Characterization of two distinct DP-related genes from *Arabidopsis thaliana*[1]

Zoltán Magyar[a,b], Ana Atanassova[a], Lieven De Veylder[a], Stéphane Rombauts[a], Dirk Inzé[a,*]

[a] *Vakgroep Moleculaire Genetica, Departement Plantgenetica, Vlaams Interuniversitair voor Biotechnologie (VIB), Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium*
[b] *Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, H-6726 Szeged, Hungary*

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Abstract E2F/DP complexes play a pivotal role in the regulation of the G1/S transition in animals. Recently, plant E2F homologs have been cloned, but DP-related sequences have not been identified so far. Here we report that *Arabidopsis thaliana* contains at least two different DP-related genes, *AtDPa* and *AtDPb*. They exhibit an overall domain organization similar to that of their animal counterparts, although phylogenetic analysis demonstrated that they form a separate subgroup. AtDPs efficiently heterodimerize in vitro with the *Arabidopsis* E2F-related proteins, AtE2Fa and AtE2Fb through their dimerization domains. *AtDPa* and *AtE2Fa* are predominantly produced in actively dividing cells with highest transcript levels in early S phase cells.

Key words: DP; E2F; G1/S transition; *Arabidopsis thaliana*

1. Introduction

The G1/S transition is an important checkpoint of the eukaryotic cell division cycle, in which the cells decide to continue or to stop dividing. In animals, the E2F/DP heterodimeric transcription factor plays a central role in the regulation of this control point through integration of the activities of important regulators of the cell cycle with the transcription apparatus [1–3]. One of the best characterized regulators is retinoblastoma (Rb) and related pocket proteins p107 and p130 that bind and negatively regulate the transcriptional activity of E2Fs [4]. Rb-E2F complexes are formed mainly in quiescent or early G1 cells, where they act as transcriptional repressor because Rb masks the E2F transactivation domain. They can also repress transcription due to the ability of pocket proteins to recruit histone deacetylase at certain promoters [5]. Phosphorylation of Rb proteins by G1-specific cyclin-dependent kinases (CDKs) releases the E2F heterodimer from the Rb in late G1 to S phase [6]. The resulting 'free E2F' can induce the expression of many genes implicated in cellular proliferation, including cell cycle regulators and enzymes required for DNA synthesis. To date, at least six E2F and two DP proteins have been identified from mammals. The E2Fs and DPs are distantly related proteins, which show weak homology in their DNA-binding and heterodimerization domains, necessary for the recognition of the consensus E2F-binding site (TTTc/gGCGCc/g) [7–10].

The animal DP-1 and DP-2 proteins are highly homologous (approximately 70%) with significant sequence differences being observed outside their DNA-binding and dimerization domains [11]. Human DP proteins have very similar biochemical and functional properties as they both form functional heterodimers with any E2F family member [12–14]. However, mammalian DP-1 and DP-2 differ in their ability to localize to the nucleus because only some splicing forms of DP-2 contain nuclear localized signal (NLS) motifs [15–17] and the production of DP-2 is more tissue-restricted than that of DP-1 [18].

Although plants have unique developmental strategies, evidence is accumulating that the G1/S control point in plants is at least partly under the control of regulators similar to those found in animals, such as D-type cyclins [19], Rb-related proteins [20], and the most recently identified E2F-like proteins [21–23]. The existence of plant E2F homologous proteins predicts the presence of DP-related proteins in plants as well. Recent data from the molecular characterization of carrot DcE2F strongly supports this view because its high-affinity binding to the canonical DNA-binding sites and the ability to transactivate either in plant or in human cells were only observed in the presence of human DP-1 proteins [21].

Here, we show that the model plant *Arabidopsis thaliana* contains at least two distinct DP-related genes (*AtDPa* and *AtDPb*). We demonstrate that the in vitro formation of AtDP–AtE2F complexes depends on the presence of their dimerization domains. Moreover, we show that the *AtDPa* and *AtE2Fa* genes are transcribed in a cell cycle-dependent manner, being most abundant in early S phase cells.

2. Materials and methods

2.1. Yeast two-hybrid experiments

For library screening, vectors and strains (HF7c) were provided with the Matchmaker two-hybrid system (Clontech). The dimerization and DNA-binding domains of the AtE2Fa (amino acids 226–356) were amplified by polymerase chain reaction (PCR) and subcloned

*Corresponding author. Fax: (32)-9-264 5349.
E-mail: diinz@genegenp.rug.ac.be

[1] The sequences reported in this paper have been deposited in the GenBank database under accession numbers AJ294531, AJ294532, AJ294534, and AJ294533 for AtDPa, AtDPb, AtE2Fa, and AtE2Fb, respectively.

Abbreviations: CDK, cyclin-dependent serine/threonine protein kinase; HA, influenza hemagglutinin; NLS, nuclear localized signal; Rb, retinoblastoma; RT-PCR, reverse transcription-polymerase chain reaction

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in-frame with the GAL4 DNA-binding domain of pGBT9 (Clontech) to create the bait plasmid pGBTE2FaBD. Screens were performed as described previously [24].

A second library screening was performed with the AtE2Fb construct (pGBTE2Fb-Rb) lacking the Rb-binding domain (amino acids 1–385). Plasmids from interacting clones were isolated and sequenced.

For the yeast two-hybrid interaction experiments, a number of yeast two-hybrid prey (in pAD-GAL424) plasmids were created by PCR amplification of fragments from the *AtDPa* (DPa^{1-292}, DPa^{1-142}, DPa^{42-142}, DPa^{42-292}, $DPa^{121-292}$, $DPa^{121-213}$, and $DPa^{172-292}$) and *AtDPb* (DPb^{1-385}, DPb^{1-193}, $DPb^{182-263}$, and $DPb^{182-385}$) genes and confirmed by sequencing. Different combinations between bait (pGBTE2FaBD, pGBTE2Fb-Rb, or $pGBTE2Fb^{1-127}$) and prey constructs were transformed into yeast cells and assayed for their ability to grow on His^- minimal media after 3 days of incubation at 30°C. Bait plasmids co-transformed with empty pAD-GAL424 and prey plasmids co-transformed with empty pGBT9 were assessed along as controls for the specificity of the interaction.

2.2. PCR amplification of AtDPb

Based on available sequence data of putative plant DP-related partial clones from the databank (soybean DP (AI939068), tomato DP (AW217514), and cotton DP (AI731675)), three oligonucleotides, corresponding to the most conserved part of the DNA-binding and E2F heterodimerization domains (MKVCEKV, LNVLMAMD and FNSTPFEL), were synthesized and designated A (ATAGAATTCATGAAAGTTTGTGAAAAGGTG), B (ATAGAATTCCTGAATGTTCTCATGGCAATGGAT) and C (ATAGGATCCCAGCTCAAAAGGAGTGCTATTGAA), respectively. PCR was performed on an *Arabidopsis*/yeast two-hybrid suspension culture cDNA library. The PCR products were purified, digested with *Eco*RI and *Bam*HI, and ligated into pCR-XL-TOPO vector (Invitrogen). The cloned inserts were sequenced by double-stranded dideoxy sequencing.

2.3. Construction of AtDP and AtE2F mutants, in vitro transcription–translation system and immunoprecipitation

Influenza hemagglutinin (HA)-tagged versions of the wild-type and mutant *AtE2Fa* and *AtE2Fb* were constructed by cloning into the pSK plasmid (Stratagene) containing the HA-tag (kindly provided by L. Bakó, Szeged, Hungary). The *AtE2F* mutants, namely $AtE2Fa^{1-420}$, $AtE2Fa^{162-485}$, and $AtE2Fb^{1-385}$, were obtained by PCR and cloned into the *Eco*RI and *Bam*HI sites of HA-pSK. The *c-myc*-tagged versions of wild-type and AtDP mutants ($AtDPa^{1-292}$, $AtDPa^{121-292}$, $AtDPa^{1-142}$, $AtDPa^{172-291}$, $AtDPa^{121-213}$ and $AtDPb^{1-385}$, $AtDPb^{182-385}$, $AtDPb^{1-263}$, $AtDPb^{1-193}$, and $AtDPb^{182-263}$) were generated by PCR and cloned into the *Eco*RI and *Pst*I sites of the pBluescript plasmid (Stratagene) containing a double *c-myc* tag. All cloning steps were carried out according to standard procedures, and the reading frames were verified by direct sequencing.

In vitro transcription and translation experiments were performed using the TNT T7-coupled wheat germ extract kit (Promega) primed with appropriate plasmids for 90 min at 30°C. For immunoprecipitation, 10 μl of the total in vitro translated extract (50 μl) was diluted at 1:5 in Nonidet P40 buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin/aprotinin/pepstatin) and incubated for 2 h at 4°C with anti-*c-myc* (9E10; BabCo) or anti-HA (16B12; BabCo) antibodies. Protein-A-Sepharose (40 μl 25% (v/v)) was added and incubated for 1 h at 4°C, then the beads were washed four times with Nonidet P40 buffer. Immune complexes were eluted with 10 μl 2× sodium dodecyl sulfate (SDS) sample buffer and analyzed by 10% or 15% SDS–PAGE and by autoradiography.

2.4. RNA isolation and reverse transcription- (RT-) PCR analysis

A. thaliana (L.) Heynh. cell suspension cultures were maintained as described previously [25]. The cells were partially synchronized by the consecutive addition of aphidicolin (5 μg/ml) and propyzamide (1.54 μg/ml). The aphidicolin block was left for 24 h. The cells were washed for 1 h in B5 medium before the addition of propyzamide. Samples were taken at the end of the 24 h aphidicolin block, at the end of a 1 h washing step, and at 1, 2, 3, and 4 h after the addition of propyzamide to the culture medium. Total RNA was isolated from the *Arabidopsis* cell suspension culture according to Magyar et al. [26] and with the Triazol reagent (Gibco/BRL) from different organs.

Semi-quantitative RT-PCR amplification was carried out on reverse-transcribed mRNA, ensuring that the amount of amplified product stayed in linear proportion to the initial template present in the reaction. 10 μl from the PCR was transferred onto Hybond-N^+ membrane, hybridized to fluorescein-labeled gene-specific probes (GeneImages random prime labeling module; Amersham Pharmacia Biotech), detected with the CDP-Star detection module (Amersham), and visualized by short exposure to Kodak X-OMAT autoradiography film.

The following primer pairs (forward and reverse) were used for the amplification: 5′-ATAGAATTCATGTCCGGTGTCGTACGA-3′ and 5′-ATAGGATCCCACCTCCAATGTTTCTGCAGC-3′ for AtE2Fa (AJ294533); 5′-ATAGAATTCGAGAAGAAAGGGCAATCAAGA-3′ and 5′-ATACTGCAGAGAAATCTCGATTTCGACTAC-3′ for AtDPa (AJ294531); 5′-GCCACTCTCATAGGGTTCTCCATCG-3′ and 5′-GGCATGCCTCCAAGATCCTTGAAGT-3′ for Arath;CDKA;1 (X57839); 5′-GGGTCTTGGTCGTTTTACTGTT-3′ and 5′-CCAAGACGATGACAACAGATACAGC-3′ for Arath;CDKB1;1 (X57840); 5′-ATAAACTAAATCTTCGCTGAA-3′ and 5′-CAAACGCGGATCTGAAAAACT-3′ for histone H4 (M17132); 5′-TCTCTCTTCCAAATCTCC-3′ and 5′-AAGTCTCTCACTTTCTCACT-3′ for ROC5 (*AtCYP1*, U072676) [27]; 5′-CTAAGCTCTCAAGATCAAAGGCTTA-3′ and 5′-TTAACATTGCAAAGAGTTTCAAGGT-3′ for actin 2 gene (U41998) [28].

3. Results

3.1. Isolation of Arabidopsis DP-related cDNA clones

Previously, we have cloned two *Arabidopsis* E2F-related cDNA clones (AJ294534 and AJ294533). They share the highest homology to each other and to other recently identified plant E2Fs (tobacco NtE2F [23], wheat TmE2F [22], and carrot DcE2F [21]). The domain organization of the two AtE2Fs is analogous to other E2Fs including the DNA-binding domain, dimerization domain, marked box, and putative Rb-binding motif (data not shown). Based on their sequential features, we could not classify them as either animal E2F1–3 or E2F4/5 homologs, therefore we named them *AtE2Fa* and *AtE2Fb*.

The observed high conservation in the heterodimerization

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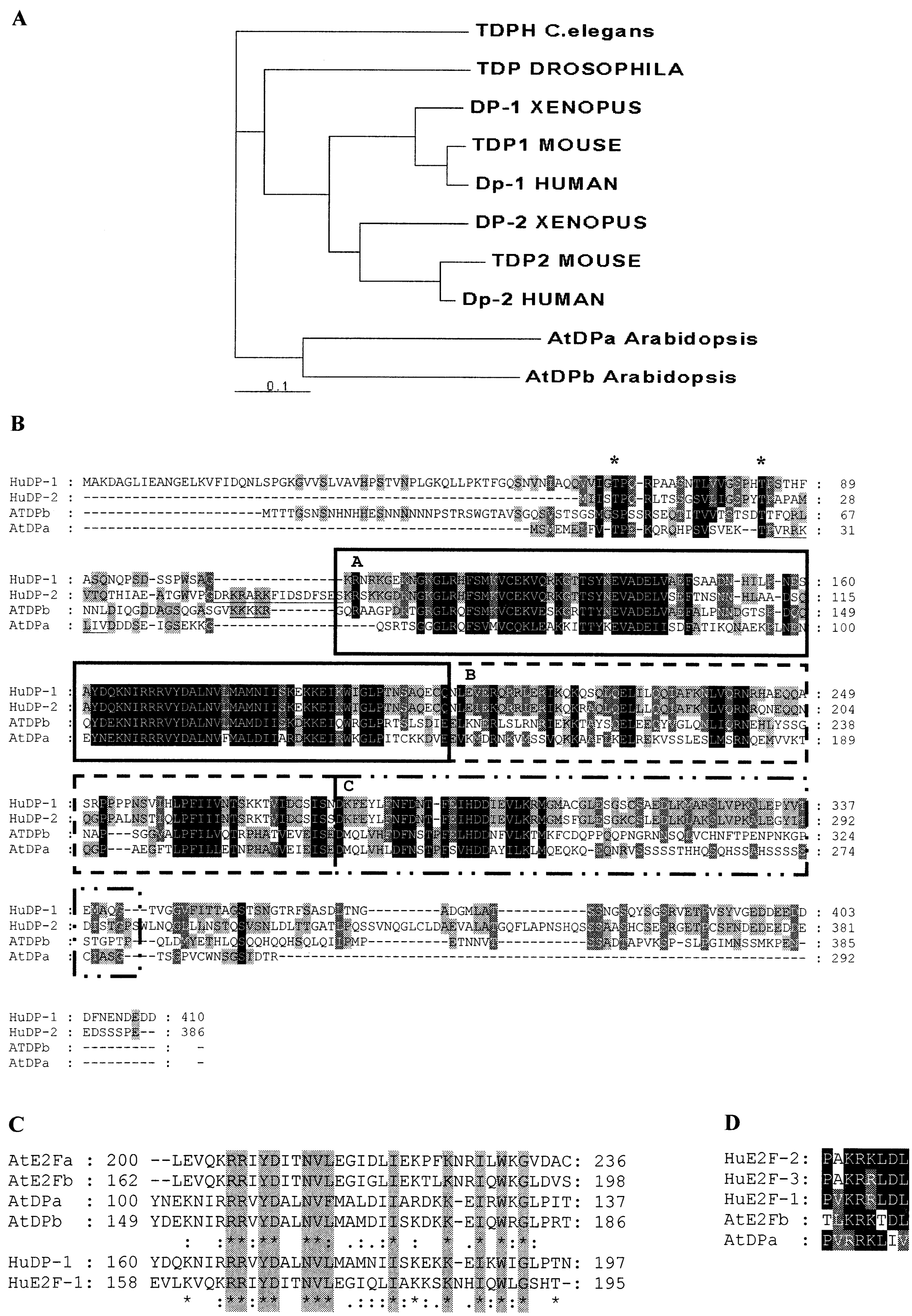
Fig. 1. Comparative analysis of AtDPa and AtDPb protein sequences. A: The phylogenetic tree describes the relationship between *Arabidopsis* DP-related proteins and animal DPs, including *Drosophila* TDP (Q24318), *Caenorhabditis elegans* TDPH (Q22703), *Xenopus* DP-1 (AAB33538) and DP-2 (AAB33539), mouse TDP-1 (Q08639) and TDP-2 (Q64163), and human DP-1 (Q14186) and DP-2 (Q14188). The phylogenetic tree was made with the local version of ClustalsW [37] based on the multiple alignment and the graphical output was prepared using the local version of TreeView [38]. Scale bar represents 10% estimated difference in amino acid sequences. B: Predicted amino acid sequence of AtDPs aligned with human HuDP-1 and HuDP-2. Numbering corresponds to the human and *Arabidopsis* amino acid sequences; dashes represent gaps introduced to allow for the best alignment. Identical and similar residues appear with black and gray background, respectively. The DNA-binding domain (A), dimerization domain (B) and the downstream element (C) are boxed. Asterisks mark the CDK phosphorylation sites in AtDPa; the NLS site of HuDP-2 and the potential NLS sites of AtDPs are underlined. C: Amino acid sequence alignment of AtDPs and AtE2Fs in regions of highest similarity and compared to the corresponding domains of human HuE2F-1 and HuDP-1. Asterisks mark conserved and colons similar amino acid residues. Identical residues are indicated by shading. D: Conserved motifs of the cyclin A/CDK2-binding region in human HuE2F-1, HuE2F-2, and HuE2F-3 aligned with the similar motifs of AtDPa and AtE2Fb. Residues highlighted in black are conserved, and those in gray are similar.

domains of the plant E2F homologs indicated that they could interact with plant DP-related proteins, which prompted us to try to identify their dimerization partners using the yeast two-hybrid strategy. Because the full-length AtE2Fa and AtE2Fb sequences could not be used as baits, due to self-activation, only parts of the proteins were utilized (see Section 2). The two AtE2F baits were used in a functional screening of the *Arabidopsis* cDNA library generated from cell suspensions. The screening with pGBTE2Fb-Rb resulted in the identification of a positive clone that turned out to be the AtE2Fb itself. The pGBTE2FaBD screen yielded a clone containing an approximately 1.1 kb cDNA insert showing considerable homology with animal DP sequences. The full-length cDNA of *AtDPa* isolated by rapid amplification of 5′ cDNA ends


A
TDPH C.elegans
TDP DROSOPHILA
DP-1 XENOPUS
TDP1 MOUSE
Dp-1 HUMAN
DP-2 XENOPUS
TDP2 MOUSE
Dp-2 HUMAN
AtDPa Arabidopsis
AtDPb Arabidopsis
0.1
B
HuDP-1
HuDP-2
ATDPb
AtDPa
C
AtE2Fa : 200 --LEVQKRRIYDITNVLEGIDLIEKPFKNRILWKGVDAC: 236
AtE2Fb : 162 --LEVQKRRIYDITNVLEGIGLIEKTLKNRIQWKGLDVS: 198
AtDPa : 100 YNEKNIRRRVYDALNVFMALDIIARDKK-EIRWKGLPIT: 137
AtDPb : 149 YDEKNIRRRVYDALNVLMAMDIISKDKK-EIQWRGLPRT: 186
HuDP-1 : 160 YDQKNIRRRVYDALNVLMAMNIISKEKK-EIKWIGLPTN: 197
HuE2F-1: 158 EVLKVQKRRIYDITNVLEGIQLIAKKSKNHIQWLGSHT-: 195
D
HuE2F-2: PAKRKLDL
HuE2F-3: PAKRRLDL
HuE2F-1: PVKRRLDL
AtE2Fb : TLKRKTDL
AtDPa : PVRRKLIV

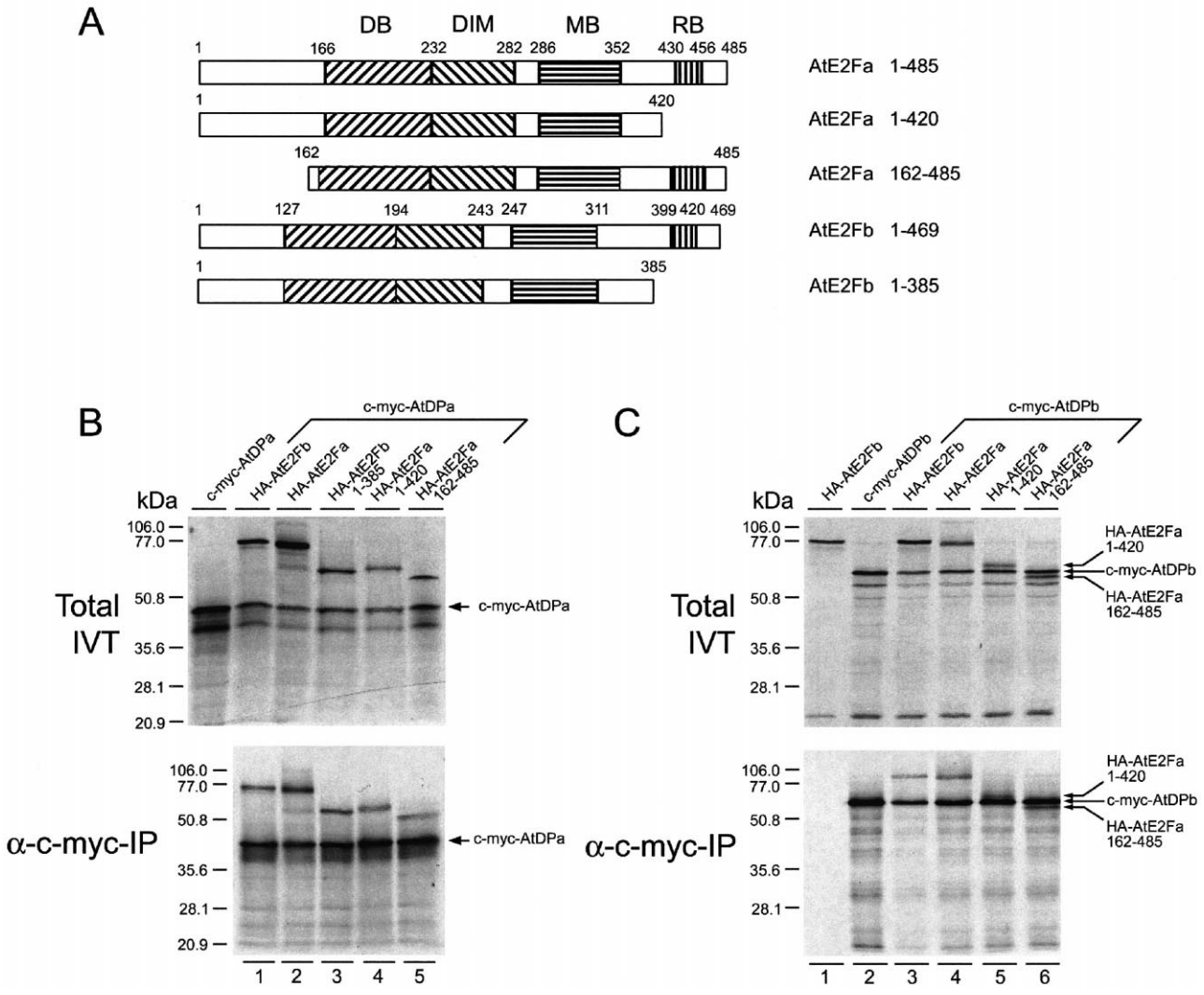


Fig. 2. AtDPa and AtDPb in vitro interactions with AtE2Fa and AtE2Fb. A: Schematic representation of the domain organization of AtE2Fa and AtE2Fb. The DNA-binding domain (DB), the dimerization domain (DIM), the marked box (MB), and the Rb-binding domain (RB) are boxed. Numbering on the right refers to the amino acid sequence contained in the different AtE2F constructs, which were used in the in vitro binding assays. The *c-myc*-tagged AtDPa (*c-myc*-AtDPa; B) and the HA-tagged AtE2Fb (HA-AtE2Fb; C, lane 1) as well as the *c-myc*-tagged AtDPb (C, lane 2) were in vitro translated and used as controls. The lower migrating proteins observed in the case of *c-myc*-AtDPa and *c-myc*-AtDPb are most probably due to initiation of translation at internal methionine codons (B and C, upper, respectively). The *c-myc*-AtDPa and the *c-myc*-AtDPb were in vitro co-translated with HA-AtE2Fb (B, lane 1, and C, lane 3), HA-AtE2Fa (B, lane 2, and C, lane 4), the C-terminal deleted form of HA-AtE2Fb (B, lane 3), HA-AtE2Fa$^{1-420}$ (B, lane 4, and C, lane 5) and the N-terminal truncated form of HA-AtE2Fa$^{162-485}$ (B, lane 5, and C, lane 6) as indicated. Numbers in the case of the mutant AtE2Fs refer to the amino acid sequence contained in these constructs. An aliquot of each sample was analyzed directly by SDS–PAGE and autoradiographed (upper). Total IVT, total in vitro translation. Another aliquot of the same samples was subjected to immunoprecipitation with anti-*c-myc* monoclonal antibodies (lower), lanes indicated by numbering. The *c-myc*-AtDPb co-migrated almost exactly with the mutant HA-AtE2Fa$^{1-420}$ (C, lane 5) and HA-AtE2Fa$^{162-485}$ (C, lane 6) in the gel system. These polypeptides as well as the position of *c-myc*-AtDPa and *c-myc*-AtDPb proteins are marked by arrows. Molecular mass markers are indicated.

encoded a protein of 292 amino acids with predicted molecular mass of approximately 33 kDa. The 5′ non-translated region contained a stop codon in-frame with a putative initiator methionine, indicating that the clone was full-length.

In parallel with this work, we attempted to isolate DP-related cDNA clones using a PCR-based method. Primers were designed based on the conserved regions of partial plant DP-related expressed sequence tag sequences from the databank (see Section 2). After sequence determination of the PCR-amplified inserts, it appeared that a distinct *Arabidopsis* DP homologous protein AtDPb had been identified. While the work was in progress, we found an *Arabidopsis* clone (AL162751) that contained the same sequence as our PCR fragments. We used the databank sequence to amplify the full-length AtDPb. It contained an open reading frame of 385 amino acids.

Pairwise comparison of the protein sequences encoded by the *Arabidopsis* clones with animal DP sequences showed that AtDPa was slightly more closely related to the human DP-2 protein (approximately 30.5%) than to the human DP-1 (approximately 29.7%), while the closest homolog of AtDPb is HuDP-1 (approximately 32%). However, phylogenetic analysis revealed that the two AtDPs did not group with either the DP-1 or DP-2 subfamilies, but rather formed a separate branch (Fig. 1A). Additionally, the overall amino acid identity

between the *Arabidopsis* DPs is much lower (approximately 35%) than the identity between the animal DP-1 and DP-2 proteins (approximately 60–70%).

Alignment analysis with human HuDPs revealed that both *Arabidopsis* proteins exhibit an overall domain organization close to that of animal DPs (Fig. 1B). The most conserved region was in the DNA-binding domain and a significant degree of conservation was also found within the dimerization domains. A small region of sequence similarity between the *Arabidopsis* DPs and E2Fs can be found in the second half of this domain (Fig. 1C). The C-terminal part of AtDPs also contained the homologous, so-called downstream element, whose function is unknown [14]. In contrast to mammalian DP-1 and DP-2, both *Arabidopsis* DP-related proteins were shorter in their C-terminal ends, especially the AtDPa (Fig. 1B) and neither of them contained a small sequence region with high proportion of acidic amino acids [11].

Interestingly, a potential NLS could be found in the N-terminal region of both AtDPs (KKKR or KKKK in AtDPb; amino acid residues 86–89 or 87–90, respectively, and PVRRKLIV in AtDPa amino acid residues 26–32) (Fig. 1B). However, neither of the AtDPs contained a region homologous to the E domain of HuDP-2, which had been found responsible for the nuclear localization of this DP protein [15,17]. Surprisingly, the potential NLS motif of the AtDP-2 as well as that of AtE2Fb was very similar to the cyclin A/CDK2-binding sites of human E2F1–3, which also includes the NLS site (Fig. 1D) [29,30]. Additionally, AtDPa, but not AtDPb, contained two consensus sites for CDK phosphorylation at its amino-terminal end (Fig. 1B), suggesting a regulatory role for plant CDKs in the function of this DP protein.

3.2. The AtDPa and AtDPb can efficiently interact in vitro with AtE2Fa and AtE2Fb

As a first step in comparing the biochemical properties of AtDPa and AtDPb, we tested their ability to heterodimerize with AtE2Fa and AtE2Fb. For this purpose, we used the coupled in vitro transcription–translation system in which the *c-myc*-tagged AtDPa or AtDPb was co-expressed with the HA-tagged AtE2Fa or AtE2Fb. One part of each sample was resolved by SDS–PAGE (Fig. 2B,C, upper), while another part was subjected to immunoprecipitation with monoclonal anti-*c-myc* antibodies (Fig. 2B,C, lower). In the absence of DP proteins, no AtE2F2a or AtE2F2b was precipitated by the anti-*c-myc* antibodies (data not shown; Fig. 2C, lane 1). However, both HA-AtE2F proteins co-precipitated reproducibly with *c-myc*-tagged AtDPa (Fig. 2B, lanes 1 and 2) and AtDPb (Fig. 2C, lanes 3 and 4). Identical results were obtained in a reciprocal experiment with anti-HA monoclonal antibodies (data not shown). These data revealed that both *Arabidopsis* DP-related proteins interacted in vitro with the different *Arabidopsis* E2F-related proteins. The obtained results were confirmed by two-hybrid interaction analysis. pGBTE2FaBD and pGBTE2Fb-Rb were co-transformed in an appropriate yeast reporter stain with a plasmid producing the full-length AtDPa or AtDPb protein fused to the GAL4 transactivation domain. The specific reconstitution of GAL4-dependent gene expression measured as the ability to grow in the absence of histidine confirms the interaction between the two DP and E2F proteins (Table 1).

The conserved dimerization domain of the AtE2Fs seemed to be important for the interaction with the AtDPs, because mutational analysis showed that deletion neither of the N-terminal extension nor the C-terminal part of AtE2Fa and AtE2Fb impaired the interaction with the DPs (Fig. 2B,C, lower). Similar results were obtained by two-hybrid analysis (Table 1).

To test whether the structural requirements for heterodimerization of the AtDPs were similar to those of their animal homologs, we constructed several deletion mutants of AtDPa and AtDPb (for a schematic illustration, see Fig. 3A,C), tagged with the *c-myc* epitope (Fig. 3B,D, upper). The interactions between the mutant AtDPs and AtE2Fb were analyzed in immunoprecipitation experiments with the specific anti-HA or anti-*c-myc* antibodies (Fig. 3B,D, middle and lower, respectively). As shown in Fig. 3, mutant AtDP proteins with deleted DNA-binding domain could bind sufficiently to the co-translated HA-AtE2Fb proteins (Fig. 3B, lane 2, and Fig. 3D, lane 2). No detectable interaction was found between the AtE2Fb protein and mutant DP proteins containing the complete DNA-binding domain, but lacking the putative dimerization domain (Fig. 3B, lane 3; Fig. 3D, lane 4). Thus, the N-terminal part of both AtDP proteins, including the conserved DNA-binding domain, was not sufficient for the in vitro interaction to occur. In contrast, a mutant form of AtDPb (amino acids 1–263) could bind to AtE2Fb (Fig. 3D, lane 3), indicating that the region of AtDPb between amino acids 182 and 263 was required for interaction with AtE2Fb. To confirm this hypothesis, a deletion mutant of AtDPb (182–263) was constructed and, as expected, it could bind to AtE2Fb (Fig. 3E). The requirement for the homologous dimerization domain of AtDPa for the interaction with AtE2Fb was supported by a binding assay in which the mutant AtDPa$^{172-292}$, with the N-terminal part of the dimerization domain deleted, failed to bind to AtE2Fb (Fig. 3B, lane 4, middle and lower). However, when we tested the E2F-binding

Table 1
AtDPs and AtE2Fs interaction in yeast two-hybrid assays

Baits	Preys												
	DPa$^{1-292}$	DPa$^{1-142}$	DPa$^{42-142}$	DPa$^{42-292}$	DPa$^{121-292}$	DPa$^{121-213}$	DPa$^{172-292}$	DPb$^{1-385}$	DPb$^{1-193}$	DPb$^{182-263}$	DPb$^{182-385}$	E2FaBD	pAD-GAL424
pGBT E2FaBD	+	−	−	+	+	−	−	+	−	+	+	−	−
pGBTE2Fb-Rb	+	−	−	+	+	−	−	+	−	+	+	−	−
pGBTE2Fb$^{1-127}$	−	NT	NT	NT	NT	NT	NT	−	NT	NT	NT	−	−
pGBTDPa$^{1-292}$	−	NT	NT	NT	NT	NT	NT	−	NT	NT	NT	+	−
pGBTDPb$^{1-385}$	NT	NT	NT	NT	NT	NT	NT	−	NT	NT	NT	+	−
pGBT9	−	−	−	−	−	−	−	−	−	−	−	−	−

Different combinations between AtE2Fs bait and AtDPs prey constructs were tested for growth on His$^-$ minimal media. −, no interaction; +, positive interaction; NT, not tested.

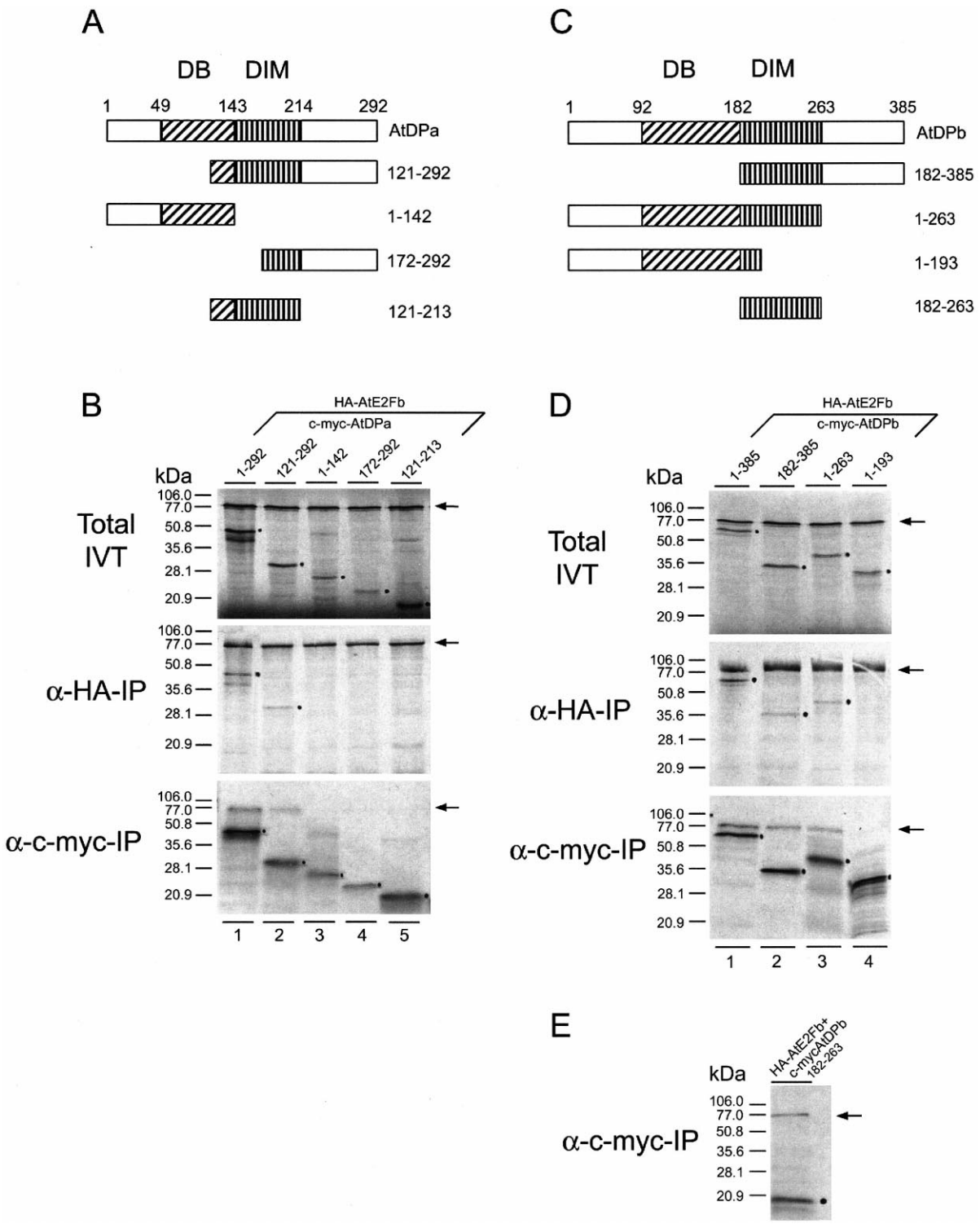


Fig. 3. Determination of protein domains in AtDPa and AtDPb required for in vitro binding to AtE2Fb. Schematic representation of AtDPa and AtDPb mutants (A and C, respectively). The DNA-binding domain (DB) and the dimerization domain (DIM) are marked by boxes. Numbering on the right side refers to the amino acid sequence contained in the different AtDP constructs, which were used in the in vitro binding assays (B, D and E). Mapping of regions in AtDPa and AtDPb required for in vitro binding to AtE2Fb (B and D, respectively). HA-AtE2Fb was co-translated with series of *c-myc*-AtDPa and *c-myc*-AtDPb mutants. An aliquot of each sample was analyzed directly by SDS–PAGE and autoradiographed (upper). Another aliquot of the same samples was subjected to immunoprecipitation with anti-HA (middle) or anti-*c-myc* (lower) monoclonal antibodies. HA-AtE2Fb was co-translated with *c-myc*-AtDPb$^{182-263}$ (E). Because of the small size of this protein, it was hardly detectable when it was directly analyzed by SDS–PAGE (data not shown). An aliquot of this sample was subjected to immunoprecipitation with anti-*c-myc* monoclonal antibodies. The *c-myc*-AtDP mutants are marked by dots. Positions of the HA-AtE2Fb proteins are indicated by arrows. Molecular mass markers are indicated.

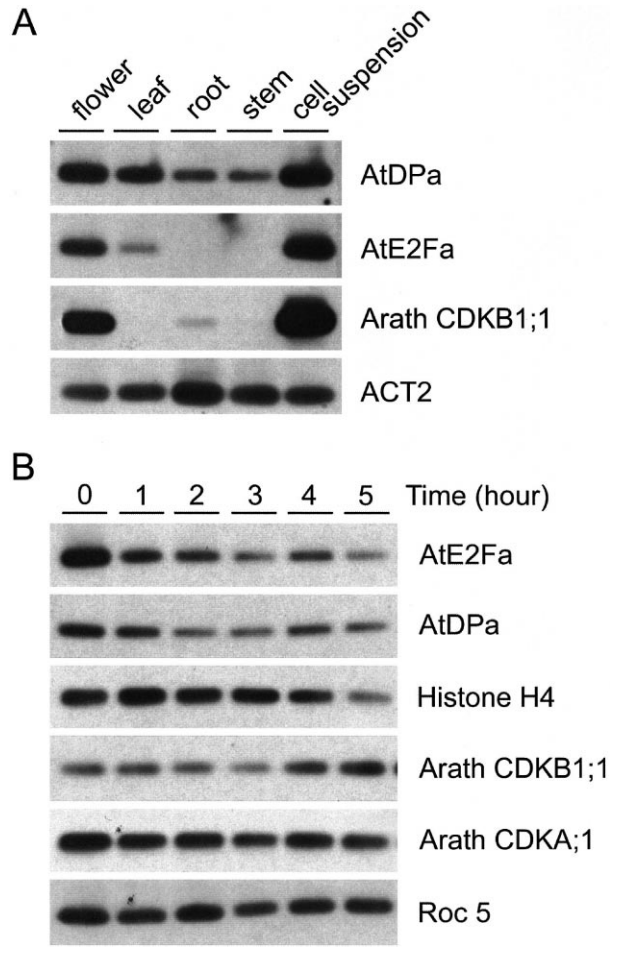


Fig. 4. Organ- and cell cycle-specific expression of *AtE2Fa* and *AtDPa*. A: Tissue-specific expression of AtDPa and AtE2Fa genes. cDNA prepared from the indicated tissues was subjected to semi-quantitative RT-PCR analysis. The Arath;*CDKB1;1* gene was used as a marker for highly proliferating tissues. The actin 2 gene (ACT2) was used as loading control. B: Co-regulated cell cycle phase-dependent transcription of *AtE2Fa* and *AtDPa*. The cDNA was prepared from partially synchronized *Arabidopsis* cells harvested at the indicated time point after removal of the cell cycle blocker was subjected to semi-quantitative RT-PCR analysis. Histone H4 and Arath;CDKB1;1 were used as markers for S and G2/M phase, respectively, and ROC5 and Arath;CDKA;1 as loading controls.

activity of the predicted dimerization domain of the AtDPa (amino acid positions 121–213), no interaction could be detected between this region and the AtE2Fb protein (Fig. 3B, lane 5). These data indicate that other carboxyl-terminal regions of AtDPa are required for the stable interaction with AtE2Fb. It is worth mentioning that the analogous region of human DP-1 had been mapped to associate with E2F1, but additional C-terminal sequences were needed for the high-affinity binding [14].

3.3. The AtDPa and the AtE2Fa genes are co-expressed in a cell cycle phase-dependent manner

The identification of the *AtDPa* in a yeast two-hybrid screen as a gene encoding an AtE2Fa-associating protein suggests that it might act cooperatively in the plant cells as a functional heterodimer. To strengthen this hypothesis, we investigated whether both genes were co-regulated at the transcriptional level. Tissue-specific expression analysis revealed that both genes were clearly up-regulated in flowers and were very strongly transcribed in actively dividing cell suspension cultures (Fig. 4A). Expression in these tissues could be a sign for the correlation between the actual proliferation activity of a given tissue and the transcript accumulation, as can be seen from the Arath;*CDKB1;1* gene. *AtDPa* transcripts were also detectable in leaf and, to a lesser extent, in root and stem tissues, whereas *AtE2Fa* transcripts were virtually undetectable in roots and stem with only slight levels of expression in leaf tissues.

Cell cycle phase-dependent gene transcription was studied using an *Arabidopsis* cell suspension that was partially synchronized by the sequential treatment with aphidicolin and propyzamide (see Section 2). The *Arabidopsis* histone H4 and the Arath;*CDKB1;1* gene were included to monitor the cell cycle progression (Fig. 4B) [31,32]. Bearing in mind the partial synchronization of the culture, it can be observed that histone H4 transcript levels peaked immediately after the removal of the inhibitor and decrease gradually thereafter (Fig. 4B). The opposite expression pattern could be observed for the Arath;*CDKB1;1* gene, illustrating that cells entered the G2-M phases with partial synchrony. Within this experimental setting, the *AtDPa* and the *AtE2Fa* genes show a very similar expression pattern. Both exhibit higher transcript accumulation before the peak of histone H4 gene expression and quickly decay in the following samples (Fig. 4B). The similarity in the expression patterns of *Arabidopsis AtDPa* and *AtE2Fa* supports the possibility that they act cooperatively as a heterodimer during the S phase.

4. Discussion

The view that the plant G1/S transition is similar in its regulation to that in animals, in which the E2F transcription factor plays a central role, has been further supported by the discovery of plant E2F-related genes. Experimental data from the molecular characterization of plant E2Fs indicate that their basic functional properties might be very close to these of animal E2Fs [21–23]. Here, we present the isolation and characterization of two plant cDNAs encoding distinct *Arabidopsis* DP-related proteins, *AtDPa* and *AtDPb*. Together with previously identified *Arabidopsis* E2F homologous genes, *AtE2Fa* and *AtE2Fb* (unpublished data), these results show that plants, like animals, contain a family of E2F and DP proteins. Because additional E2F homologs from *Arabidopsis* were identified from databanks, it is not possible to preclude that the two DPs described here will be the only ones existing in plants. Although the two plant DPs share similar domain organization with that of animal DPs, they are distinct members of the DP family because they group separately from the animal DP-1 and DP-2 proteins in the evolutionary tree. The DNA-binding domain of both *Arabidopsis* DP-related proteins is highly conserved and alignment of this region with the *Arabidopsis* E2F protein sequences revealed a small region of similarity, matching the residues conserved between animal DPs and E2Fs, which participate in the recognition of the DNA sequences [9,10]. Therefore, the *Arabidopsis* E2F- and DP-related proteins may also recognize the same DNA-binding sequences. The animal DP-1 and DP-2 behave in a similar fashion in their interaction with any E2F family member, implying that their dimerization domains are functionally equivalent [8,14]. Because the AtDPa and AtDPb are much

more distinct on primary amino acid level, it was particularly interesting to analyze their dimerization ability. Our in vitro protein–protein interaction assays demonstrated that AtDPa and AtDPb can efficiently heterodimerize with two different *Arabidopsis* E2F-related proteins. Because no significant differences could be detected between the in vitro binding activity of AtDPs towards AtE2Fs, their dimerization domains might be functionally equivalent. Furthermore, we mapped the region in AtDPa and AtDPb required for heterodimerization with AtE2Fb and found that it contains the homologous heterodimerization domain, but not the DNA-binding domains as demonstrated for animal DP proteins [12–14]. Our results also indicate that the region of AtE2Fa and AtE2Fb required for heterodimerization includes the conserved leucine zipper region, but not the Rb-binding domain and the amino-terminal extension [13]. Therefore, we propose that the structural requirements for heterodimerization of *Arabidopsis* E2F- and DP-related proteins are similar to those of their animal counterparts.

In agreement with the proposed role for plant E2Fs in the regulation of S phase progression, expression analyses have revealed that they are up-regulated during the S phase of the plant cell cycle [21–23]. Coincidentally, our partial synchronization experiment provides another clue that plant E2F homologs play a role in the S phase of the cell cycle because higher *AtE2Fa* transcript levels could be observed in the aphidicolin-treated (S phase block) cells. Although the *AtDPa* shows a more ubiquitous expression pattern in different organs than *AtE2Fa*, indicating that this regulator might not function in cell division only, they have a similar expression level during cell cycle. The similarity in the expression patterns supports the exciting possibility that they may act cooperatively as a functional heterodimer in the regulation of genes required for S phase progression. Some plant genes with putative G1/S function have been reported to contain conserved E2F-binding sites in their promoter regions, such as the tobacco ribonucleotide reductase genes [33] and Arath;*CycD3;1* gene [20]. Because the animal DP proteins participate actively in the selection of E2F-binding site [34], it will be interesting to analyze the DNA-binding properties of plant E2F heterodimers and to determine whether they have different promoter preferences.

Animal DP and E2F proteins can differ in the regulation of their subcellular localization [15,17]. Because AtDPs possess different potential NLS motifs (Fig. 1B), we suggest that they might be localized in the nucleus independently of interactions with other nuclear factors. The presence of a potential NLS motif in AtDPa (Fig. 1D) is highly intriguing as this motif is not found in animal DPs but rather in the human E2F1–3. This region is required for stable binding to cyclin A/CDK2 complexes [29,30]. In the case of the human HuE2F-1/DP-1 complex, the E2F-1 serves as an adaptor for DP-1 phosphorylation by cyclin A/CDK2 complexes and this reaction contributes to the negative regulation of E2F-1 DNA-binding and the transactivation function during S and G2 phases. Considerable evidence suggests now that plant A-type CDKs play a pivotal role in the regulation of G1/S as well as G2/M control points of the plant cell cycle [35]. Furthermore, an A-type cyclin from *Medicago sativa* (Medsa;CycA2) has recently been shown to interact in vitro with alfalfa cdc2MsA and maize pRB1 proteins, indicating that plant cyclin A/CDK complexes could also participate in the regulation of the plant Rb-E2F function [36]. This possibility is further supported by the fact that AtDPa contains consensus CDK phosphorylation sites (Fig. 1B). Whether the E2F/DP transcription factors are indeed substrates for plant cyclin A/CDK and/or other cyclin/CDK complexes and how the phosphorylation of plant DP and/or E2F proteins can influence the function of the E2F transcription factors remain to be established.

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