Interaction of the Arabidopsis E2F and DP Proteins Confers Their Concomitant Nuclear Translocation and Transactivation

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E2F transcription factors are required for the progression and arrest of the cell cycle in animals. Like animals, plants have evolved to conserve the E2F family. The Arabidopsis genome encodes E2F and DP proteins that share a high similarity with the animal E2F and DP families. Here, we show that Arabidopsis E2F and DP proteins are not predominantly localized to the nucleus in analyses with green fluorescent protein, and that the complete nuclear localization of some members is driven by the co-expression of their specific partner proteins. Both AtE2F1 and AtE2F3 were translocated to the nucleus and transactivate an E2F reporter gene when co-expressed with DPa but not DPb. In contrast, AtE2F2 was inactive for both nuclear translocation and transactivation even when Dpa or DPb was co-expressed. Because the DNA binding activities of the three E2Fs are equally stimulated by the interaction with DPa or DPb in vitro, the observed transactivation of AtE2F1 and AtE2F3 is DPa specific and nuclear import dependent. A green fluorescent protein fusion with an AtE2F3 mutant, in which a conserved nuclear export signal-like sequence in the dimerization domain was deleted, was localized to the nucleus. Thus, the concomitant nuclear translocation seems to be conferred by the DPa interaction to release an activity that inhibits an intrinsic nuclear import activity of AtE2Fs. Furthermore, the nuclear translocation of AtE2F3 stimulated by DPa was abolished by the deletion of the N-terminal region of AtE2F3, which is conserved among all the E2F proteins identified in plants to date. Replacement of the N-terminal region of AtE2F3 with a canonical nuclear localization signal only partially mimicked the effect of the DPa co-expression, demonstrating the function of plant E2F distinct from that observed for animal E2Fs. These observations suggest that the function of plant E2F and DP proteins is primarily controlled by their nuclear localization mediated by the interaction with specific partner proteins.

The E2F family of transcription factors plays an important role in cell cycle control by regulating the transcription of the genes involved in the progression to the S phase from the G_1 (G_0) phase of animal cells (for review, see Dyson, 1998; Lavia and Jansen-Dürr, 1999). Yeast (*Saccharomyces cerevisiae*) lacks this protein family, whereas plants possess it. A recent finding has shown that tobacco (*Nicotiana tabacum*) ribonucleotide reducatse and proliferating cell nuclear antigen promoters contain E2F-binding sites, which function as cis-elements essential for the cell cycle-regulated expression of the gene (Chaboute et al., 2000; Egelkrout et al., 2001), suggesting that the E2F gene family is functional in the control of the plant cell cycle.

In animals, a number of the E2F family members have been identified; six E2F and two DP proteins for mammals, and two E2Fs and one DP for *Drosophila melanogaster* (Dynlacht et al., 1994; Dyson, 1998; Helin, 1998; Sawado et al., 1998). The E2F members form heterodimers with the DP members and activate transcription from genes responsible for cell cycle control, initiation of replication, and DNA synthesis, as well as several proto-oncogenes such as c-*myb*, B-*myb*, and c-*myc* in mammalian cells (Lavia and Jansen-Dürr, 1999). On the other hand, pocket proteins (pRb, p107, and p130) interact with the E2F/DP complex to repress transcription of the E2F-regulated genes by masking the E2F transcriptional activation domain that overlaps with the Rb-binding region and/or recruitment of histone deacetylase activity to promoter sites that the E2F complex binds (Dyson, 1998; Lavia and Jansen-Dürr, 1999).

The human E2F proteins (E2F-1–6) are functionally different from each other. E2F-6 lacks transcriptional activation and Rb-binding domains and is thought to function as a competitive inhibitor for E2F-binding sites independently of pocket proteins (Cartwright et al., 1998; Gaubatz et al., 1998; Trimarchi et al., 1998). A subgroup composed of E2F-1, E2F-2, and E2F-3 greatly contributes to cell cycle progression to S phase, whereas the other subgroup members E2F-4 and E2F-5 are mainly involved in a G_1 (G_0) arrest mediated by interaction with p107 or p130 (Dyson, 1998; Lindeman et al., 1998; Lavia and Jansen-Dürr, 1999; Gaubatz et al., 2000; Paramio et al., 2000; Rampel, 2000; Wang et al., 2000).

The functions of the E2F-4/5 subgroup members are regulated through changes of their subcellular localization. In contrast to the E2F-1/2/3 subfamily members, which are predominantly localized to the

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nucleus, the subcellular localization of the E2F-4/5 subfamily is regulated dependent on the cell cycle; they are found in both the nucleus and cytoplasm in the G_0 and early G_1 phases and are mainly cytoplasmic in the S phase (Allen et al., 1997; Lindeman et al., 1997; Müller et al., 1997; Verona et al., 1997). The difference in the subcellular localization is because the E2F-1/2/3 subfamily possesses a nuclear localization signal (NLS) in the N terminus and the E2F-4/5 subfamily does not. The nuclear translocation of E2F-4 and -5 seems to be mediated by interacting proteins because it can be enhanced by the coexpression of DP-2, p107, or p130 (Magae et al., 1996; Lindeman et al., 1997). It has been reported that some spliced forms of DP-2 have a NLS and their overexpression leads to nuclear translocation of coexpressed E2F-4 (de la Luna et al., 1996). The lack of nuclear localization activity in the E2F-4/DP-1 complex appears to be mainly because of the absence of the NLS in DP-1 (de la Luna et al., 1996). This is consistent with the observation that the E2F-4 protein fused with the NLS derived from E2F-1, E2F-2, or simian virus 40 large T antigen is predominantly localized to the nucleus (Müller et al., 1997; Verona et al., 1997).

In plants, a number of cDNAs encoding E2F or DP homologs have been isolated and characterized (Ramírez-Parra et al., 1999; Sekine et al., 1999; Albani et al., 2000; Magyar et al., 2000; Ramírez-Parra and Gutierrez, 2000). The plant E2Fs share high sequence similarity but have no distinguishable similarity with the animal E2F proteins although they slightly resemble E2F-4 and E2F-5. Like the E2F family from animals, the plant E2F proteins can bind to the consensus binding sites of the animal E2F (Albani et al., 2000) and their DNA-binding activities can be stimulated by the plant DP proteins and human DP-1 (Albani et al., 2000; Magyar et al., 2000; Ramírez-Parra and Gutierrez, 2000). It has been also shown that they can bind human Rb or Rb-like proteins from plants (Ramírez-Parra et al., 1999; Sekine et al., 1999). However, little is known about the properties of the plant E2Fs, including transactivation, subcellular localization, and functional differences.

Three E2Fs (referred to as AtE2F1, AtE2F2, and AtE2F3 in this study) and two DPs (DPa and DPb) have been identified in Arabidopsis (Magyar et al., 2000). A search of the Arabidopsis genome sequence has revealed that these five proteins that show an overall similarity to the animal E2F and DP proteins are main members of the Arabidopsis E2F family, although there are three other genes likely encoding more distantly related E2F-like proteins that share a limited similarity only with the DNA binding domain. In this study, we examined the properties of the five Arabidopsis proteins. The results have shown that the function of the E2F and DP family members of plants could be regulated through a nuclear localization process, which proceeded apparently by a cooperative and interaction-dependent action of both members.

RESULTS

Stimulation of DNA-Binding Activity of Arabidopsis E2F Proteins by Interaction with Arabidopsis DP Proteins

We isolated three E2F (AtE2F1 and AtE2F3) and two DP (DPa and DPb) cDNAs from Arabidopsis by reverse transcriptase-PCR based on the sequence information in databases, and their deduced amino acid sequences were identical to those from cDNAs registered in the databases, except for AtE2F2 (accession no. AB050114; see "Materials and Methods"). It seemed that all the cDNAs contain the complete open reading frames, judging from the cDNA sequences including the 5′-untranslated region in the database and the mRNA length estimated from our northern analyses (data not shown). To avoid confusion about designations for the three E2Fs or other animal E2Fs, we have referred to E2F1 (accession no. AF242580) and AtE2Fb (Magyar et al., 2000) as AtE2F1; E2F2 (accession no. AF242581) as AtE2F2; and E2F3 (accession no. AF242582), E2F-4 (accession no. AJ276619), and AtE2Fa (Magyar et al., 2000) as AtE2F3, respectively. The DNA binding ability of AtE2Fs and DPs was tested by electrophoretic mobility shift assay (EMSA) with the te2f-1 DNA probe containing an E2F-binding site in a tobacco proliferating cell nuclear antigen promoter (accession no. AB041727). In vitro-translated AtE2Fs or DPs alone did not bind to the probe DNA (Fig. 1, lanes 2–6), although a high concentration of the recombinant thioredoxin fusion proteins with AtE2F2, AtE2F3, or DPa did (data not shown). In contrast, the AtE2F1, AtE2F2, or AtE2F3 product cotranslated with DPa or DPb exhibited DNA binding with a high affinity (lanes 7–12), indicating an increase in the DNA binding activity by the interaction of AtE2Fs with DPs.

Specificity in the Interaction of E2Fs with DPa or DPb

We further measured the strength of the interaction of each E2F with DPa or DPb by the yeast two-hybrid assay (Fig. 2). Although in the DNA binding assay AtE2Fs exhibited no differential increase in DNA binding between the complex with DPa and DPb, the yeast assay revealed a differential specificity in the dimerization affinity. AtE2F1 and AtE2F3 interacted more preferentially with DPa than with DPb, whereas AtE2F2 did better with DPb.

Potent Transactivational Activities of AtE2F1 and AtE2F3 Are Dependent on the Interaction with DPa

To determine the transactivational activity dependent on the interaction of AtE2Fs and DPs, we carried out cotransfection assays with an E2F reporter gene,

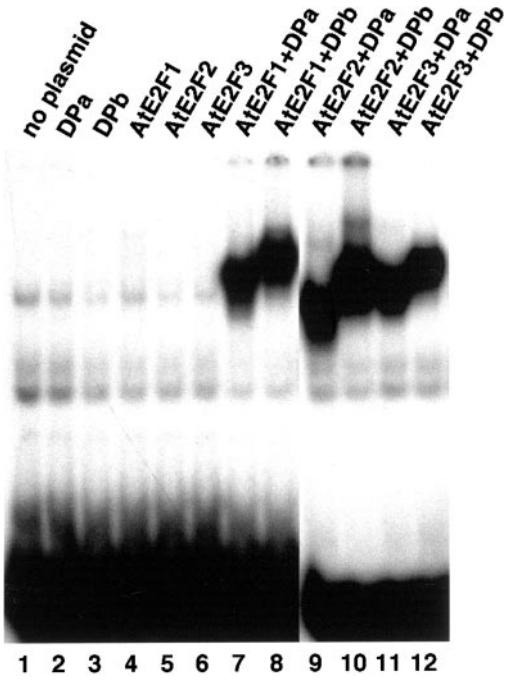

Figure 1. EMSAs for DNA binding activity of Arabidopsis E2Fs and DPs. Radiolabeled double-stranded te2f-1 oligonucleotides (100 fmol) were incubated with 1.5 μL of in vitro-coupled transcription-translation reaction products using the indicated input plasmids. A control (lane 1) was performed with the reaction product without plasmids.

te2fR4-luc, which contains four copies of the E2F-binding site (te2f-1) in the tobacco proliferating cell nuclear antigen promoter, by microprojectile bombardment of suspension-cultured tobacco cells. When the reporter plasmid was cotransfected with either expression plasmid containing the AtE2F1, AtE2F2, AtE2F3, DPa, or DPb cDNA under the control of the cauliflower mosaic virus (CaMV) 35S promoter to tobacco cells, no significant activation of the reporter gene was observed. For AtE2F1 and AtE2F3, only a 3- and 2-fold activation was found, respectively, compared with the control with the reporter plasmid alone (Fig. 3). When the AtE2F1 or AtE2F3 plasmid was transfected with the DPa plasmid, activation of the reporter gene was increased about 34- and 50-fold, respectively. The average increase in activation for AtE2F3/DPa, based on data from 17 independent experiments, was 64-fold with an SD of 53-fold. The fluctuation of the transactivation ratio is dependent on the growth state of the cells used; more actively dividing cells tended to have a higher background level of reporter gene activity, leading to a low activation ratio. The increase in the Luc activity caused by these factors was dependent on the E2F sites in the reporter gene because a mutant reporter gene, mte2fR4-luc, was activated only at low level when cotransfected with the AtE2F3 and DPa plasmids. Although the mutant E2F site did not bind both the cotranslation product of AtE2F3/DPa and the recombinant AtE2F proteins (data not shown), the weak transactivation observed for the mutant reporter gene may be because of an increased binding activity of the multimerized mutant site or some weak binding sites present in the vector or the core promoter sequences. Cotransfection with the DPb, in contrast to DPa, plasmid led to only about a 10-fold activation for AtE2F1 or AtE2F3 (Fig. 3). Furthermore, unlike AtE2F1 and AtE2F3, AtE2F2 had only a minor effect on the transactivational activity even when co-expressed with DPa or DPb (Fig. 3). These observations indicate distinct functions in transactivation of the Arabidopsis E2F and DP members.

Weak Transactivation Activity of AtE2F2 Is Attributable in Part to the Activity of the C-Terminal Activation Domain

We then tested whether the weak transactivational activity of AtE2F2 is because of the transcriptional activation domain. In yeast, the expression of AtE2F1 (AF1) and AtE2F3 (AF3), which were fused with the GAL4 DNA-binding domain, resulted in a significant activation of the β-galactosidase reporter gene, whereas that of AtE2F2 (AF2) had no effect (Fig. 4A). This is consistent with the transactivational properties of these proteins in tobacco cells as shown in Figure 3. In the animal E2Fs, the transcriptional activation domains are located in the C-terminal region. The truncated form of AtE2F3 (AF3ΔC), which lacked

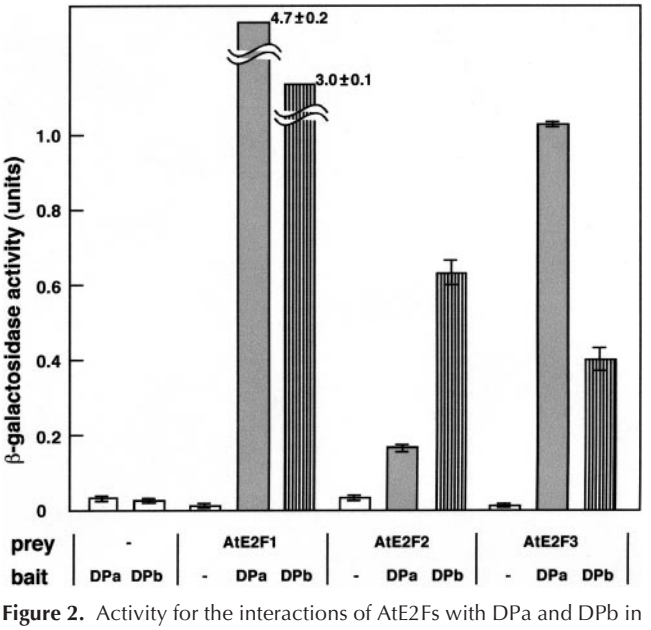

Figure 2. Activity for the interactions of AtE2Fs with DPa and DPb in the yeast two-hybrid assay. AtE2F1, AtE2F2, or AtE2F3 cDNA cloned in the pAD vector as prey was introduced into the yeast SFY526 with the DPa or DPb cDNA cloned in the pGBKT7 vector as bait. Galactosidase activity from the *GAL4 UAS-LacZ* reporter gene was measured. Data are shown as means ± SD (bars) of two independent experiments, which were carried out in triplicate.

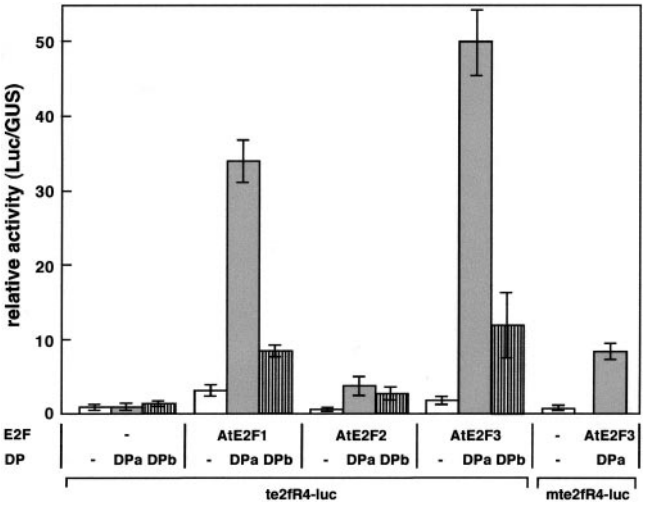

Figure 3. Activation of the E2F reporter gene by cotransfection of AtE2F and DP expression constructs. Suspension-cultured tobacco cells were transfected with 1 μg of the E2F reporter plasmid (te2fR4-luc or its mutant, mte2fR4-luc) and a total of 2 μg of the indicated expression plasmid by microprojectile bombardment. Plasmid quantities were equalized with a control expression plasmid, p35S-nptII, which consists of the neomycin phosphotransferase II (nptII) gene under the control of the 35S promoter. In addition, 0.4 μg of the 35S-β-glucuronidase (GUS) plasmid was included in each transfection as an internal control for transfection efficiency. In these and the subsequent experiments, the reporter activities were normalized as Luc/GUS activity in each transfected sample, and the relative activities were calculated as a fold activation relative to that of the reporter construct alone.

the C-terminal region corresponding to the animal E2F transactivation domain, had no transactivational activity when cotransfected with DPa in tobacco cells, indicating the presence of the transcriptional activation domain in the C terminus as well as the animal E2Fs (Fig. 4B). The corresponding C-terminal region of AtE2F2 was translationally fused with AF3ΔC and the resulting construct, AF3-F2C, was tested for transactivation. The reporter gene activation of AF3-AF2C mediated by the co-expression of DPa in the tobacco cells was lowered to about one-third of the wild-type AtE2F3, and AF3-F2C had no transactivational activity in yeast. These observations indicate that the low efficiency for the reporter gene activation of AtE2F2 is partly attributable to a weak activity of the transcriptional activation domain. Although the transactivation domain of AtE2F2 appeared to be considerably weaker than that of AtE2F3, AF3-F2C retained a moderate level of transactivational activity in plant cells, suggesting that the strength of the transactivation domain of AtE2F2 alone does not reflect the activity for transactivation. We suspected that the transactivation effect observed for AtE2F1, AtE2F3, and AF3-AF2C includes an effect because of sequestration of Rb species that interact with endogenous E2F proteins to repress the E2F reporter gene, rather than a direct transactivation effect through the activation domain of AtE2F3. To test this possibility, we used an expression construct for the C-terminal region of AtE2F3 containing a potential Rb-binding domain, fused with SV40 NLS, the NLS from simian virus 40 large T antigen (AF3C, Fig. 4B). When AF3C was expressed with the reporter gene, however, less activation of the reporter gene was observed (Fig. 4B), indicating that the activation is a direct effect of transactivation by overexpressed E2F and DP proteins.

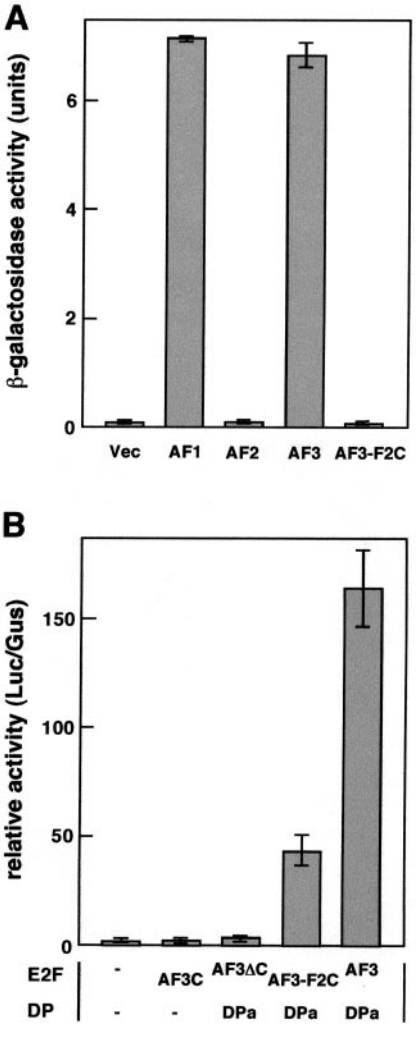

Figure 4. Analyses for the transactivation potencies of AtE2F proteins in yeast and the tobacco cells. A, Yeast assay. The indicated AtE2F cDNAs cloned in the GAL4 DNA binding domain expression vector pGBKT7 were introduced into the yeast SFY526, and β-galactosidase from the *GAL4UAS-LacZ* reporter gene was assayed. B, Transfection assay with tobacco cells. The cultured tobacco cells were cotransfected with the te2fR4-luc reporter construct and the indicated expression constructs of DPa and the wild-type AtE2F3 (AF3) or its derivatives with a deletion (AF3ΔC) or a replacement (AF3-F2C) of the C-terminal transactivation domain of AtE2F3. AF3C represents an expression plasmid for the C-terminal transactivation domain of AtE2F3 containing the SV40 NLS.

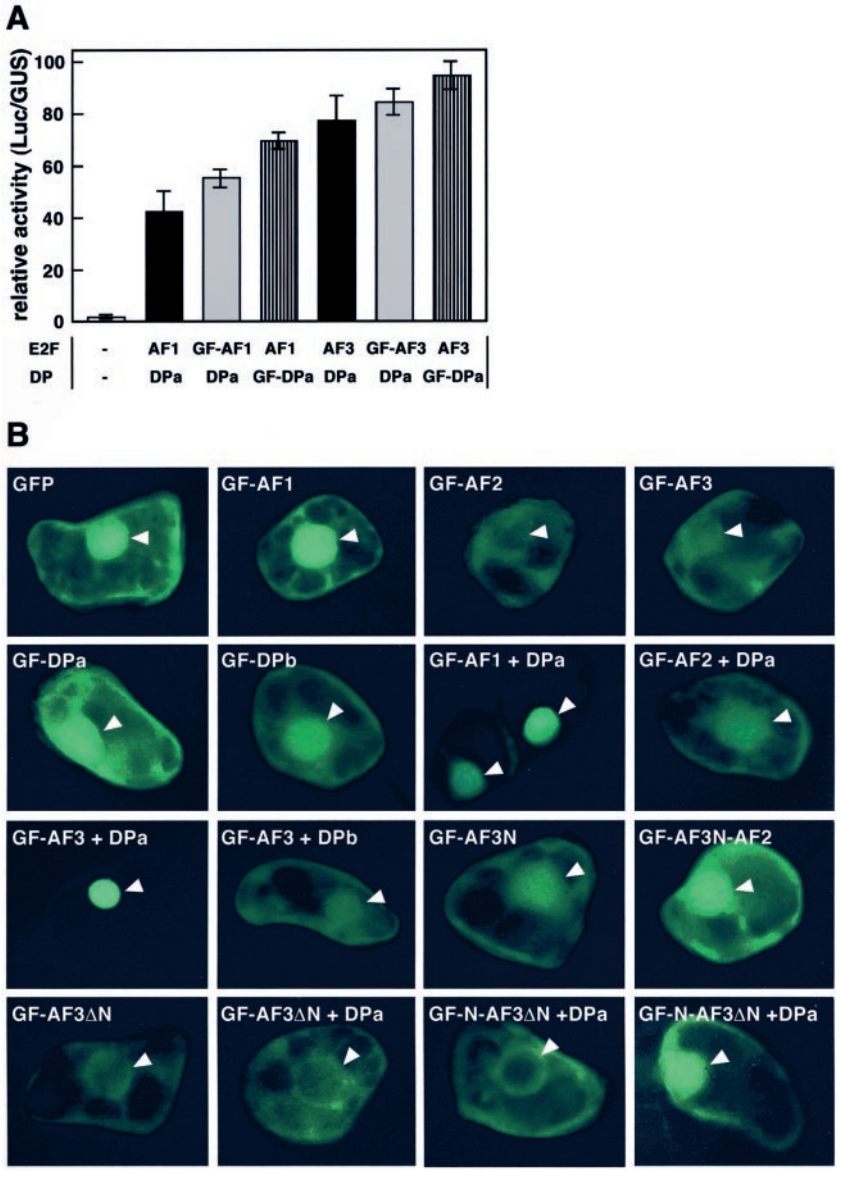

Figure 5. Subcellular distribution of GFP fusions with AtE2F and DP proteins. A, Activation of the E2F-reporter gene by GFP fusions with AtE2F and DPa proteins. The cultured tobacco cells were cotransfected with the te2fR4-luc reporter construct and the indicated expression constructs. B, Subcellular localization of GFP fusion proteins. The cultured tobacco cells were transfected with the indicated GFP fusion constructs of AtE2Fs and DPs, or combinations of both the GFP fusion and the non-fusion expression constructs. Arrowheads indicate positions of nuclei. These photos are representatives for each of the transfected constructs.

E2F and DP of Arabidopsis Exhibit No Predominant Nuclear Localization, and the Interaction of DPa with AtE2F1 or AtE2F3 Stimulates Their Nuclear Translocation

Although both DPa and DPb efficiently stimulated the DNA binding activity of AtE2Fs, only DPa mediated a high level of transactivation. Thus, it was expected that there is another mechanism controlling the transactivation of the E2F/DP complex besides the regulation of DNA binding activity.

The subcellular distribution of AtE2Fs and DPs was examined using proteins fused with the green fluorescent protein (GFP). We first confirmed that GFP-AtE2F1 (GF-AF1), GFP-AtE2F3 (GF-AF3) and GFP-DPa (GF-DPa) had a transactivation potential similar to their native proteins when they were coexpressed with the corresponding partner proteins in the cultured tobacco cells (Fig. 5A). It seemed that the addition of the GFP tag rather increased the transactivation efficiency, probably because of an efficient translation initiation or increased protein stability of the GFP fusions. Observation of GFP fluorescence using an epifluorescence microscope revealed that the GFP-AtE2Fs (GF-AF1, -AF2, and -AF3) and GFP-DPs (GF-DPa and GF-DPb) proteins were localized to the cytoplasm or both the cytoplasm and nucleus (Fig. 5B). In about 30% and 50% of the cells expressing GF-AF2 and GF-AF3, respectively, the fluorescent signals were observed mainly in the cytoplasm (Table I). When we used a GFP fusion protein fused at the C terminus of AtE2F3 to generate AtE2F3-GFP, a similar pattern of the subcellular distribution was observed (data not shown), indicating that the intracellular movement of AtE2F3 is less affected by the

Table I. *Subcellular localization of GFP-AtE2F and -DP proteins*[a]

Construct		Subcellular Localization[b]		
E2F	DP	N	C	N+C
			%	
GF-AF1	–	3	0	97
GF-AF1	DPa	94	0	6
GF-AF1	DPb	0	0	100
GF-AF2	–	7	29	64
GF-AF2	DPa	11	6	83
GF-AF2	DPb	4	4	92
GF-AF3	–	3	48	49
GF-AF3	DPa	82	3	15
GF-AF3	DPb	6	36	58
–	GF-DPa	0	0	100
AF3	GF-DPa	69	0	31
–	GF-DPb	0	0	100
AF3	GF-DPb	1	0	99
GF-AF3ΔD	–	67[c]	0	33
GF-AF3ΔD	DPa	66[c]	0	64

[a] Cultured tobacco cells were transfected with 0.5 mg of the indicated expression constructs of GFP fusion proteins or together with 0.5 μg of the expression constructs containing no GFP coding region. Plasmid quantities were equalized with the p35S-nptII plasmid. One hundred to 200 GFP-positive cells were scored for protein localization. [b] Percentage of cells displaying exclusive nuclear (N), relatively cytoplasmic (C), or both nuclear and cytoplasmic fluorescence (both). [c] The extent of the nuclear localization was weaker than that of GF-AF3 (or GF-AF1) plus DPa.

fusion of GFP. In contrast, GF-AF1 was observed in both the nucleus and cytoplasm in a majority of the cells, which would reflect the higher transactivational activity on expression of AtE2F1 relative to the other two E2Fs, as shown in Figure 3. We then examined the change in the subcellular localization of the GF-AF1, -AF2, and -AF3 proteins by cotransfection with the 35S-DPa or -DPb plasmid. The coexpression of the DPa protein effectively induced exclusive nuclear localization of GF-AF1 and GF-AF3 but not GF-AF2, whereas the DPb protein did not significantly affect the localization (Fig. 5; Table I). These results were confirmed by a reciprocal exchange through the addition of the GFP tag to DPa and DPb (Table I, the last four lanes). The nuclear translocation of AtE2F1 and AtE2F3 mediated by DPa was highly correlated with the strong transactivational activities of these combinations (Table I; Fig. 3). This concomitant nuclear localization process seems to be mediated by a novel mechanism because neither AtE2Fs nor DPs were nuclear. Moreover, we wondered whether this process involves a recruitment of other cellular factors such as NLS-containing factors or protein kinases that have been stimulated in actively dividing cells. However, when we used cultured tobacco cells incubated with a hormone-free medium as semiquiescent cells, the nuclear localization of GF-AF1 and GF–AF3 mediated by DPa occurred at an efficiency similar to that in the actively dividing tobacco cells (data not shown). We then examined whether the nuclear translocation of AtE2F/DPa involves their direct interaction, using a GFP fusion with a dimerization domain mutant of AtE2F3 (GF-AF3ΔD), in which 45 amino acid residues of the dimerization domain were deleted. It was unexpected that GF-F3ΔD was localized to the nucleus by itself, and the efficiency of the nuclear localization was not affected by the co-expression of DPa (Table I). These results suggest that AtE2F3 has an autonomous NLS and that the dimerization domain of AtE2F3 contains a nuclear export signal or a sequence that inhibits the nuclear localization, which may be masked by the binding of DPa to release the inhibition of the nuclear localization.

The N-Terminal Conserved Region of AtE2F3 Is Necessary for Nuclear Localization Mediated by DPa

Plant E2F proteins isolated to date and the mammalian E2F-1 share a conserved region containing three consecutive basic amino acids in their N-terminal end (Fig. 6A). To evaluate the role of the conserved region in the nuclear localization mediated by the interaction with DPa, we constructed an AtE2F3 mutant (AF3ΔN), in which the N-terminal 37 amino acids (92–128) of AtE2F3 were deleted. No nuclear localization of the GFP fusion protein was observed even when DPa was co-expressed (Fig. 6B). This mutant protein was localized mainly in the cytoplasm in a greater number of cells than the wild-type AtE2F3 (Figs. 5 and 6B). The loss of the nuclear localization activity was only partly complemented by the fusion of SV40 NLS to the N terminus when the construct was co-expressed with DPa but not DPb (N-AF3ΔN, Figs. 5 and 6B). The nuclear localization activities of these mutants were well correlated with

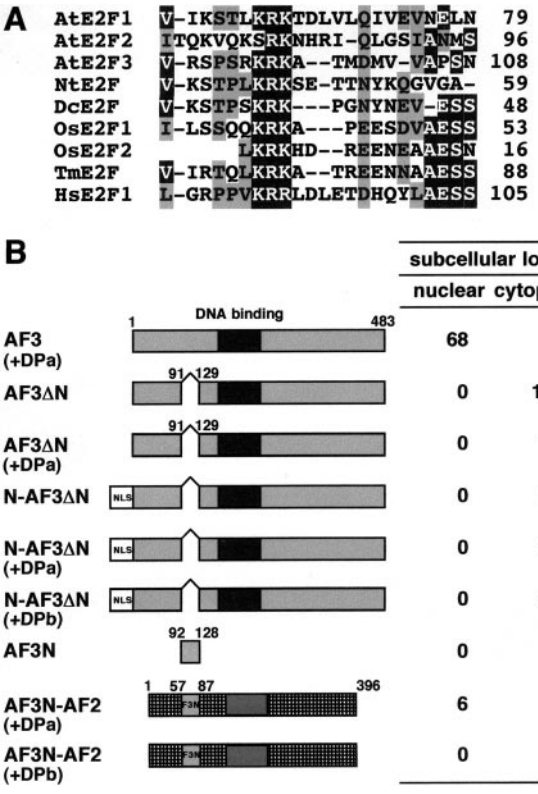

Figure 6. Effects of the N-terminal conserved region of AtE2F3 on nuclear localization and transactivation. A, Amino acid sequence alignment of the N-terminal conserved regions of the human E2F-1 and plant E2F proteins. The N-terminal conserved sequences of E2F proteins from Arabidopsis (AtE2F1–3), tobacco (NtE2F), carrot (*Daucus carota*; DcE2F), rice (*Oryza sativa*; OsE2F1 and OsE2F2), wheat (*Triticum aestivum*; TmE2F), and human (HsE2F1) were aligned. Solid highlighting with white characters indicates amino acids identical in at least five proteins, whereas gray-highlighted characters represent amino acids conserved among five to nine proteins. Dots indicate gaps inserted to optimize the alignment. Numbers indicate positions of the last amino acids of each sequence. B, For subcellular localization, the cultured tobacco cells were transfected with 0.5 μg of the indicated constructs for the GFP expression or together with 0.5 μg of the expression constructs indicated with parentheses. One hundred to 200 GFP-positive cells were scored for protein localization, and the percentage of cells displaying exclusive nuclear, relatively cytoplasmic, or both nuclear and cytoplasmic fluorescence was determined. Similarly, a transactivation assay was carried out with the te2fR4-luc reporter plasmid and the indicated expression constructs without GFP. Degrees of transactivation are represented as relative mean values of Luc/GUS activities to that of the reporter construct alone. Light-gray and dark-hatched boxes represent the coding region of AtE2F3 and AtE2F2, respectively, in which black boxes represent the regions corresponding to the DNA binding domains of both proteins. NLS indicates the NLS derived from the SV40 large T antigen. nt, Not tested.

the transactivational activity (Fig. 6B, right column). Moreover, we examined the autonomous NLS function of the N-terminal sequence of AtE2F3 (AF3N) by fusing the sequence to the C terminus of GFP. The GF-AF3N construct, however, did not exhibit an exclusive nuclear localization of the GFP protein, similar to the original GFP protein (Fig. 5), indicating that the N-terminal basic region has no NLS activity by itself. AtE2F2 contains an N-terminal basic region with a structure slightly different from those of other plant E2Fs (Fig. 6A), which is a possible cause of the deficiency in the nuclear localization activity of AtE2F2. Thus, we replaced the N-terminal region consisting of 31 amino acids (57 and 87) of AtE2F2 with the AF3N sequence of AtE2F3 to generate AF3N-AF2, and fused it with GFP. This fusion protein did not exhibit an exclusive nuclear localization even when co-expressed with DPa or DPb (Figs. 5 and 6B), suggesting that the N-terminal conserved sequence is important but not sufficient for mediation of the nuclear localization of plant E2Fs.

DISCUSSION

The Arabidopsis genome encodes three E2F and two DP proteins that show an overall similarity to the animal E2F and DP proteins. Although three more E2F-like proteins are encoded in the genome, they exhibit a limited similarity only with the DNA-binding domain of E2F proteins and have no ability to interact with DP proteins (S. Kosugi and Y. Ohashi, unpublished data), and thus can be categorized to a distantly related subgroup of E2F. Like the animal E2F, the three Arabidopsis E2F proteins in

this study can form a complex with each of the two DP proteins, thereby gaining the ability to bind DNA with a high affinity. None of the Arabidopsis E2F proteins are, however, predominantly localized to the nucleus, in contrast to the animal E2Fs, among which only the E2F-4/5 subfamily has no autonomous NLSs. The nuclear localization of E2F-4/5 is driven by the co-expression of some variants of DP-2 or p107, which contain an NLS and provide their NLSs in trans to E2F-4/5. A similar regulation appears to be true of the Arabidopsis E2Fs, AtE2F1 and AtE2F3, whose nuclear translocations are conferred by the co-expression of DPa but not DPb. However, the GFP fusion protein with DPa as well as DPb did not exhibit an exclusive nuclear localization except when AtE2F1 or AtE2F3 was co-expressed, indicating that neither the Arabidopsis E2Fs nor DPs have an autonomous activity for nuclear localization. Thus, the nuclear localization of AtE2F1/AtE2F3 and DPa dependent on their interaction occurs concomitantly and apparently in a cooperative manner. This further suggests that the regulation of subcellular localization of the Arabidopsis E2F and DP family involves an unknown novel mechanism by which the nuclear translocation is mediated via the interaction between the two proteins that have no activity for nuclear import in themselves.

A possible mechanism for the nuclear localization of the Arabidopsis E2Fs is that a nuclear export signal (NES) or a sequence (structure) that inhibits the nuclear import present in the dimerization domain inhibits the nuclear localization of AtE2Fs, as exemplified by the dimerization domain mutant of AtE2F3 (GF-AF3ΔD). A recent finding on the nuclear localization of the human E2F-4 demonstrates that E2F-4 has two NES and exhibits a cell cycle-dependent subcellular distribution through the coordination between the NES activity and the NLS activity provided by DP-2 or the pocket proteins such as p107 (Gaubatz et al., 2001). The two NESs are conserved in plant E2Fs as well as animal E2Fs and the second NES-like sequence (LQAEIENLAL, in which underlined Leu and iso-Leu indicate conserved amino acids) present in the N terminus of the dimerization domain of AtE2F3 has been completely deleted in the AtE2F3 mutant. The likely involvement of an NES activity in the AtE2F localization is supported by the observation that even the addition of the SV40 NLS to AtE2F3 did not cause the nuclear localization, whereas it improved the efficiency of the nuclear localization on co-expression of DPa, as shown in Figure 6B. Thus, it is plausible that the nuclear localization of AtE2F3 or AtE2F1 involves a mechanism by which the binding of DPa to AtE2Fs masks the putative NES to release the cytoplasmic retention of AtE2Fs and translocate the bound DPa together to the nucleus. This hypothesis, however, contradicts the observation that DPb has no ability to induce the nuclear localization of AtE2Fs, despite that it can interact with AtE2Fs and stimulate the DNA binding activity at an efficiency similar to that of DPa. We speculate that DPb itself may have an NES activity to retain the ATE2F/DPb complex in the cytoplasm. Alternatively, the interaction of DPb with AtE2Fs might ineffectively block the putative NES of AtE2Fs because of a conformational difference between AtE2F/DPa and AtE2F/DPb.

For the concomitant nuclear localization of AtE2F3 and DPa to proceed, the N-terminal conserved region of AtE2F3 is required. All plant E2Fs identified to date exhibit high amino acid sequence similarities to each other and cannot be classified based on the distinct subdomain organization observed in the mammalian E2Fs. The plant E2Fs share an N-terminal conserved region, which contains a stretch of three consecutive basic amino acids. Although the corresponding region of AtE2F3 had no activity as an autonomous NLS, the deletion of the region from AtE2F3 led to a significant loss in the nuclear localization activity conferred by the co-expression of DPa. This is consistent with the poor conservation of the N-terminal region of AtE2F2, whose nuclear translocation is, unlike other Arabidopsis E2F members, not conferred even by the co-expression of DPa or DPb. Thus, also in other plant species, subcellular localization of E2F and DP members would be regulated by their concomitant nuclear localization process through the conserved N-terminal region of the plant E2Fs. It is interesting that this conserved region exhibits similarity also with the region necessary for the nuclear localization and the cyclin A binding of mammalian E2F-1 (Krek et al., 1994; Xu et al., 1994; Müller et al., 1997). The three consecutive basic amino acids in the region are conserved in the mammalian E2F-1/2/3 subfamily and all the plant E2F members except AtE2F2. It is conceivable that the nuclear localization of the Arabidopsis E2Fs may be affected by activities of some growth-associated protein kinases including cyclin A and cyclin-dependent kinases. The nuclear translocation, however, appears to be an autonomous function of the complex of AtE2F1/3-DPa because cells incubated with a culture medium depleted of a growth promoting hormone, auxin, exhibited a similar efficiency for the nuclear localization of the E2F complexes. Because the dimerization domain mutant of AtE2F3 shows relatively efficient nuclear localization, an intrinsic sequence of AtE2F1/3 containing the N-terminal conserved region would function as an NLS.

It is clear that the DPa-mediated nuclear localization of AtE2F1 and AtE2F3 is essential for their transactivation function because the degree of their nuclear translocation was correlated with the efficiency of the transactivation. Therefore, the DPa protein would be an effective dimerization partner of both AtE2F1 and AtE2F3. On the other hand, a specific partner of AtE2F2 is likely to be DPb. AtE2F2 lacks

both the abilities of transactivation and nuclear translocation to be conferred by the co-expression of DPa or DPb. Because AtE2F2 can efficiently bind to DNA by forming a complex with DPa or DPb, the deficiency in the transactivation function seems to be primarily because of a weak or nonfunctional transactivation domain. Hence, the complex of AtE2F2 with DPb may function as a negative regulator of E2F-regulated genes by competing for E2F-binding sites in the nucleus, where it might be localized through an interaction with another protein such as an Rb-related protein containing an NLS, as observed in the interaction of E2F-4/5 with p107 or p130 (Magae et al., 1996; Lindeman et al., 1997). In animals, E2F-6, which lacks a transactivation domain, has been shown to function as a dominant negative repressor and/or an active repressor through recruiting the polycomb transcriptional repressor complex (Cartwright et al., 1998; Gaubatz et al., 1998; Trimarchi et al., 1998, 2001). Whether AtE2F2 acts as an active repressor awaits further analysis.

In summary, we have made some novel observations on the functions of the plant E2F and DP proteins. First, Arabidopsis E2F and DP proteins do not exhibit a predominant nuclear localization by themselves. Second, the interaction of AtE2F1 or AtE2F3 with DPa but not DPb induces concomitant nuclear localization apparently in a cooperative manner. This nuclear localization is mediated by the N-terminal region of AtE2Fs, which is conserved among all the plant E2F proteins identified to date, suggesting that the nuclear localization of the plant E2Fs is controlled by an interaction with some DPs or other proteins. Because these findings differ from those for the animal E2Fs, it is interesting to know how the difference in the regulation of the E2F/Rb pathway between plants and animals reflects cell cycle regulation in the two kingdoms.

MATERIALS AND METHODS

Plant Materials

Suspension-cultured tobacco (*Nicotiana tabacum*) cells were established from calli of tobacco cv Sumsun NN. The calli were initiated from leaf strips grown on Murashige and Skoog salt medium (Murashige and Skoog, 1962) supplemented with 200 mg L^{-1} of NaH_2PO_4 and 1.0 mg L^{-1} of 2,4-dichrolophenoxyacetic acid, followed by culturing with liquid medium containing the same constituents with shaking at 100 rpm at 28°C and maintained by weekly subculturing in fresh medium.

cDNA Cloning

The coding regions of the AtE2F1, AtE2F2, AtE2F3, DPa, and DPb cDNAs from Arabidopsis were cloned by reverse transcriptase-PCR amplification with mRNA isolated from young whole plants (Arabidopsis cv Columbia), based on sequences in the databases. All the N-terminal and C-terminal primers were designed to contain *Xba*I (or *Spe*I) and *Xho*I sites at their 5′ ends, respectively, and the amplified fragments were digested with *Xba*I or *Spe*I and *Xho*I and cloned into the corresponding sites of the pBluescript SK^+ vector (Stratagene, La Jolla, CA). The isolated cDNAs were identical to the cDNA sequences already registered in the databases, but the amino acid sequence of AtE2F2 (accession no. AB050114) differed at position 297 (T) from the amino acid (A) of E2F2 on the database.

Construction of Plasmids

The E2F-reporter constructs, te2fR4-luc and mte2fR4-luc, were generated by the insertion of dimerized fragments of double-stranded oligonucleotides (5′-TCGACAAGGCGGGAAAAAAGGCGGGAAAAC-3′ and 5′-TCGAGTTTTCCCGCCTTTTTTCCCGCCTTG-3′ for te2fR4-luc; 5′-TCGACAAGGTTGGAAAAAAGGTTGGAAAAC-3′ and 5′-TCGAGTTTTCCAACCTTTTTTCCAACCTTG-3′ for mte2fR4-luc) into the *Xho*I site of the 35S-54-luc vector, which contains the firefly (*Photinus pyralis*) luciferase gene located downstream of the CaMV 35S minimal promoter region (Kosugi et al., 1995). The effector plasmids for the cDNA expression, p35S-AF1, -AF2, -AF3, -DPa, and -DPb, were generated by the insertion of the cDNAs of AtE2F1, AtE2F2, AtE2F3, Dpa, and DPb, respectively, into the *Spe*I and *Xho*I sites of the CaMV 35S promoter-based expression vector pCEP5 (Kosugi and Ohashi, 2000). The C-terminal deletion derivative of AtE2F3, p35S-AF3ΔC, which contains a deletion of 119 amino acids of the C terminus, was constructed by the deletion of an *Sph*I-*Xho*I fragment from p35SAF3. For p35S-AF3-F2C, a blunt-ended AlwNI-*Xho*I fragment of AtE2F2, corresponding to 88 amino acids of its C terminus, was replaced with the *Sph*I-*Xho*I fragment of AtE2F3. The *Sph*I-*Xho*I fragment was translationally fused at the 5′ end with the NLS from the simian virus 40 large T antigen (SV40 NLS), which was generated by annealing oligonucleotides (5′-CTAGTTCCATGGCTCCAAAGAAGAAGAGAAAGG and 5′-GATCCCTTTCTCTTCTTCTTTGGAGCCATGGAA), and cloned into the *Spe*I and *Xho*I sites of the pCEP5 vector to generate p35S-AF3C. The N-terminal deletion derivative of AtE2F3, p35S-AF3ΔN, was generated by the deletion of a *Bgl*II-EcoNI fragment, which corresponded to 37 amino acids in its N-terminal region, from p35S-AF3. For p35S-N-AF3Δ 78, the SV40 NLS sequence was fused with a *Bam*HI-*Xho*I fragment from p35S-AF3ΔN, and cloned into pCEP5.

Plasmids for the expression of GFP fusion proteins were constructed with a pCEP-GFP vector, a slightly modified version of the 35S-sGFP vector (provided by Yasuo Niwa, University of Shizuoka, Japan), which contained the sGFP (S65T) gene (Chiu et al., 1996) with *Eco*RI, *Bam*HI, *Spe*I, *Xho*I, and *Sac*I sites for fusion at the C terminus under the control of the CaMV 35S promoter. Fragments from the pCEP5 derivatives described above were cloned into the *Spe*I and *Xho*I sites to generate p35S-GF-AF1, -AF2, -AF3, DPa, DPb, AF3ΔN, and N-AF3ΔN. For p35S-GF-AF3N, a *Bgl*II-*Xho*I fragment from double-stranded oligonucleotides (5′-AGAGGAATTCAAGATCTCCTTCACGAAAGAGAAAGGCGACAATGGA and 5′-AGAGCTCGA-

GGGAATTCCAGATGGAGCAACAACCATATCCATTG-TC) was inserted into the *Bam*HI and *Xho*I sites of the pCEP-GFP vector. An *Eco*RI fragment of the above double-stranded oligonucleotides was replaced with an *Eco*RI fragment from the N-terminal region of AtE2F2 in p35S-GF-AF2 to generate p35S-GF-AF3N-AF2. A GFP fusion with a dimerization domain mutant of AtE2F3, p35S-GF-F3ΔD, was generated by deleting a 135-bp *Pvu*II-*Eco*RV fragment from the AtE2F3 cDNA and cloning to the pCEP-GFP vector.

For yeast (*Saccharomyces cerevisiae*) vectors, fragments from the above GFP fusion constructs and p35S-AF3-F2C were cloned into the *Eco*RI or *Nco*I and *Sal*I sites of the pGBKT7 vector (CLONTECH, Palo Alto, CA) to generate pGB-AF1, -AF2, -AF3, -AF3-F2C, -DPa, and DPb. The *Xba*I-*Xho*I fragments of the AtE2F cDNAs were cloned into the *Nhe*I and *Xho*I sites of the pAD-GAL4–2.1 vector (Stratagene) to generate pAD-AF1, -AF2, and -AF3.

In Vitro-Coupled Transcription-Translation Reactions

Proteins were synthesized by coupled transcription-translation with a TNT wheat germ extract kit (Promega, Madison, WI) and T7 RNA polymerase using the pGB-AF and -DP plasmids. Reactions were performed with a total of 1 μg of plasmid DNA in 25 μL of solution.

EMSAs

The conditions for the DNA-binding reaction and electrophoresis were as described (Kosugi and Ohashi, 1997), except that 100 ng of salmon sperm DNA per 10 μL of reaction solution was used in place of poly(dI-dC) as a nonspecific DNA competitor. DNA probes were generated by annealing the oligonucleotides used in the construction of the reporter genes and filling cohesive *Sal*I or *Xho*I sites at the 5′ ends with α-^{32}P-dCTP using the Klenow enzyme.

Yeast Two-Hybrid Assays

Yeast manipulation and transformation were performed as described (Kosugi and Ohashi, 1997). A combination of prey and bait plasmids were introduced into the yeast strain SFY526 (CLONTECH) and β-galactosidase activity was measured using *o*-nitrophenyl β-D-galactopyranoside as described (Yocum et al., 1984).

Transient Transfection Assays

Transfection of plasmid DNA into the cultured tobacco cells was performed by microprojectile bombardment. The suspension-cultured tobacco cells at an early stationary phase were collected, and 10 mL of the packed volume was resuspended with 40 mL of 0.5 M mannitol and incubated for 1 h at room temperature. Approximately 1 mL of the cell suspension was blotted on filter paper (2.5 × 2.5 cm), placed on layers of paper towels, and allowed to stand for 10 to 30 min. DNA-coated gold particles were prepared as described (Christou et al., 1991). Approximately 2.0 mg of gold microparticles (Bio-Rad Laboratories, Hercules, CA) was coprecipitated with a total of 8 μg of the effector plasmids, 4 μg of the reporter plasmids, and 2 μg of a reference plasmid of p35S-GUS, the GUS gene under the control of the 35S promoter. One-fourth (4 μL) of the particles were transferred to the screen of a syringe filter unit of the GIE-III particle delivery system (Tanaka Co., Ltd., Sapporo, Japan), a type of particle in-flow gun. The filter paper coated with the cells was placed on a culture plate solidified with 0.3% (w/v) gelrite (Wako Pure Chemicals Industries, Osaka) immediately before bombardment, and the plate was placed on a shelf in the chamber at a distance of 10 cm from the screen in the syringe filter unit. A vacuum of 70 in Hg was applied and the particles were discharged when He gas (at 5.5 kgf/cm^2) was released during 25 μsec. Bombarded samples were cultured at 28°C for 16 to 17 h in the dark. The samples were harvested, extracted with a buffer composed of 0.1 M NaH_2PO_4 (pH7.8), 5% (w/v) glycerol, 1 mM EDTA, and 0.1% (w/v) Triton X-100, and assayed for luciferase and GUS activity using luciferin and 4-methylumbelliferyl β-D-glucuronide as substrates, respectively. All luciferase values were normalized using GUS activities. All experiments were carried out in triplicate and independently performed at least two times.

Observation of GFP Fluorescence

Transfection of the GFP constructs into the suspension-cultured tobacco cells was conducted by microprojectile bombardment with 0.5 mg of gold particles coated with a total of 1 μg of plasmid, as described above. GFP expression in the cells was observed 17 to 20 h after the transfection using an epifluorescence microscope, model AX70 (Olympus, Tokyo), with an MWIA/GFP filter cube (excitation filter, 460–490 nm; barrier filter, 510–550 nm).

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