E2Ls, E2F-like Repressors of Arabidopsis That Bind to E2F Sites in a Monomeric Form*

E2F transcription factors are major regulators of cell proliferation, and each factor contributes differently to cell cycle control. Arabidopsis contains six E2F homologs, of which three are proteins that exhibit an overall similarity to animal E2Fs and interact with DPα and DPβ to stimulate DNA binding. Here we describe the other three E2F-like proteins from Arabidopsis, E2L1–3, which have two copies of a domain with a limited similarity only to the DNA binding domain of E2F. Unlike known E2Fs, the three E2L proteins failed to interact with DPα and DPβ and could efficiently bind E2F sites in a monomeric form through the dual-type domain. Transfection assays revealed that E2Ls repress the transactivation of reporter genes under the control of E2F-regulated promoters, indicating that E2Ls function to antagonize transactivation mediated by E2F-DP. When fused to green fluorescence protein, E2L1 and E2L3 were predominantly localized to the nucleus whereas E2L2 was detected in both the nucleus and cytoplasm. Because the transcripts of E2Ls were abundant in meristematic rather than fully differentiated tissues, E2Ls may balance the activities of E2F-DP and play a role in restraining cell proliferation.

The E2F family of transcription factors plays a pivotal role in the control of cell proliferation. E2F proteins form heterodimers with DP proteins to bind the E2F sites conserved in promoters of DNA synthesis and replication-associated genes (e.g. RNR and PCNA, which encode ribonucleotide reductase and proliferating cell nuclear antigen, respectively) and cell cycle control genes (e.g. cyclin D and cdc2) that are induced mainly during the G1(G0)-to-S transition and activate or repress these promoters (reviewed in Refs. 1 and 2). Recent studies using DNA microarray analysis suggest that E2F regulates also the expression of genes involved in differentiation, apoptosis, and mitosis (3, 4). A repressor function of E2F is modulated also the expression of genes involved in differentiation, proliferation, and each factor contributes differently to cell cycle control.
conserved sequence and a conserved nuclear export signal-like sequence present in the dimerization domain of AtE2Fs (37). This contrasts to the regulation of the mammalian E2F family, in which only the E2F-4/5 subfamily exhibits a regulated nuclear import mediated by interaction with DP-2, p107 or p130 (38–42). On the other hand, AtE2F2 has no transactivation potential, because it lacks an intrinsic transactivation domain (36, 37), suggesting a role for AtE2F2 in transcriptional repression, like E2F-6 from mammals.

Here we describe that Arabidopsis expresses three more E2F-like proteins that contain two separate domains exhibiting a similarity limited to the DNA binding domain of E2F. Unlike known E2Fs, these E2F-like proteins efficiently bind E2F sites in the monomorphic form but not as a heterodimer with DP proteins and repress E2F-regulated promoters. Additionally, these proteins have an ability to localize to the nucleus, and all of their mRNAs are expressed mainly in meristematic tissues. These findings suggest that plants regulate the cell cycle through both the E2F-DP-Rb pathway and the E2L repressors unique to plants.

**EXPERIMENTAL PROCEDURES**

*Plant Materials—* Suspension-cultured tobacco cells were established from calli of Nicotiana tabacum cv. Sumsun NN and maintained as described previously (30). Arabidopsis plants (A. thaliana ecotype Columbia) were grown in pots in growth chambers at 20 °C under 16 h of illumination per day.

* cDNA Cloning—* The E2Ls cDNAs were cloned by RT-PCR amplification with mRNA isolated from young whole Arabidopsis plants, based on open reading frames predicted from Arabidopsis genomic sequences. The AtE2F2 (GenBank™ accession numbers AF242581/AFB051014), AtE2F3 (AJ294534/AF242582), DpA (AJ294531), and DpB (AJ294532) cDNAs were isolated by RT-PCR based on the cDNA sequences in the database. The N-terminal primers were designed to contain SpeI/NcoI sites for E2L1 and BamHI sites for E2L2 and E2L3, and all the C-terminal primers contained XhoI sites at their 5′-ends. The amplified fragments were subcloned into the pBluescript SK+ vector (Stratagene, La Jolla, CA). The sequence integrity of all the amplified fragments was confirmed by sequencing with ABI DNA sequencers (Applied Biosystems).

*Plasmid Constructions—* The cDNA fragments excised from the subcloned plasmids were inserted into the pET-32a vector (Novagen, Madison, WI) and used for production of recombinant proteins. Deletion mutants of E2L1, E2L1ΔC, E2L1DB1, and E2L2DB2 were generated by insertion of the following fragments into the pET-32a or -32c vector; an Ncol-HindIII fragment from the E2L1 cDNA (for AL1–E2L1DB2). For AF3DB, a fragment amplified by PCR with the AtE2F3 cDNA and a primer set (5′-CTACGCAGGATCCTGACATCCTCACAT-3′), was inserted into the pET-32a vector. The yeast two-hybrid assays, the E2L1, AtE2F2, AtE2F3, DpA, and DpB cDNAs were inserted into the pGBK7 vector (CLONTECH, Palo Alto, CA) to generate bait constructs, pGB-E2L1, -AF2, -AF3, -DpA, and -DpB. The N-terminal primers contained SpeI sites at their 5′-ends. The primer sets used for luciferase and GUS assays as described (34). All GUS values were normalized using luciferase activities. All experiments were carried out in triplicate and independently performed at least two times.

*Visualization of GFP Fluorescence—* Transfection of the GFP constructs into the suspension-cultured tobacco cells was conducted by microinjection 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 17–20 h after the transfection using an epifluorescence microscope, model AX70 (Olympus, Tokyo, Japan), with a MWIA/GFP filter cube (excitation filter, 460–490 nm; barrier filter, 510–550 nm).

*Reverse Transcription-PCR—* Total RNA was isolated using the TRIzol procedure, according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized with 5 μg of total RNA, 0.5 μg of an oligo dT primer, and ReverTra Ace (Toyobo, Osaka, Japan). RT-PCR was performed using 0.5–2.0 μl of a 1:30 dilution of the cDNA products and the primer set used for isolation of the E2L cDNAs. For analyses of Arabidopsis 18S RNA expression, we used the primers 5′-ACAATGAACTCCCTAAGGATCTG-3′ and 5′-TCTGACGATTACGATGCCTCTC-3′. All the primers were labeled with [γ- 32P]ATP and T4 polynucleotide kinase. PCR products were amplified for 32 cycles for E2L1, 36 cycles for E2L2 and E2L3, and 16 cycles for 18S RNA and separated by electrophoresis on 4% polyacrylamide gel. Gels were dried and autoradiographed using intensifying screens.

**RESULTS**

*Domain Organization of E2L Proteins—* The Arabidopsis genome encodes three E2F proteins that exhibit an overall similarity to animal E2F proteins (36, 37). Surveying the Arabidopsis genome sequence has revealed that the genome contains more than three E2F-like genes exhibiting a limited similarity to E2F. We isolated the cDNA encoding the three E2F-like proteins, designated E2L1, E2L2, and E2L3, by RT-PCR based on the open reading frames predicted from the genome sequence. The E2L1–3 cDNAs encoded proteins with 359, 354, and 403 amino acids, respectively, which exhibited 57, 38, and 42% amino acid identity between E2L1/E2L2, E2L1/E2L3, and E2L2/E2L3, respectively. Strikingly homologous regions among these proteins, DB1 and DB2, are homologous to the DNA binding domain of animal and plant E2Fs (Fig. 1). The DB1 and DB2 domains of E2L1 share 36 and 31% identical (60 and 46% similar) amino acids, respectively, with the DNA binding domain of human E2F-1. No other region of the E2Ls exhibited similarity to conserved domains of E2F, including the dimerization domain, marked box, and Rb binding domain.

*Binding of E2Ls to E2F Sites—* The similarity of E2Ls to the DNA binding domain of E2F suggests the ability of E2Ls to bind DNA. We analyzed the DNA binding activity by electrophoretic mobility shift assays (EMSAs) using recombinant thioredoxin (Trx) proteins fused with E2Ls and double-stranded oligonucleotide probes containing E2F sites. All the E2L fusion proteins bound to a te2F1 probe, which contains an E2F site present in the tobacco PCNA promoter (30), but not to an E2F2/pro:mutate2F1 (Fig. 2). A telF2/pe:mutate2F1 probe, a mutant of telF2-1 site (30), also bound these proteins with a slightly decreased activity. The observed DNA binding specificity of E2Ls was the same as that of rice E2F proteins (OsE2F1 and OsE2F2) and an OsE2F1-DP complex (30). Furthermore, E2Ls...
appeared to be efficient in binding DNA at low concentrations, suggesting no requirement of other factors for the activity.

Two Conserved Domains of E2Ls Are Required for the DNA Binding—To determine the role of the conserved domains of E2Ls in DNA binding, we tested the ability of E2L1 to bind DNA by EMSA using recombinant Trx-E2L1 proteins with a deletion (Fig. 3A). E2L1/H9004C, an E2L1 mutant with a deletion of the C-terminal 127 amino acid residues, retained the ability to bind the te2f-1 probe with no apparent loss of activity (Fig. 3B). Incidentally, the mobilities of these shifted bands were similar partly due to a C-terminally fused portion derived from the vector sequence in the pET-E2L/H9004C construct. When either the DB2 or DB1 domain was deleted from E2L1 (E2L1DB1 or E2L1DB2), a complete loss of the activity was observed. Similarly, the DNA binding domain from AtE2F3 (AF3DB), an Arabidopsis E2F protein, had no ability to bind the DNA probe. The Trx-E2L1DB1, -E2L1DB2, and -AF3DB proteins did not bind to the probe even at a higher concentration (data not shown). These results indicate that E2L1 requires both DB1 and DB2 to bind the E2F site.

E2L1 Does Not Interact with DP Proteins and Binds DNA as a Monomer—E2F proteins interact with DP proteins to bind DNA whereas E2Ls appear to efficiently bind DNA by themselves. To determine whether E2Ls interact with DP proteins and the DNA binding activity is affected by the interaction, we first measured the activity of E2L1 for the interaction with Arabidopsis DP proteins, DPa and DPb, by the yeast two-hybrid assay. When E2L1 was used as prey, it did not activate an LacZ reporter gene by the coexpression of DPa and DPb used as bait, indicating that E2L1 interacts with neither DPa nor DPb (Fig. 4A). Moreover, the E2L1 constructs as prey and bait did not interact with each other, indicating no ability to form a homodimer. In contrast, AtE2F3 used as prey had the ability to interact with both DPa and DPb, as demonstrated by high levels of β-galactosidase activity. But AtE2F3 as prey did not interact with E2L1 used as bait. Similarly, when AtE2F1 and AtE2F2 were used as prey, no β-galactosidase activity was observed for the interaction with E2L1 used as bait (data not shown), indicating that E2L1 also can not interact with AtE2Fs.

By EMSA with in vitro translation products of E2L1 and Arabidopsis E2Fs and DPs, we next examined a possible change in the DNA binding activity of E2L1. An in vitro translation product of E2L1 efficiently bound to the te2f-1 probe whereas no other related proteins (AtE2F2, AtE2F3, DPa, and DPb) exhibited activity to bind the probe (Fig. 4B). Cotranslation products of E2L1 exhibited neither changes in DNA binding activity for E2L1 nor any newly shifted bands other than the original band of E2L1. Although AtE2F3 formed a complex with DPs to bind the probe, as shown by a low mobility shift band in the last lane, this complex did not appear to cause a significant change in the high mobility band of E2L1. It is noted...
that the calculated molecular mass of the AtE2F3-DPa complex (~86 kDa) roughly corresponds to a homodimeric form of E2L1 (82 kDa: 2 times 41 kDa), indicating that the high mobility band of the E2L1-DNA complex is derived from the monomeric E2L1 bound to DNA but not the dimeric protein. These results indicate that E2L1 does not interact with DP and E2F proteins and bind to DNA in a monomeric form.

E2Ls Repress E2F-regulated Promoters—To examine the transcriptional regulatory function of E2Ls, we carried out transfection assays by microprojectile bombardment of suspension-cultured tobacco cells. Reporter constructs containing the bacterial β-glucuronidase (GUS) gene fused with the tobacco (NtPCNA) or rice PCNA promoter (OsPCNA), which contain functional E2F sites (30), exhibited a moderate GUS expression in transfected tobacco cells (Fig. 5A). When either expression construct containing the E2L1, E2L2, or E2L3 cDNA under the control of the CaMV35S promoter was transfected with the reporter gene, the expression of the reporter genes, especially those fused with the green fluorescence protein (GFP). When the fusion proteins (GFP-E2L1, -E2L2, and -E2L3) were expressed in cultured tobacco cells, GFP fluorescence was observed mainly in the nucleus, although GFP-E2L1 was detected in both the cytoplasm and nucleus (Fig. 6). The incomplete nuclear localization correlate with a less effective repression of the PCNA promoters compared with E2L1 and -3, as shown in Fig. 5. In contrast, GFP fused with the bacterial β-glucuronidase (GUS), which is a cytoplasmic protein in plant cells, was detected mainly in the cytoplasm (Fig. 6D). These results suggest that the E2L proteins are nuclear and carry an intrinsic nuclear localization signal. Consistently, there is a potential bipartite nuclear localization signal (KRX11KRXK) in the C-terminal region, which is conserved in all the E2L proteins.

Expression of mRNAs for E2Ls—Finally, we performed RT-PCR analysis to examine the cell specificity of the accumulation of E2L transcripts (Fig. 7). First-strand cDNAs were synthesized with total RNA extracted from mature leaves at a stage before flower initiation (ML), young immature leaves containing leaf primordia and meristems (YL), young developing stalks located under immature inflorescence (YS), mature growing stalks (MS), young developing flower buds (YP), and mature open flowers (MF). cDNA synthesis was assessed by RT-PCR with primers specific for the 18 S rRNA gene. RT-PCR analysis using primer sets specific for the E2L cDNAs showed that the E2L1 transcript was abundant in young developing organs and tissues such as the meristematic leaves, young stalks, and immature flower buds, whereas it was less common in mature organs such as adult leaves and stalks, as shown by the amplified fragments (Fig. 7). Similar patterns were observed for the accumulation of the E2L2 and E2L3 transcripts,
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Control of the 35S promoter, were transfected to cultured tobacco cells translationally fused at the C terminus with the E2L cDNAs under the PCNA reporters. A rice cotransfected with the indicated expression constructs (0.5 \( \mu \)g) to suspension-cultured tobacco cells by microprojectile bombardment. Plasmid quantities were equalized with 35S-nptII, which consists of the neomycin phosphotransferase II (nptII) gene under the control of the 35S promoter. In addition, 0.2 \( \mu \)g of the 35S-luc plasmid, under the control of the 35S promoter, was included in each transfection as an internal control for transfection efficiency. The reporter activities were normalized as GUS/Luc activity in each transfected sample, and the relative activities were calculated as -fold activation relative to that of the reporter construct alone.

A rice PCNA promoter truncated to −263, OsPCNAΔ-GUS, was similarly used as a GUS reporter. The reporter plasmid (0.5 \( \mu \)g) was cotransfected with the indicated expression constructs (0.5 \( \mu \)g) to suspension-cultured tobacco cells by microprojectile bombardment. Plasmid quantities were equalized with 35S-nptII, which consists of the neomycin phosphotransferase II (nptII) gene under the control of the 35S promoter. In addition, 0.2 \( \mu \)g of the 35S-luc plasmid, under the control of the 35S promoter, was included in each transfection as an internal control for transfection efficiency. The reporter activities were normalized as GUS/Luc activity in each transfected sample, and the relative activities were calculated as -fold activation relative to that of the reporter construct alone. B, cotransfection assay of the PCNA-GUS reporter genes with E2Ls and AtE2F1-DPs expression constructs. Tobacco cells were transfected with the PCNA reporter (0.5 \( \mu \)g) and AtE2F1-DPs expression (0.25 \( \mu \)g each) plasmids, and the indicated E2L (L1, L2, and L3) expression plasmid (0.5 \( \mu \)g). As an internal control, 35S-luc plasmid was included in each transfection. The calculation of relative activities was conducted as described in A.

Repression of PCNA promoters by E2Ls. A, transfection assay with PCNA-GUS reporter genes and E2L expression plasmids. The tobacco and rice PCNA promoters fused to the uidA gene encoding the bacterial β-glucuronidase, NtPCNA-GUS and OsPCNA-GUS, were used as reporters. A rice PCNA promoter truncated to −263, OsPCNAΔ-GUS, was similarly used as a GUS reporter. The reporter plasmid (0.5 \( \mu \)g) was cotransfected with the indicated expression constructs (0.5 \( \mu \)g) to suspension-cultured tobacco cells by microprojectile bombardment. Plasmid quantities were equalized with 35S-nptII, which consists of the neomycin phosphotransferase II (nptII) gene under the control of the 35S promoter. In addition, 0.2 \( \mu \)g of the 35S-luc plasmid, under the control of the 35S promoter, was included in each transfection as an internal control for transfection efficiency. The reporter activities were normalized as GUS/Luc activity in each transfected sample, and the relative activities were calculated as -fold activation relative to that of the reporter construct alone. B, cotransfection assay of the PCNA-GUS reporter genes with E2Ls and AtE2F1-DPs expression constructs. Tobacco cells were transfected with the PCNA reporter (0.5 \( \mu \)g) and AtE2F1-DPs expression (0.25 \( \mu \)g each) plasmids, and the indicated E2L (L1, L2, and L3) expression plasmid (0.5 \( \mu \)g). As an internal control, 35S-luc plasmid was included in each transfection. The calculation of relative activities was conducted as described in A.

Subcellular localization of GFP proteins fused with E2Ls. Expression constructs, containing the sGFP coding region translationally fused at the C terminus with the E2L cDNAs under the control of the 35S promoter, were transfected to cultured tobacco cells (A, GFP-E2L1; B, GFP-E2L2; C, GFP-E2L3). For comparison, the 35S-GFP-GUS construct, in which the GUS coding region was fused to the C terminus of sGFP, was also transfected (B, GFP-GUS). GFP expression was observed 17–20 h after the transfection using an epifluorescence microscope. Arrowheads indicate positions of nuclei. These photos are representatives for each of the transfected constructs.

Expression pattern of E2Ls transcripts analyzed by RT-PCR. Total RNA was extracted from mature leaves (ML), young leaves < 5 mm in length containing apical meristematic regions (YL), young (YS), and mature (MS) stalks, flower buds (young flower, YF) and open flower (mature flower, MF), and analyzed for the expression of the E2L1 (top row), E2L2 (second row), and E2L3 (third row) transcripts. To verify equal amounts of cDNA templates containing each sample, RT-PCR was conducted with primers specific for the Arabidopsis 18S rRNA gene (bottom row). Amplified fragments fractionated on gels exhibited expected sizes, and their integrity was confirmed by sequencing.

indicating that the three E2L transcripts are preferentially expressed in growing tissues. Unlike the E2L1 transcript, however, the E2L2 and E2L3 transcripts were less expressed at the developing stages in stalk and abundant at both stages of flower development, indicating that both the E2L2 and E2L3 genes are regulated in a manner different from the E2L1 gene.

DISCUSSION

Structural Features of E2L Proteins for Binding to E2F Sites—The three E2L proteins found in Arabidopsis contain two copies of a domain that resembles the DNA binding domain of plant and animal E2Fs and can effectively bind to E2F sites as a monomer. General E2F proteins, including AtE2F1–3 form a complex with DP proteins to stimulate the binding of DNA. E2F itself also can bind DNA as a homodimer at high concentrations. For recombinant rice E2F proteins, OsE2F1 and -2, a concentration of 10 ng/ml is required for the binding to DNA whereas heterodimeric complexes of OsE2F1 or DPβs can effectively bind DNA at lower concentrations (30). We have observed that at high concentrations DPs and DPβ proteins also can bind E2F sites apparently as homomers.2 Because DP proteins also contain a region homologous to the DNA binding domain of E2F, a DP homodimer seems to bind E2F sites through this homologous domain, as well as a E2F homodimer and E2F-DP heterodimer. It is most likely that the repeat of the DNA binding domain-like region of E2Ls mimics two DNA binding domains in the homodimer or heterodimer of E2F and DP and constitutes a DNA binding domain. This is supported by the observation that deletion of either of the two homologous regions of E2L1 resulted in a complete loss of the ability to bind DNA. This novel DNA binding domain allows E2Ls to effectively bind DNA even at a low concentration. Because the strong DNA binding activity of the E2F-DP heterodimer relative to that of each homodimer is due to a greater affinity for heteromeric interaction, the DNA binding domain of E2Ls could be in the form of a covalently linked homodimer or heterodimer of E2F and DP.

E2Ls can bind at least two different E2F-binding sites, which are conserved in predicted E2F-regulated promoters in Arabidopsis (30) but not a mutated E2F site. This specificity is similar to that of a heterodimer of OsE2F1 and DPβ (30). Although the E2F and DP family has evolved to be conserved between the animal and plant kingdoms, only plants seem to

2 S. Kosugi and Y. Ohashi, unpublished observation.
It has been shown that the human E2F-6 and *Drosophila* dE2F2 proteins function as repressors for E2F-regulated genes (17–22). AtE2F2, one of three *Arabidopsis* E2F homologs that exhibit an overall similarity to animal E2Fs, has been shown to have no transcriptional function due to the lack of an activation domain (37), suggesting that AtE2F2 is structurally and functionally similar to E2F-6 and dE2F2. E2Ls are thus distinct from these E2F repressors in that they can bind to E2F sites as a monomer and apparently do not require any factors to exert the repression effect despite the fact that they share a similar transcriptional repressing function.

The function of E2Ls seems to primarily depend on the regulation of their transcription, because both the DNA binding and nuclear localization are conferred by the autonomous function of the E2Ls, in contrast to AtE2Fs, which are regulated through interaction with DP proteins (37). The expression patterns of the three E2L transcripts are similar. The transcripts are more abundant in developing young leaves and stems than in fully differentiated mature ones, indicating that these genes are preferentially expressed in dividing cells. In stems than in fully differentiated mature ones, indicating that these genes are preferentially expressed in dividing cells. In stems, these genes are preferentially expressed in dividing cells.