) between the HSP promoter and the NOS terminator in a plant vector using a pair of synthetic oligonucleotides (5′-CATGCCCCAAGAAGACGCCAGCAAAAGAGCAGCGCCTCTGGTGGGCCCGCCG-3′ and 5′-GATCCCAGGGCCCGCCACAAGCGGCCCTCCTTCTGGGCGGCTTCTTCTTGG-3′) encoding a basic region of Dof1 (aa 118–135). Fluorescence photographs of protoplasts transfected with the HSP-sGFP or HSP-Dof1(119–135)-sGFP construct were taken with an Olympus microscope (CX40) equipped with a fluorescence condenser.

Yeast expression vector, transformation, and β-gal assay
To construct a yeast expression vector, the EcoRI-PstI fragment encoding the activation domain of Dof1 (aa 175–238) was recovered from a plant expression vector and inserted between the GAL4-BD coding region and the ADH1 terminator of the yeast expression vector, pGBT9 (Clontech). The KpnI-BamHI fragment of pGAD424 (Clontech) that encoded GAL4-AD was also inserted between the GAL4-BD coding region and the ADH1 terminator of pGBT9 using two oligonucleotides (5′-AATTTCCCGGCGCT-3′ and 5′-GGCCGCT-3′) to produce the GAL4-BD-AD construct. Transformation of budding yeast Saccharomyces cerevisiae was carried out according to the lithium acetate method (Ito et al. 1983). The strain used was Y-190 [MATa, ura3-52, his3-200, ade2-101, lys2-801, trpl-901, leu2-3, 112, gal4Δ, gal80Δ, cyh2Δ, LYS2::GALIUS-GALI-TAD-HIS3, URA3::GALIUS-GALI-TAD-lacZ (Flick and Johnston 1990)]; β-Gal assay with a chemiluminescent substrate (4-methylumbelliferyl-β-D-galactoside) was performed according to the fluorometer instructions for the DyNA Quant 200 (Hoefer Pharmacia Biotech Inc., U.S.A.). Relative β-gal activity was obtained after normalization with the optical density at 600 nm.

Co-transfection into human HepG2 cells and reporter enzyme assays
To construct the mammalian expression vectors, the HindIII-PstI DNA fragments encoding GAL4-BD, GAL4-BD-AD, or a fusion protein were recovered from yeast expression vectors. These fragments were then inserted into the multicloning sites of pcDNA3.1/Zeo (Invitrogen, U.S.A.). For the reporter plasmid for the mammalian system, the HindIII-BamHI DNA fragment containing GAL4-binding sites fused to the 35S minimal promoter was ligated to the region immediately upstream of the CAT gene in pBLCAT6 (Boshart et al. 1992). The 35S promoter has been precisely transcribed in a human in vitro transcription system, suggesting that its TATA box and initiation site are functional even in animal cells (Katagiri et al. 1990). Human HepG2 cells were co-transfected with the indicated expression vector, the reporter plasmid, and pSV-β-Galactosidase (Promega, U.S.A.) as a reference plasmid using Lipofectin reagent (Gibco BRL, U.S.A.) and normalized with the optical density at 600 nm.

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