The E2F transcription factors are key components of the cyclin D retardinoblastoma/E2F pathway. Here we demonstrate that Arabidopsis thaliana contains six functional AtE2F genes that are all expressed in cell suspension culture but show different patterns of expression during cell cycle progression. According to their structural and functional features, the six AtE2Fs can be divided into two distinct groups; although the three members of the first group, AtE2Fa, AtE2Fb and AtE2Fc, possess all the conserved domains found in other plant and animal E2Fs, the remaining AtE2Fs are novel proteins, which reveal a duplication of the DNA binding domain but lack any other conserved region. Furthermore, the AtE2Fs of the first group are functional transcription factors that in association with AtDP proteins can recognize specifically an E2F cis-element and can transactivate an E2F-responsive reporter gene in plant cells. In contrast, the AtE2Fs of the second group can bind specifically the E2F site without interacting with DP partners but cannot activate gene expression and, instead, are able to inhibit E2F-dependent activation of gene expression in Arabidopsis cells. These findings suggest distinctive roles for the plant E2F proteins and point to a complex concerted regulation of E2F-dependent gene expression in plant cells.

Recent studies have shown that the basic regulatory circuits governing cell cycle progression in animal cells are remarkably conserved in higher plants. In particular, plant cells possess all the key components of the cyclin D/retinoblastoma/E2F pathway, which in animal cells is a major regulator of cell proliferation and is part of a critical checkpoint controlling the progression from G1 to S phase of the cell cycle (1). The cyclin D-cdk2 or cyclin D-cdk4 complexes regulate the activity of the pRB pocket proteins, which in their hypophosphorylated state bind to the E2F family of transcription factors and block their transactivating potential (2–4). Furthermore, the pocket proteins have been shown to recruit to the E2Fs chromatin remodeling activities, such as histone deacetylases, histone methyltransferase, or SWI-SNF complexes, which once tethered to the DNA can actively repress the transcription of E2F-regulated genes by blocking the access of other transcription factors in the proximity of the E2F binding sites (5–9). It is now widely believed that in quiescent cells or during the early G1 phase of the cell cycle the E2Fs are mainly involved in the repression of several cell cycle-regulated promoters, whereas during the transition from G1 to S phase the release of transcriptionally active E2Fs, resulting from the phosphorylation of the pocket proteins, leads to an E2F-dependent activation of several genes coding for regulatory proteins and for enzymes involved in nucleotide and DNA synthesis. This dual function of the E2F complexes clearly explains why these transcription factors, depending on the cellular and developmental context, can be either positive or negative regulators of cell proliferation and can act as both oncogenes and tumor suppressors (2, 4). Moreover, highlighting the remarkable functional complexity of animal E2Fs, in Drosophila the DmE2F1 protein has been shown to affect directly the localization and the DNA-activating activity of the origin replication complex (10) and in animal cells this family of proteins is believed to participate in the regulation of several cellular processes (3). Indeed, according to the broad range of mammalian genes, which change their expression in response to the activation of some of the E2Fs, these transcription factors have been shown to be involved in the control of differentiation, development, proliferation, and apoptosis (11). In mammalian cells some E2Fs have been actually shown to induce apoptosis through the regulation of p53 activity as well as in a p53-independent pathway (3, 4).

The various E2F proteins can recognize specific DNA cis-elements forming heterodimers with partially related proteins called DP. So far, six E2Fs and two DPs have been found in human cells, and, according to a comparative analysis of the genome of Arabidopsis thaliana, at least six putative E2F genes and two DP genes appear to be present in Arabidopsis cells as well. Plant E2F genes have been described in carrot, tobacco, and wheat (12–14), and three of the Arabidopsis E2Fs have been recently described (15, 16), whereas DP homologues have been reported in wheat and Arabidopsis (15, 17). All the E2F proteins described so far possess a highly conserved DNA-binding domain, forming a winged helix motif, which is flanked toward the C-terminal side by a DP dimerization domain containing a leucine heptad repeat. Next to the dimerization domain, all the E2Fs possess another conserved region called marked box, which in human cells is recognized by the adeno-
virus E4 protein and may be involved in heterodimerization and DNA bending (18, 19). Based on their primary structures and on their functional features, the six mammalian E2Fs can be divided into three groups. E2F1, E2F2, and E2F3, which belong to the first group, possess a conserved cyclin A-binding domain in their N-terminal region and a transactivating domain, overlapping a conserved pRB-binding region, at their C-terminal end. These three E2Fs are believed to be direct activators of S phase genes; accordingly, their expression is not detectable in quiescent cells but it is strongly up-regulated during G1/S transition. In contrast, E2F4 and E2F5, the members of the second group, are expressed in quiescent cells as well as throughout the cell cycle and lack the cyclin-A-binding domains but possess pRB-binding and transactivating C-terminal regions. E2F6, the last known member of the mammalian E2F family, lacks the N-terminal cyclin-A-binding domain as well as the transactivating C-terminal region and it has been shown to bind directly polycomb group proteins and to act as a repressor of E2F-dependent transcriptional activation (20).

The plant E2Fs described so far, do not possess evident cyclin A-binding domains in the N-terminal region but show a conserved pRB-binding domain in their C-terminal region. In this respect, wheat, tobacco, and Arabidopsis E2Fs have been shown to interact with plant pRBR proteins (13, 14, 16) and the carrot E2F factor has been shown to be a transcriptional activator that, heterodimerizing with a DP partner, can bind a consensus E2F cis-element and can transactivate a synthetic E2F-responsive promoter in both plant and animal cells (12).

In this work we report on the isolation and functional characterization of the cDNA clones of all six E2Fs of A. thaliana. Although the previously described AtE2F sequences show the distinctive roles for these plant E2F proteins pointing to a functional classification that can be divided into three groups. E2F1, E2F2, and E2F3, which belong to the first group, possess a conserved cyclin A-binding domain and DNA-binding assays and transactivation experiments suggest a complex concerted regulation of E2F-dependent gene expression in plant cells.

**EXPERIMENTAL PROCEDURES**

**Plant Materials—**Arabidopsis plants (ecotype Columbia) and two different cell lines were used as a source of plant material. For protoplast preparation and transient expression studies, a habituated cell suspension line was grown at 23 °C under continuous light in Murashige and Skoog pH 5.8 medium (Duchefa) supplemented with 3% sucrose and was subcultured weekly by transferring a 2-ml packed cell volume inoculum to 50 ml of fresh medium. For synchronization experiments the cell line T87 of Arabidopsis (21) was maintained at 23 °C with a 16-h photoperiod in B5 Gamborg's medium (Sigma), pH 5.8, supplemented with 30 g/liter sucrose and 1 μmol NAA, and was subcultured weekly transferring 4 ml into 40 ml of fresh medium. Suspension cells were synchronized by a 24-h treatment with 20 μg/ml aphidicolin as described previously (22). At different time points, the cells were harvested and quickly frozen in liquid nitrogen before isolation of total RNA.

**Isolation of AtE2F and AtDP cDNAs—**To amplify the cDNA sequences of the various E2F and DP genes, poly(A) + RNA was isolated from Arabidopsis suspension cells and from plants at the flowering stage using oligo(dT) celluose (Roche Molecular Biochemicals) following a standard batch procedure (22). The RNA was then reverse transcribed using an anchored oligo(dT) primer or oligonucleotides derived from the putative 3'-untranslated region of each gene. The RT primers were: ATCGGTCTAGATGAGTCTTTAG (AtE2Fa/RT primer), GAGAATTAACATCTGGTTGA (AtE2Fb/1 primer), CTGAAATTTCAGTTACAGAAA (AtE2Fb/RT primer), CTGTTTATAACACATCTGGTTCA (AtE2Fb/RT primer), AGAATTTTGGAATAGAAGCTC (AtDPa/RT primer), and CTTTACAGTTTTA (AtDPb/RT primer).

The reverse transcription reactions were performed for 1 h at 45 °C using 400 ng of poly(A) + RNA in 20 μl of reaction containing 10 pmol of each primer, 40 units of RNasin ribonuclease inhibitor (Promega), 0.5 mM dNTPs, and 200 units of Moloney murine leukemia virus reverse transcriptase (Promega). The cDNA samples were then used as templates for PCR reactions, which were performed with the RT primers coupled with the following oligonucleotides derived from the known AtE2Fb upstream region, as determined by 5'-rapid amplification of cDNA ends (23), or from the putative 5'-untranslated region of the other genes: TCGACTCTCTGCTCACTAGGA (AtE2Fa/1 primer), TCTGCAATCGCAGGATCTC (AtE2Fb/1 primer), TCTGCAATCGCAGGATCTC (AtE2Fb/3 primer), ATCGGTCTAGATGAGTCTTTAG (AtE2Fb/1 primer), AATATTCTGCTGGCTTTTT (AtE2Fb/1 primer), CGTGTGAGCTAATCTGCAT (AtE2Fb/1 primer), and AGAATTTACATCTGGTTCTCA (AtE2Fb/1 primer). Nested reactions were performed using a second set of gene-specific primers with the following sequences: CATAAGATCTCGATGACTTCTGCTGTA (AtE2Fb/3Xho primer), ACCGGATC-CAACCCCGCGTTACAGGC (AtE2Fb/5Bam primer), CCCCTCGAGATATCTGGTT (AtE2Fb/5Bam primer), AATGCGATCTGGTTAAG (AtE2Fb/1 primer), GGGGATTTTAATTTCTCAGAATATG (AtE2Fb/3Xho primer), TCTGCAATCGCAGGATCTC (AtE2Fb/1 primer), and AGAATTTACATCTGGTTCTCA (AtE2Fb/1 primer). As shown in italics, the 5’ end AtE2F oligonucleotides contained an anchored BamHI site and the 3’ end primers contained an EcoRI or a XhoI cloning site, whereas the DP oligonucleotide sequences included a XhoI and an EcoRI cloning site. After electrophoretic analysis of the nested reactions, the resulting PCR fragments were either digested with the suitable restriction enzymes and ligated into the pluBluescriptII KS+ plasmid (Stratagene), in the case of the AtE2Fa, AtE2Fb, AE2Fd, and AtE2Fe cDNAs, or directly cloned into the pGEM-T Easy vector (Promega). All the clones were sequenced using the Big Dye Terminator cycle sequencing on an ABI sequencer (PerkinElmer Applied Biosystems).

**Expression Studies by Semiquantitative RT-PCR Analysis**—After dividing and synchronizing Arabidopsis cells in a medium, total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For the RT-PCR reactions, oligo(dT)-primed first-strand cDNA was synthesized from 5 μg of total RNA using SuperscriptII RNAseH− Reverse Transcriptase (Invitrogen) following the manufacturer's protocol and aliquots of 2.5 μl of the cDNA were then used for each PCR reaction. 20 cycles of PCR amplification were performed in a final volume of 20 μl with 25 pmol of gene-specific primers: CCAATCGCGGTCTGCTGACGATC and CCTGAGTCAAGAATTCCAGG (AtE2Fe), ATGGAGTCTGAGGATCTGAGA and CGTGAATTTAGAGATCAGTTCT (AtE2Fb), TCTGGAACTTGGGACTTGGA and CTGTTTATTGCTGTTCTCA (AtE2Fd), TCTGGAACTTGGGACTTGGA and CTGTTTATTGCTGTTCTCA (AtE2Fd). The PCR products were resolved on a 1% agarose gel and transferred onto a Hybond N + membrane (Amersham Biosciences, Inc.). Hybridization was carried out overnight at 62 °C with 32P-labeled probes corresponding to the coding regions of the specific genes. The membranes were washed twice at 62 °C in 1× SSC containing 1% SDS and once in 0.1× SSC containing 1% SDS and then autoradiographed.

**Proline oligomers of E2F and DP Proteins**—To produce recombinant AtE2F proteins, the cDNA fragments of five AtE2Fb clones (AtE2Fa, AtE2Fc, AtE2Fd, AtE2Fe, and AtE2Ff) were isolated after digestion with BamHI and KpnI and were inserted into the polynucleotide of suitable pSET5 vectors (Invitrogen). pSETA was used for AtE2Fa and AtE2Fd, pSETB for AtE2Fb, AtE2Fc, and AtE2Fe, and pSETC for AtE2Ff. The resulting plasmids were introduced into Escherichia coli.
coli BL21(DE3) or BL21(DE3)pLysE, and the corresponding proteins carrying a histidine-tagged N-terminal extension (His-AtE2Fα, His-AtE2Fβ, His-AtE2Fδ, His-AtE2Fε, His-AtE2Ff) were purified under non-denaturing conditions by metal affinity chromatography on nickel-nitrotriacetate acid resin (Qiagen) as previously reported (24). Because a similar approach was unsuccessful for AtE2Fb, the recombinant protein was produced as a GST-AtE2Fb fusion by ligating into the Smal site of the pGEX-4T-2 vector (Amerham Biosciences, Inc.) the blunt-ended fragment isolated after digestion of the AtE2Fb cDNA clone with XhoI and NotI. Similarly, to obtain recombinant AtDP proteins, the relative cDNA fragments, isolated after digestion of the clones with XhoI and NotI, were cloned into the corresponding sites of the pGEX-4T-3 vector and pGEX-6P-1 vector respectively. The resulting plasmids were introduced into E. coli XL1-blue cells, and the GST-AtDPα, GST-AtDPβ, and GST-AtE2Fb recombinant proteins were purified by chromatography on glutathione-agarose (Amerham Biosciences, Inc.) according to the manufacturer’s instructions. Electrophoretic analysis of the eluted proteins confirmed the purification and the expected size of the recombinant proteins.

**Electrophoretic Mobility Shift Assays—**Electrophoretic mobility assays were performed essentially as previously described (12). The DNA binding reactions of the purified recombinant AtE2F and AtDP factors were conducted incubating 50–300 ng of recombinant proteins with 50,000 cpm of radiolabeled canonical EC probe (5’-aaattcTTTTCCATCGCTTTTgaatt-3’) in 15 l of buffer containing 100 mM KCl, 1 mM MgCl2, 1 mM EDTA, 5% glycerol, and 1 mM dithiothreitol for 30 min at room temperature. For the competition experiments, increasing amounts of annealed unlabeled canonical EC probe or unlabeled mutant EM probe (5’-aaattcTTTTCCATCGCCTTTTgaatt-3’) were included in the reactions. The protein-DNA complexes were electrophoresed for 3 h at 4 °C on 4% polyacrylamide gels in 0.5× TBE.

**Transactivation Assays—**For the construction of the effector plasmids, various AtE2F and AtDP cDNA fragments were isolated after digestion with BamHI and XhoI and were ligated into a BamHI/SalI-digested pFF19 plasmid (25) to give rise to the p35S-AtE2Fα, p35S-AtE2Fβ, p35S-AtE2Fδ, p35S-AtE2Fε, p35S-AtE2Ff, p35S-AtE2Fβ, and p35S-AtDPβ plasmids. The pB221-2-E2F reporter gene assays has been described previously (21). Transactivation experiments were conducted with protoplast isolated from 3-day-old Arabidopsis cell suspension cultures. The cells were incubated for 3 h with 1% cellulase and 0.2% pectinase in protoplast isolation solution (27.2 mg/liter KH2PO4, 101 mg/liter KNO3, 1.4 g/liter CaCl2, 246 mg/liter MgSO4, 0.16 mg/liter KI, 0.025 mg/liter CuSO4, 10 mM MES, and 1.7 mM sorbitol, pH 5.5) and the resulting protoplasts were pelleted, washed three times in the same solution without enzymes, and then resuspended at a density of 0.5 × 106 protoplasts ml−1. After incubation for 2 h at room temperature under dim light, the protoplasts were pelleted again, resuspended at a density of 1 × 106 protoplasts ml−1 in W5 solution (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 5 mM succrose, pH 5.8), and left for 20 min at room temperature. After this incubation, the protoplasts were finally pelleted, resuspended at a density of 1.5 × 106 protoplasts ml−1 in MaMg solution (0.5 mM mannitol, 15 mM MgCl2, 5 mM MES, pH 5.6), and divided into aliquots of 0.3 ml. For the transactivation reactions, each aliquot of the protoplast suspension was mixed with 10 μl of each test plasmid and with sonicated calf thymus DNA, included to bring the total DNA content to 50 μg. After adding 300 μl of FEG solution (40% polyethylene glycol 6000, 0.1 mM Ca(NO3)2, 0.4 mM mannitol, pH 9), the samples were incubated at room temperature for 20 min; then 5 ml of F solution (140 mM NaCl, 125 mM CaCl2, 5 mM KCl, 0.75 mM NaH2PO4, 5 mM succrose, pH 7.2) were added to each aliquot, and the reactions were incubated at room temperature for an additional 20 min. The protoplasts were finally pelleted, resuspended in 6 ml of culture medium (Gamborg’s B 5 medium supplemented with 300 mg/liter CaCl2, 825 mg/liter NH4NO3, 100 mg/liter sodium pyruvate, 200 mg/liter malic acid, 200 mg/liter citric acid, 300 mg/liter casamino acids, 200 mg/liter yeast extract, 20 g/liter saccharose, 76 g/liter mannitol, 0.1 mg/liter 2,4-dichlorophenoxyacetic acid, 0.2 mg/liter benzydaminopirine, 10−6 M NAA, 5 × 10−7 M zeatin riboside, pH 5.6) and incubated for 40 h in the dark at 25 °C. GUS activity was measured as described by Gallie et al. (26).

**RESULTS**

The Arabidopsis E2F Family Is Composed of Three Typical E2Fs and of Three Distinctive Members with Unique Structural Features—The recent isolation of plant E2F homologues has revealed the striking conservation of a portion of the DNA-binding domain in plant and animal E2Fs. Computer searches on the A. thaliana genomic sequences in the GenBank database have revealed the presence of at least six putative E2F-like genes: one each on chromosome 1 and 2, two on chromosome 3, and two on chromosome 5. For one gene, corresponding to the recently described AtE2Fb and AtE2Ff clones (15, 16), the sequence of the 5′ region was missing in the data base and 5′-rapid amplification of cDNA ends experiments were performed to obtain the full coding region. For the remaining genes, complete genomic sequences were available and, following a predictive analysis of the putative open reading frames, specific primers were selected and used to isolate the corresponding cDNAs by RT-PCR. Bearing in mind the possibility that some of the AtE2F genes could be expressed only in specific cell types or during particular developmental stages, the RT-PCR reactions were conducted using pooled mRNA isolated from an actively dividing Arabidopsis cell culture as well as from entire flowering plants. These reactions led to the isolation of cDNA clones corresponding to the transcripts of all six AtE2F genes. In agreement with the nomenclature already proposed for the two recently reported AtE2Fa and AtE2Fb clones (15), we named the four remaining AtE2F genes with letters from c to f. Fig. 1A shows the alignment of the six AtE2F proteins, derived from the translated cDNA sequences, and reveals the existence of two different groups of E2F-like members in Arabidopsis. The AtE2Fa and AtE2Fb cDNAs, which confirm the reported clones, code for proteins of 485 and 469 amino acids, respectively, with predicted molecular masses of 52.8 and 51.7 kDa. The highly related AtE2Fc cDNA, which belongs to the same group, codes for a smaller product of 396 amino acids with a putative molecular mass of 44.5 kDa. The sequence of these three cDNAs is identical to the recently reported AtE2Ff, AtE2Ff, and AtE2Ff clones, respectively (16). As shown in Fig. 1A, these three AtE2F proteins possess all the typical conserved domains, including the marked box and the pRBR-binding region, previously found also in other plant E2Fs. On the other hand, AtE2Fd, AtE2Fe, and AtE2ff, which are the members of the second group of proteins, code for unique E2F-like products that, as highlighted in Fig. 1B, possess two E2F-like DNA-binding domains but none of the other conserved regions. The AtE2F and AtE2Fe cDNAs code for larger products of 359 and 379 amino acids, respectively, with putative molecular masses of 40.6 and 42.4 kDa. In contrast, the AtE2Ff cDNA sequence retained a predicted intron that introduces a stop codon at amino acid position 201 and gives rise to a putative translation product of 22.4 kDa truncated in the middle of the second E2F-like DNA-binding domain. As shown in Fig. 1A, the AtE2Fa protein possesses a putative nuclear localization signal (NLS) of the SV40 large T antigen type at position 92 in its N terminus but this sequence (PSRKKR) is only partially conserved in the AtE2Fb and AtE2Fc proteins and is not observed in the AtE2Fes of the second group. However, both AtE2Fd and AtE2Fe possess a homologous bipartite NLS sequence in their C-terminal region, starting at position 241 and 270, respectively, and in the AtE2Fd protein this putative NLS is additionally overlapped by a SV40 large T antigen type NLS (PKKR) located at position 239. The AtE2Ff protein, being truncated in the second DNA-binding domain, does not possess any of these sequences, and other putative NLS sequences cannot be defined. Overall, it is clear that the AtE2F family of Arabidopsis comprises two distinct types of E2F-like proteins, and, although the members of the first group closely resemble the plant and animal E2Fs so far reported, the second group of AtE2Fs possess remarkable structural features that distinguish them from all the E2F proteins so far described.

**The Arabidopsis E2F and DP Genes Are Differentially Ex-
pressed during Cell Cycle Progression—Because the RT-PCR reactions that led to the isolation of the various cDNA clones were conducted using pooled mRNA isolated from both flowering plants and proliferating cell culture, it was not clear whether all six AtE2F genes could be expressed in suspension cells. However, a preliminary Northern blot analysis with mRNA isolated from an Arabidopsis cell culture in exponential growth revealed that, although to different extents, the AtE2F genes are all expressed in proliferating cells (data not shown). Consequently, it was particularly interesting to verify whether some of them could be differentially regulated during cell cycle progression. For this purpose, we performed semiquantitative RT-PCR reactions to detect the expression of the AtE2F transcripts in an Arabidopsis cell culture partially synchronized by treatment with aphidicolin, an inhibitor of DNA polymerase. Similar analyses were conducted for the AtDPα and AtDPβ transcripts, as well as for histone H4 and actin transcripts. The expression of histone H4 transcripts allowed us to verify the efficiency of synchronization and to monitor the passage through S phase, whereas the expression of actin transcripts, which are believed to be constitutive, was analyzed to verify the uniformity of the various mRNA samples. The efficiency of synchronization of plant cells and its extension in time are cell culture-dependent and, based on our experience with several independent experiments, only 40–50% of the cells of our Arabidopsis cell culture can acquire synchronization (data not shown). Nevertheless, as reported in Fig. 2, in our partially synchronized cell culture the steady state levels of histone H4 transcripts are low immediately after the release from block and increase in the following hours, marking a clear peak of S phase 8–10 h after release. Histone H4 expression then decreases after 12 h, suggesting that most of the synchronized Arabidopsis cells have moved into G2 by that time. Even though our synchronized cells move into S phase only several hours after the release from block, possibly because of the relatively high concentration of aphidicolin used to obtain a good synchronization, it is clear that the expression of the various AtE2F and AtDP genes is strongly regulated during cell cycle progression from G1 to G2. Furthermore, as fully described in Fig. 2, the various genes show very distinctive patterns of expression. The AtE2Fa gene is maximally expressed shortly before the peak of S phase, and its expression decreases remarkably during the passage into G2, whereas the AtE2Fb transcripts accumulate maximally at the G1/S transition and show a lower but constant steady state level in the following phases. The AtE2Fc and AtE2Fd genes,
which code for proteins of the two different AtE2F groups, are expressed in a similar way, and their transcripts are not detectable at the G1/S transition, but their steady state levels increase during the progression into S phase and peak after the passage into G2. On the other hand, AtE2Fe and AtE2Ff, the other members of the second group of AtE2Fs, are expressed at both the G1/S and S/G2 boundaries, but their transcripts are not detectable during early S phase. As described in Fig. 2, the two AtDP genes also show different patterns of expression in relation to cell cycle progression. The AtDPa transcripts appear to accumulate constitutively throughout the progression into S phase, whereas the AtDPb gene shows very low expression at the G1/S boundary and increases considerably its expression during the progression into S phase reaching a peak after passage into G2.

All the Arabidopsis E2F Proteins Recognize Canonical E2F cis-Elements, but the Two Groups of Proteins Have Different DNA Binding Requirements—Although the AtE2Fa and AtE2Fb proteins have been shown to interact in vitro and in vivo with both AtDPs (15) and AtE2Fb has been recently shown to be able to recognize E2F cis-elements forming a complex together with a recombinant human D1P1 partner (16), the DNA binding features of AtE2Fa and AtE2Fc have not yet been described. Furthermore, in view of the remarkable difference in primary structure between the two groups of AtE2F proteins, it was particularly interesting to investigate whether all of these Arabidopsis proteins can specifically recognize a canonical E2F DNA probe in the absence of DP partners. As shown in Fig. 3 (A and B), the results of these EMSA analyses revealed that His-AtE2Fa binds to the canonical E2F probe in the absence of DP proteins. Moreover, addition of recombinant AtDPs did not affect the efficiency of DNA binding or the relative mobility of the AtE2Fd or AtE2Fe protein-DNA complexes (data not shown), suggesting that an interaction between these two unique AtE2Fs and the AtDP proteins is unlikely to occur. This possibility is further supported by the results of EMSAs performed with recombinant AtE2Ff, which was tested together with an excess of AtDP proteins. As shown in Fig. 3F, despite being truncated in the middle of the second DNA-binding domain, the His-AtE2Ff protein can still bind to the E2F probe by itself and addition of both GST-AtDPa or GST-AtDPb does not affect its DNA binding efficiency. Moreover, as mentioned before, recombinant AtDPa alone gives rise to protein-DNA complexes of slower mobility if compared with those obtained with His-AtE2Ff and coinubcation of GST-AtDPa and His-AtE2Ff does not generate new complexes of intermediate mobility, further ruling out the formation of heterodimers between these proteins. As seen in Fig. 3, in several of these EMSAs multiple retarded DNA-protein complexes are formed, which could indicate either the binding of multimeric protein complexes or the presence of truncated isoforms of the recombinant proteins. To verify this point, the purified AtE2F proteins produced in E. coli were subjected to Western blot analyses performed with anti-histidine tag antibodies. Although all the samples contained a major recombinant protein of the expected molecular mass, minor amounts of truncated products were also detected (data not shown) and it is likely that they could form smaller protein-DNA complexes.
FIG. 3. DNA binding features of the AtE2F proteins. All the EMSAs show the specific binding of the AtE2Fs to a canonical E2F binding site (EC), which is not competed by a 30–200-fold excess of unlabeled mutated probe (EM) as reported. The presence and the relative amount of the recombinant proteins included in the various samples are indicated schematically by bars on the top of each panel. A, His-E2Fa binds specifically the E2F site only in the presence of either GST-AtDPa (left panel) or GST-AtDPb (right panel). The DNA binding capacity of the His-E2Fa-GST-AtDPa complex increases with higher amounts of the AtE2Fa protein, and, conversely, the binding of the His-E2Fa-GST-AtDPb complex is dependent on the amount of GST-AtDPb included in the sample. B, GST-AtE2Fb binds specifically the E2F site together with GST-AtDPb. C, His-AtE2Fc needs the presence of either one of the GST-AtDP proteins to bind specifically the canonical E2F site. D and E, His-AtE2Fd and His-AtE2Fe proteins bind specifically and efficiently the E2F cis-element by themselves in the absence of GST-AtDP proteins. F, AtE2Ff binds specifically the canonical probe and also GST-AtDPa alone can bind the E2F site with low efficiency, giving rise to a protein-DNA complex with slower mobility. Inclusion of both His-AtE2Ff and GST-AtDP proteins in the same samples does not form a protein-DNA complex of intermediate mobility, thus confirming the independent binding of His-AtE2Ff to the canonical DNA sequence.
Arabidopsis protoplasts. To the background activity of the pBI221-E2F reporter construct alone (control, /H11002 reported as in coexpression of the AtE2Fd, AtE2Fe, or AtE2Ff effectors, with or without AtDP effectors. The results of at least six independent experiments are reported as in A. Error bars indicate the standard deviation.

FIG. 4. Transactivating potential of AtE2Fa, AtE2Fb, or AtE2Fc and inhibiting activity of AtE2Fd, AtE2Fe, or AtE2Ff proteins in Arabidopsis protoplasts. A, transactivation of the E2F-responsive GUS construct pBI221-E2F by the various AtE2Fs alone (−AtDPs) or together with the AtDPs (+AtDPa or +AtDPb). The results of at least five independent transfections are expressed as the average -fold activation relative to the background activity of the pBI221-E2F reporter construct alone (control, −AtDPs). B, inhibition of the AtE2Fb transactivating activity by coexpression of the AtE2Fd, AtE2Fe, or AtE2Ff effectors, with or without AtDP effectors. The results of at least six independent experiments are expressed as average -fold activation, are described in Fig. 4.

Discussion

The E2F Family of Arabidopsis Includes Both Activators and Inhibitors of E2F-dependent Gene Expression—Having established the distinctive DNA binding requirements of the two groups of AtE2F proteins, it was interesting to verify whether any of these transcription factors could act as a transcriptional activator in plant cells. For this analysis, effector plasmids expressing the various AtE2F and AtDP proteins were obtained by placing the corresponding cDNA sequences under the control of the double 35 S gene promoter of cauliflower mosaic virus (CaMV) in the expression plasmid pFF19 (25). As previously described for the characterization of the DcE2F protein, the pBI221-E2F reporter gene used for these assays consisted of the GUS gene placed under the control of a minimal −67 bp CaMV 35 S promoter fused to an upstream DNA fragment containing six consecutive canonical E2F cis-elements (12). Arabidopsis protoplasts obtained from a suspension cell culture were transiently transfected with the E2F-responsive GUS construct alone or in combination with the various AtE2F and AtDP effectors and at least five independent experiments were performed for each sample combination. In contrast to the results previously observed with carrot protoplasts isolated from somatic embryos, the transient expression in Arabidopsis protoplasts of the pBI221-E2F reporter gene alone yielded a moderate level of GUS expression, which suggests that endogenous E2F activities in these plant cells are sufficient to activate the minimal promoter containing six E2F binding sites (data not shown). In each experiment, the GUS activity measured after cotransfection of the pBI221-E2F reporter gene together with the various combinations of effectors was then directly compared with the background GUS activity of the pBI221-E2F construct alone and the cumulative results, expressed as average -fold activation, are described in Fig. 4.

These data show that transient expression of AtE2Fb, either alone or together with the AtDPs, can give the strongest transactivation of the pBI221-E2F construct. Cotransfection of just the AtE2Fb effector is sufficient to increase GUS expression approximately 3-fold, but coexpression with AtDPa or AtDPb transactivates the E2F-responsive gene over 5-fold on average. On the other hand, expression of AtE2Fa and AtE2Fc gives weaker transactivation and, surprisingly, in the case of AtE2Fa this seems to occur only when AtDPa is also expressed. Transient expression of AtE2Fc together with either AtDPs can transactivate the E2F-responsive construct by −3-fold on average, whereas AtE2Fa with AtDPs can yield only a nearly 2-fold transactivation. Nevertheless, the overall results show that the three members of the first group of AtE2F proteins, although with different efficiencies, can transactivate the E2F-responsive promoter in our transient assay and their activity is strongly dependent on the presence of at least one of the AtDP partners. On the other hand, as shown in Fig. 4A, cotransfection of Arabidopsis protoplasts with the AtE2Fd, AtE2Fe, or AtE2Ff effectors, either alone or in combination with the AtDPs or AtDPb effectors, cannot transactivate the pBI221-E2F construct but, in contrast, it appears to lower the level of the background GUS activity. These results suggest that the three members of the second group of AtE2F proteins are unlikely to possess activation domains but can be targeted in vivo to the E2F-responsive promoter, where they are able to compete with the endogenous E2F activities for DNA binding. To further confirm this hypothesis, the AtE2Fd, AtE2Fe, and AtE2Ff effectors were coexpressed with AtE2Fb, the strongest activator, either alone or together with AtDPs, to verify whether they can compete with this factor for DNA binding and transactivation. As shown in Fig. 4B, coexpression of these AtE2Fs together with AtE2Fb lowers the transactivating potential of this transcription factor in Arabidopsis protoplasts, and this effect, which is particularly marked with AtE2Fd, demonstrates that these novel AtE2Fs can inhibit E2F-dependent activation of gene expression in plant cells.

Discussion

The discovery of at least six E2F genes in A. thaliana, all of which are differentially expressed during cell cycle progression, suggests that, in plant cells, like in mammalian cells, the activity of the pRB/E2F pathway relies on the concerted regulation of several E2F members. Furthermore, according to their structural and functional characteristics, these six AtE2Fs can be divided into two distinctive groups. The AtE2Fa, AtE2Fb, and AtE2Fc proteins possess all the typical regions that are conserved in other plant E2Fs and are able to bind efficiently to a E2F site forming heterodimers with AtDP proteins. Addition-
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ally, as previously described for carrot DeE2F (12), these three AtE2Fs can transactivate a synthetic E2F-responsive promoter in plant cells, confirming their ability to act as positive regulators of gene expression. However, these studies have shown that the three members of the first group possess different transactivating capabilities. Even though AtE2Fa is able to bind canonical DNA target sites interacting in vitro with either AtDPa or AtDPb, it can transactivate slightly the E2F-responsive reporter gene only when it is coexpressed with AtDPs. In this respect, it is known that in human cells the activity of E2F4 and E2F5, which lack NLS sequences, is regulated at the level of subcellular localization. Coexpression of E2F4 together with human DP2, which possess a functional NLS, but not with DP1, has been shown to enable its nuclear translocation (27, 28) and its transactivation of a E2F-responsive reporter construct (29, 30). Concerning the Arabidopsis E2F-DP complexes, it is not known whether the activity of the various AtE2F and AtDP proteins is regulated at the level of their subcellular localization but it is interesting to note that whereas AtE2Fa possesses a putative nuclear localization signal in its N terminus, this sequence is only partially conserved in the AtE2Fb and AtE2Fc proteins. It remains to be confirmed whether this region is actually involved in the nuclear targeting of these proteins.

In this study we have also demonstrated that Arabidopsis cells possess three novel and remarkable E2F members, called AtE2Fd, AtE2Fe, and AtE2Ff, that contain duplicated DNA-binding domains. This feature enables these proteins to recognize a consensus E2F site independently of a DP partner, and their DNA binding specificity proves that these factors are actual components of the Arabidopsis pRB/E2F pathway. However, although they are likely to be targeted to the nucleus, these AtE2Fs cannot activate gene expression; instead, they appear to be able to interfere with the activity of the other AtE2F members, possibly by competing for the same DNA target sites. This effect is particularly clear with the AtE2Fd effectors and is less pronounced with AtE2Ff. The latter factor, however, lacks part of the second DNA-binding domain, and EMSA analyses indicated that it binds less efficiently to the canonical E2F site. Furthermore, AtE2Ff lacks also the putative NLS sequence, which is located near the C terminus of both AtE2Fd and AtE2Fe, and its translocation to the nucleus could occur differently.

In mammalian cells, depending on their interaction with pocket proteins, the various E2Fs can behave as activators or as repressors of gene transcription. The remarkably different pattern of expression of the various AtE2F genes during the passage from the G1 to the G2 phase of cell cycle indicates that also in Arabidopsis cells at least some of the E2F activities are likely to be involved in both activation or repression of S phase-specific gene expression. This possibility has been already suggested for the carrot DeE2F protein, which is expressed ubiquitously in all plant tissues (12). A dual function of the plant E2F activities has also been suggested by the recent studies of two plant E2F-regulated promoters. In both the tobacco RNR2 promoter and the Nicotiana benthamiana PCNA promoter, two distinct E2F binding sites have been identified, and in both promoters the functional disruption of the downstream site has been shown to increase the transcriptional activity in either cycling cells or in mature leaves, respectively (31, 32).

The three AtE2Fs of the first group are good candidates for pocket protein-mediated control of E2F activity because they possess a conserved pRBR-binding region in their C terminus. In this context, AtE2Fa and AtE2Fb have been recently shown to interact with pRBR proteins in a yeast two-hybrid system (16). Additionally, wheat TmE2F and tobacco NtE2F have been shown to bind plant pocket proteins and, in the case of the wheat factor, this interaction is strictly dependent on the presence of the conserved C-terminal region (13, 14). These findings contribute to link the activity of the plant E2Fs to the mode of action of plant DNA viruses, which encode proteins that interact with pRBR proteins (33). On the other hand, the AtE2Fd, AtE2Fe, and AtE2Ff proteins lack the pRBR-binding domain and therefore are unlikely to be subjected to a control by pocket proteins. A similar situation is seen with the mammalian E2F6 protein, which is also not regulated by pocket proteins. However, unlike the three AtE2Fs of the second group, E2F6 binds a DP partner and requires this interaction to recognize efficiently E2F consensus sites. Moreover, E2F6 has been shown to actively repress gene expression by interacting directly with polycomb group proteins, which recognize specifically its marked box region (20). The AtE2Fd, AtE2Fe, and AtE2Ff proteins do not contain a marked box region, and, although the first two members of this group possess discrete C-terminal regions that could potentially harbor unknown functional domains, the AtE2Ff protein is truncated in the second DNA-binding domain and does not appear to possess other discrete regions outside those potentially involved in DNA recognition. Hence, the AtE2Ff protein and possibly the other AtE2Fs of the second group might not be able to recruit repressing activities to the E2F-regulated promoters, as in the case of human E2F6, but could act as dominant inhibitors of E2F-dependent gene activation by simply blocking the access of other AtE2Fs to shared DNA target sites.

Although the distribution of the various AtE2F proteins in Arabidopsis cells remains to be verified, considering their functional features and in view of the expression pattern of their transcripts, we can envision a cascade of events that could contribute to the regulation of the pRB/E2F pathway during the cell cycle in plants. More specifically, the peak of accumulation of the AtE2Fb transcripts at the G1/S transition and their constitutive distribution during cell cycle progression suggest that this factor, similarly to the mammalian E2F4 and E2F5 proteins, could be a key target of the pRBR proteins in early S phase. On the other hand, we speculate that AtE2Fa, like the mammalian E2F1, E2F2, and E2F3 factors, could be a direct and specific activator of S phase genes, whereas AtE2Fc could have important regulatory functions during both the S phase and the following G2 phase. Finally, the AtE2F members of the second group are clearly adding a new level of complexity to the control of E2F-dependent gene expression by possibly inhibiting, in different phases of the plant cell cycle, the DNA binding capacity of the first group of AtE2Fs. In this respect, it is worth noting that in mammalian cells overexpression of a partial E2F protein containing only the DNA-binding domain or introduction of peptides that antagonize E2F DNA binding have been shown to trigger apoptosis in a p53-independent manner (34–36). Whether or not any of the AtE2F members is involved in the control of apoptosis in plants, it is evident, from the data obtained so far, that they are likely to be complex regulators of the plant cell cycle. Future investigations will help to verify the proposed model of AtE2F activity and will reveal whether in plants, like in animals, these transcription factors are involved in the control of other cellular processes and/or can regulate unique plant developmental programs.

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