Characterization of wheat DP, a heterodimerization partner of the plant E2F transcription factor which stimulates E2F–DNA binding

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Abstract Recent studies suggest that the G1/S transition in plants depends on the activity of E2F transcription factors. In animal cells, E2Fs interact with DP proteins, whose identification in plants has been elusive, so far. Here we show that although an E2F-containing DNA-binding activity can be detected in plant cell extracts, purified E2F protein binds poorly to DNA. In a yeast two-hybrid screening, using wheat E2F as a bait, we have isolated a cDNA clone encoding a wheat DP (TmDP) protein. TmDP is expressed ubiquitously and exhibits a domain organization similar to animal DPs. Contrary to the specificity observed for the plant RBR/E2F interaction, human and plant E2F and DP proteins can interact in a heterologous manner. Purified TmDP protein stimulates E2F–DNA complex formation.

Key words: DP; E2F; Retinoblastoma; Plant; Transcription; G1/S cell cycle control

1. Introduction

The regulated expression of S-phase-specific genes plays a pivotal role at the G1/S transition. While in the yeast *Saccharomyces cerevisiae* this function is carried out by the Swi4/Swi6 and Mbp1/Swi6 transcription factors [1], in multicellular eukaryotes a different set of molecular components has evolved, namely, the E2F family of transcription factors, which act in coordination with the retinoblastoma (RB) family of proteins [2–4].

Recent findings support the idea that plants have evolved a G1/S transition control which relies on proteins unrelated to those in yeast but which are, at least in part, analogous to those acting in animal cells. Thus, cDNAs encoding RB-related (RBR) [5–9] and E2F [10–12] proteins have been recently cloned and characterized. Wheat E2F (TmE2F) interacts with plant RBR protein through a C-terminal amino acid motif, poorly conserved with animal E2Fs, its domain organization resembles that of the human E2F-1, -2 and -3 subgroup and its expression is cell-cycle-regulated [10], as it occurs with the human E2F-1, -2 and -3 proteins [4].

Plant E2Fs exhibit transactivation properties in yeast, mammalian cells and plant protoplasts [10–12]. The activity of E2F proteins depends, in mammalian cells, on their heterodimerization with either DP-1 or DP-2 (or DP-3, its murine homolog) proteins [13–16]. So far, isolation of plant DP cDNAs has been elusive. Here, we describe the isolation and characterization of a wheat cDNA clone encoding a DP protein (TmDP) and study the heterodimerization properties with plant and human E2Fs. We also show that TmDP stimulates the association of plant E2F to a canonical E2F binding site. These studies together with previous reports provide a complete set of tools to study the mechanisms of E2F-mediated transcriptional activation during the plant cell cycle.

2. Materials and methods

2.1. Plant cell cultures

Triticum monococcum [17], tobacco BY2 [18] and *Arabidopsis thaliana* [19] suspension cell cultures, provided by P. Mullineaux, C. Koncz and J.A.H. Murray, respectively, were used.

2.2. DNA manipulations and plasmid constructions

Standard DNA manipulations were carried out as described [20]. For in vitro transcription–translation, the full-length TmE2F and TmDP cDNAs were cloned into pBluescript SK+ (pBS^{SK+}-TmDP). Plasmids pGADE2F-1, pGADE2F-5, pACT2-DP1 and pACT2-DP2 containing human E2F-1, E2F-5, DP1 and DP2, respectively, were provided by N. La Thangue and S. de la Luna. Plasmid pGBT-TmE2F was generated by cloning the TmE2F cDNA in frame into the pGBT8 vector (Clontech), pGBT-TmE2F(1–373) by deleting the *Ssp*I–*Xho*I fragment of pGBT-TmE2F and pGBT-TmDP by cloning the TmDP cDNA in frame into the pGBT8 vector. Plasmid pGEX-TmE2F(1–373) was constructed by cloning the *Sma*I–*Ssp*I fragment from pGAD-TmE2F [10] plasmid in-frame into pGEX-KG vector (Pharmacia) and pMBP-TmDP by cloning the TmDP cDNA in frame into the pMal-c2 vector (New England Biolabs).

2.3. Purification and use of recombinant TmE2F and TmDP proteins

TmE2F and TmDP proteins, fused to glutathione-*S*-transferase (GST) and maltose binding protein (MBP), respectively, were expressed in *Escherichia coli* BL21(DE3) and purified using glutathione–Sepharose beads (Pharmacia) and amylose agarose beads (New England Biolabs), respectively. For the pull-down experiments, the full-length TmE2F cDNA was in vitro transcribed and translated in the presence of ^{35}S-methionine using the TNT kit (Promega).

2.4. Electrophoretic mobility shift assays (EMSA)

Protein extracts for DNA binding studies were prepared as described [19]. Binding reactions contained 20 mM HEPES, pH 7.9, 12% glycerol, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 1 mM $MgCl_2$, 1 μg of salmon sperm and 10 μg of protein extract or 200 ng of bacterially purified MBP-TmDP and/or GST–TmE2F(1–373) proteins, as indicated. Binding mixtures were incubated 20 min at 4°C and the DNA–protein complexes fractionated by electrophoresis though 4% polyacrylamide gels at 4°C in 0.5×TBE buffer. Synthetic oligonucleotides (Isogen Bioscience) were end-labelled with γ-^{32}P-ATP (top strand), annealed with an excess of the cold complementary bottom strand and used as binding substrates. For the supershift assays, 2 μl of the polyclonal serum against TmE2F [10] were added to the binding mixture and the incubation proceeded for 10 min at 4°C.

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2.5. Yeast two-hybrid screening and assays

Yeast cells, transformed first with plasmid pGBT-TmE2F(1–373) and, then, with the wheat cDNA library, were grown for a period of 3–8 days, and the cotransformants selected as previously described [10,21]. The interaction was corroborated by a β-galactosidase assay [22].

2.6. RNA extraction and Northern blot analysis

Total RNA from wheat cells, leaves and roots was prepared as described [23]. The RNA sample (10 μg) was denatured, fractionated in a 1.2% agarose gel plus 2.2 M formaldehyde, and transferred to a Zeta-Probe membrane (Bio-Rad). The full-length TmDP probe was labelled by random priming with α-^{32}P-dCTP.

3. Results

3.1. E2F–DNA complex formation using plant cell extracts and purified TmE2F

The remarkable homology within its putative DNA-binding domain to human E2Fs together with structural studies [24] strongly suggested to us that plant E2F might recognize DNA sequences that conform to the consensus E2F binding site TTTSSCGS [25]. To detect directly an E2F–DNA-binding activity in wheat cells we used EMSA. As DNA substrates for complex formation (Fig. 1A), we used a double-stranded oligonucleotide (E2wt) which contained the sequence TTTCGCGC, a canonical DNA sequence for human E2F-1 [26], as well as a control oligonucleotide (E2mut), which differs only in two nucleotides, critical for E2F binding [16]. A DNA–protein complex was clearly detected when the E2wt, but not when the E2mut DNA probe was used (Fig. 1B, lanes 1 and 7), indicating that complex formation depended on the presence of an intact E2F binding site. To confirm that TmE2F was present in this complex, we added to the binding assays a TmE2F antiserum [10], which produced a supershift of the DNA probe (Fig. 1B, lane 4). Since most of the E2F-containing complex was supershifted, we can conclude that either only one E2F protein exists in wheat suspension cell extracts or, perhaps more likely, that different wheat E2F species are recognized by the anti-TmE2F antibody. We also detected specific complexes with extracts prepared from *A. thaliana* and *Nicotiana tabacum* BY-2 cells (Fig. 1B, lanes 2, 3, 8 and 9), although the anti-TmE2F antiserum did not recognize efficiently the heterologous E2F species (Fig. 1B, lanes 5 and 6).

We also carried out EMSA using purified GST–TmE2F protein. Fig. 1C shows that, with increasing amounts of purified TmE2F, a single DNA–protein complex was detected with the E2wt probe. Formation of this TmE2F–DNA complex was specific since (i) it depends on an intact E2F binding site, and (ii) it is not formed when purified GST protein was added (Fig. 1C). Furthermore, an increasing molar excess of the unlabelled E2wt probe, but not of the mutated E2mut probe, competed out the preformed TmE2F–DNA complexes (Fig. 1D). Altogether, these data indicate that TmE2F binds specifically to a canonical E2F DNA-binding sequence.

In these binding studies the amount of purified E2F protein needed to efficiently form a complex was relatively high, suggesting that complex formation was not optimal, even under a wide variety of binding conditions (not shown). These observations prompted us to investigate whether wheat cells might contain a DP-like protein, which could stimulate TmE2F DNA binding.

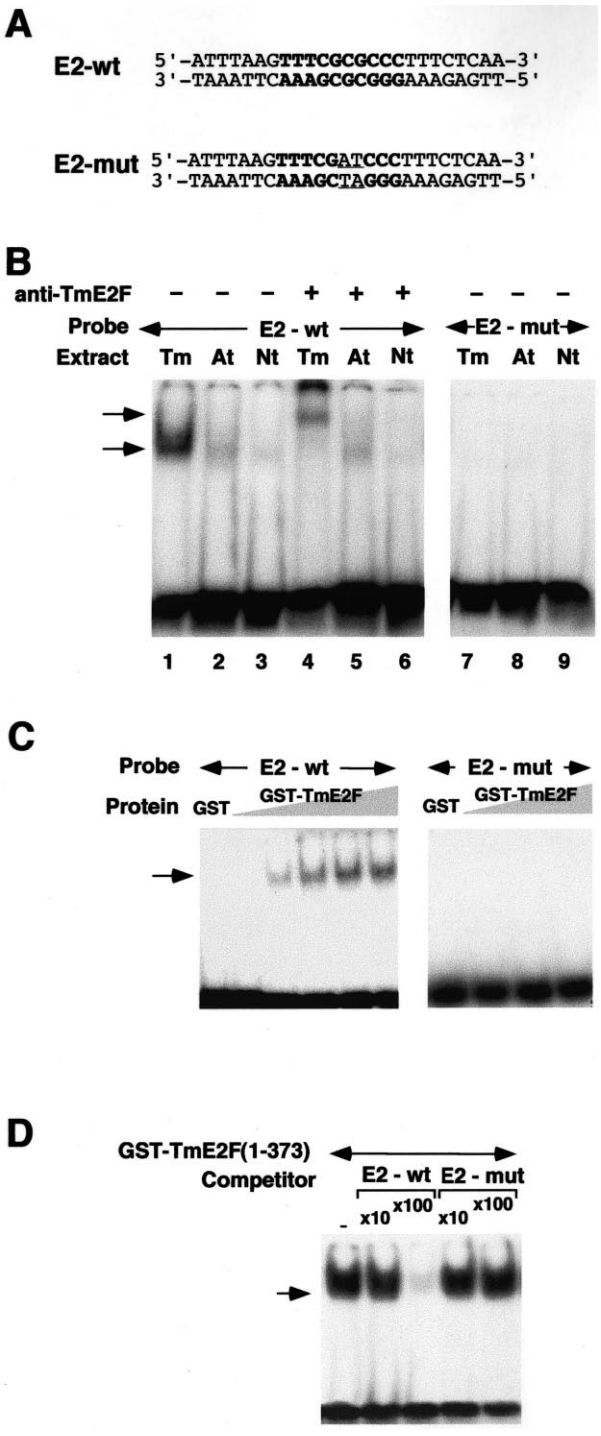

Fig. 1. DNA-binding properties of purified wheat E2F and cell extracts. A: Nucleotide sequence of the double-stranded probes used. Probe E2wt contains a canonical binding site for human E2F-1 while E2mut contains two point mutations within the core binding site. B: EMSA using wheat, *A. thaliana* and *N. tabacum* BY-2 cell extracts (10 μg per assay) and the indicated ^{32}P-labelled DNA probe. The antiserum against TmE2F (2 μl) was added to the binding mixture. C: Increasing amounts of purified GST–TmE2F fusion protein (0–2 μg) or GST alone (2 μg) were added to the E2wt or the E2mut oligonucleotides probes, as indicated. D: Specificity of complex formation on a ^{32}P-labelled E2wt probe studied by competition experiments by adding 10- or 100-fold molar excess of the unlabelled E2wt or the E2mut oligonucleotide probes.

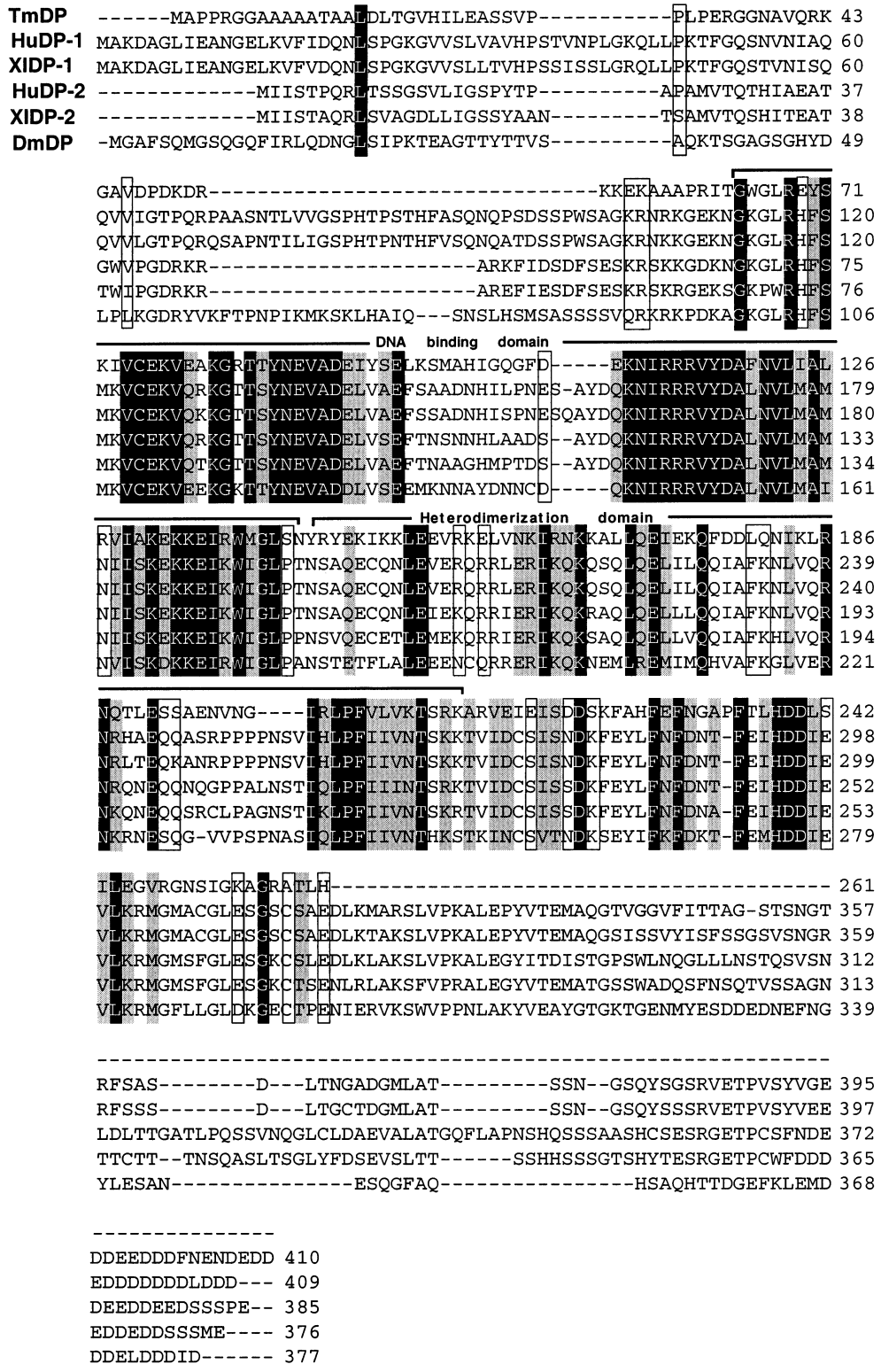

Fig. 2. Amino acid sequence alignment of TmDP with animal DP proteins. The accession number of the sequences shown are AJ271917 (*T. monococcum* DP), L23959 (Human DP-1), AAB33538 (*X. laevis* DP-1), L40386 (Human DP-2), AAB33539 (*X. laevis* DP-2) and X79708 (*D. melanogaster* DP). Invariable and conserved residues appear within black and gray boxes, respectively. Other partially conserved residues appear within an empty box.

3.2. Identification and cloning of TmDP

To identify a plant DP-like protein we screened a wheat cDNA library in a yeast two-hybrid assay using TmE2F as a bait. We chose to use a C-terminal truncated version (TmE2F(1–373)) since the intrinsic transactivation ability of TmE2F in yeast [10], precluded the use of the full-length TmE2F protein. Screening of $\sim 10^7$ yeast cotransformants was carried out in selective medium and plasmid DNA of

the stronger interactors, which were able to grow in 30 mM 3-AT and displayed a strong β-galactosidase signal, was isolated. Five of them contained a ~1.1 kb insert of which a partial DNA sequence from their 5′ ends was confirmed to be identical. When used as a query against the available databases, several members of the animal DP family were retrieved. Sequencing of the TmDP cDNA (GenBank accession number AJ271917) insert revealed that it is 1089 bp long and contains a single ORF whose conceptual translation gives rise to a predicted protein of 261 amino acids (expected molecular mass 29.3 kDa). In vitro transcription–translation of the TmDP cDNA clone yielded a protein of ~30 kDa (not shown). The sequence around the presumptive initiation codon conforms to the consensus for translation initiation (GxxAUGG; [27]).

TmDP exhibits an overall 29–33% amino acid similarity with human [28,29] and *Xenopus laevis* [30] DP-1 and DP-2 and a slightly smaller similarity (27%) with *Drosophila melanogaster* DP [31]. Plant and animal DP proteins have a similar domain organization (Fig. 2). The highest homology occurs within a 70 amino acid region (residues 64–143 in TmDP which in animal DP proteins are important for DNA binding [32]), including a region of 10 fully conserved amino acids. The heptad repeats (residues 144–213 in TmDP), involved in heterodimerization with E2F [24,32], are also conserved (not shown). Quite interestingly, TmDP lacks the C-terminal acidic region present in animal DPs. The less conserved region corresponds to the N-terminal domain whose length and amino acid sequence is, nevertheless, similar to that of animal DPs, in particular to the DP-2 group. Thus, we conclude that TmDP presents a higher sequence similarity to animal DP-2 but it has a smaller size and lacks an acidic C-terminal domain.

3.3. TmDP is expressed ubiquitously

Northern analysis of total RNA samples (Fig. 3) revealed the existence of a major ~1.9 ± 0.2 kb transcript together with another much larger transcript (3.5 ± 0.2 kb). Both transcripts were abundant in cultured cells, as well as to a lesser extent in shoots and leaves, and in roots. The larger transcript may represent a partially processed RNA species or the result of an alternative splicing, which is characteristic of mammalian DP proteins [14,32,33], although its functional signifi-

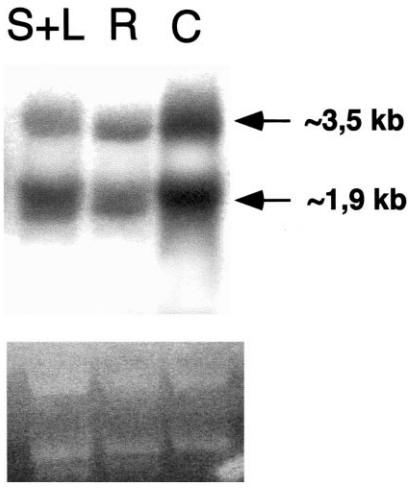

Fig. 3. Expression of TmDP in wheat cells and tissues. TmDP transcripts were detected by Northern analysis of total RNA (10 μg per lane) isolated from wheat shoots and leaves (S+L), roots (R) and cultured cells (C). An ethidium bromide staining of the gel showing ribosomal RNA is used as loading control.

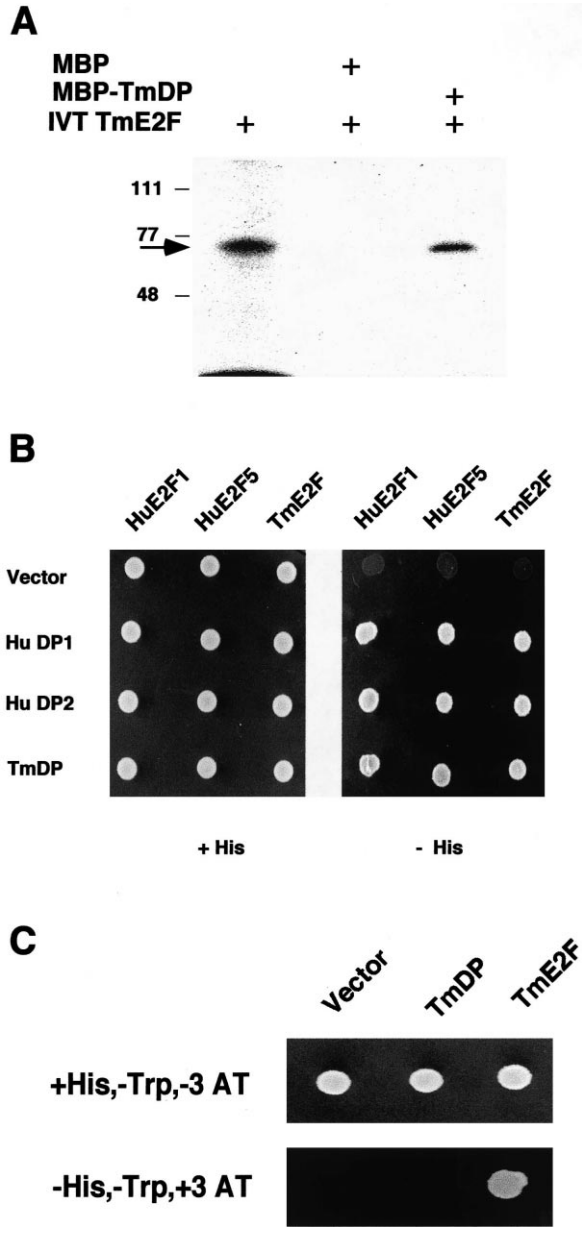

Fig. 4. Characterization of the TmE2F–TmDP interactions. A: Pull-down experiments. The TmE2F cDNA was transcribed and translated in vitro (IVT TmE2F) in the presence of ^{35}S-labelled methionine and incubated with either MBP-TmDP fusion protein (2 μg) or MBP alone (3 μg) expressed and purified from *E. coli*. Labelled material bound to a amylose resin was fractionated by PAGE. The arrow points to the retained ^{35}S-labelled TmE2F. B: Heterodimerization of plant and animal E2F and DP proteins. Yeast HF7c cells were cotransformed with plasmids expressing the Gal4 DNA-binding domain alone (vector) or fused to the human DP-1, human DP-2 or wheat DP and plasmids expressing human E2F-1, human E2F-5 or wheat E2F fused to the Gal4 activation domain, as indicated. Cotransformants were allowed to grow in selective media (−Trp, −Leu, ± His) in the presence of 30 mM 3-AT. C: TmDP does not act as a transactivator in yeast. HF7c yeast cells were transformed with plasmids expressing the Gal4 DNA-binding domain alone (vector) or fused to TmE2F or TmDP, as indicated, and plated in selective medium (± His, −Trp) in the presence of 30 mM 3-AT.

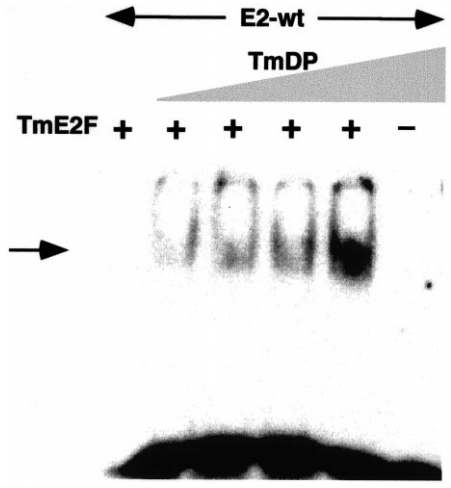

Fig. 5. TmDP stimulates binding of TmE2F to a canonical E2F binding site. The ^{32}P-labelled E2wt oligonucleotide probe was incubated with a constant amount of purified TmE2F(1–373) (50 ng, lanes 1–5) and increasing amounts of purified MBP-TmDP (lane 2, 10 ng; lane 3, 20 ng; lane 4, 50 ng; lane 5, 100 ng; lane 6, 2 μg). After incubation, DNA–protein complexes were fractionated in native polyacrylamide gels. Note that under the conditions used, neither TmE2F (lane 1) nor TmDP (lane 6) alone are able to allow detectable complex formation.

cance has not been established yet. Studies in transiently transfected onion epidermal cells revealed that TmDP, fused to the green fluorescent protein (GFP), remains in the cytoplasm (not shown), in spite of the presence of a positively charged amino acid stretch in its N-terminus. A possibility that needs to be addressed is whether the nuclear localization signal identified in TmE2F [10] is responsible for transporting the TmE2F/TmDP heterodimer to the nucleus.

3.4. Heterodimerization properties of TmE2F and TmDP

First, we determined whether TmE2F/TmDP interaction can occur in the absence of other proteins using in vitro pull-down experiments. When in vitro translated ^{35}S-labelled TmE2F was mixed with purified MBP-TmDP, but not with MBP alone, a significant amount of the input material remained bound to the resin (Fig. 4A), indicative of a direct and specific interaction between TmE2F and TmDP.

Since human DPs form stable heterodimers with any of the E2F family members [13,14,28], we investigated the heterodimerization properties of TmDP using a yeast two-hybrid approach. All the combinations tested between human (HuE2F-1, HuE2F-5, HuDP-1 and HuDP-2) and plant (TmE2F and TmDP) proteins allowed efficient growth of the cotransformants in selective medium (Fig. 4B) and were positive in the β-galactosidase assay (not shown). Therefore, we can conclude that the protein domains involved in heterodimerization are functionally conserved between organisms as divergent as human and wheat. Deletion experiments further confirmed that a central domain in TmE2F is necessary and sufficient to mediate the heterodimerization with TmDP (not shown).

It has been proposed that human DP may have its own transactivation potential perhaps associated to its C-terminal acidic domain [16]. Thus, to determine whether TmDP can itself transactivate a reporter gene, yeast cells were transformed with a plasmid expressing TmDP fused to the Gal4 DNA-binding domain. After plating them under selective conditions, yeast cells carrying the Gal4 DNA-binding domain alone (vector) were unable to grow, while yeast cells expressing TmE2F fused to the Gal4 DNA binding domain did grow (Fig. 4C), indicating that TmDP lacks transactivation potential on its own. In the same assay, human DP-1 and DP-2 alone do not transactivate either (data not shown).

3.5. TmDP stimulates binding of TmE2F to a canonical DNA binding site

To determine the effect of TmDP on TmE2F DNA binding activity, we carried out EMSA with purified proteins. Addition of purified MBP-TmDP to a DNA probe containing a canonical E2F binding site (E2wt) did not produce any retarded band, indicating that TmDP alone does not bind to DNA (Fig. 5). However, under conditions of low amounts of TmE2F, where DNA binding was virtually undetectable, adding increasing amounts of purified MBP-TmDP significantly stimulated complex formation (Fig. 5). Therefore, we conclude that TmE2F/TmDP heterodimerization contributes to increase the affinity and/or stability of DNA–protein complex formation.

4. Discussion

In recent years, studies of plant cell cycle regulators have made a significant progress [34–37] and now it seems clear the the G1/S transition relies on a RBR/E2F pathway. The information available on the components of this pathway strongly supports the prediction that DP-like proteins might exist in plants [10,12], although previous attempts to isolate plant DP-encoding cDNA clones had been unsuccessful. The use of a yeast two-hybrid screening strategy using as a bait a truncated version of plant E2F has allowed us to isolate a cDNA encoding a wheat DP protein.

Amino acid sequence analysis has revealed that TmDP, more closely related to animal DP-2 protein, shares with animal DPs some features as well as possesses unique properties. The murine homolog of human DP-2 (DP-3) is unique in that its primary transcript undergoes extensive alternative splicing giving rise to a complex mixture of four products (α, β, γ and δ [14]). Thus, based on its amino acid sequence (presence of a short N-terminal region, lack of the so-called E region present in the α and δ isoforms, and lack of the Q residue within the DNA-binding domain of the γ isoform), TmDP seems to be structurally related to the murine DP-2β isoform [33]. While this work was in process, the sequence of genomic regions of *A. thaliana* encoding putative DP-like proteins has been released. The predicted sequences of *A. thaliana* DP-like proteins (DP1-like, accession number AL162751, gene 'F12E4_160' and DP2-like, accession number AL162971, gene 'T22P11_60') indicate that they also have a short N-terminus and lack the acidic C-terminal end characteristic of the animal DPs.

In human cells, the transcriptional function of E2F depends on its heterodimerization with DP proteins [13,15]. TmDP associates with TmE2F and also with human E2F-1 and E2F-5. Conversely, TmE2F interacts efficiently with human DP-1 and DP-2. This is in contrast to the more stringent interaction reported for TmE2F and pocket proteins [10], most likely due to the unique RB-binding motif in plant E2Fs. The molecular basis for the heterologous interaction between E2F and DP proteins may reside in the structural

conservation of the heterodimerization domain in both types of proteins [24]. Therefore, this could explain the ability of E2F to form homo and heterodimers with DP proteins from different origins. Furthermore, although TmDP contains a DNA binding domain relatively similar to that of human E2F [24], TmDP alone did not bind to DNA, as it is the case with human DPs [13,15]. On the contrary, TmE2F alone did bind to a DNA sequence containing a canonical E2F-1 binding site, although with low affinity. We have also demonstrated that TmDP stimulates E2F–DNA complex formation, an activity which may contribute to stabilize E2F/DP complexes bound to their target promoter sequences.

The isolation of a TmDP cDNA clone extends the number of components of the pathway controlling gene expression at the G1/S transition and, perhaps, the re-entry into the cell cycle. Thus, our current view includes a key RBR protein [3,37,38] which regulates the function of the E2F transcription factors [10–12], whose site-specific DNA binding activity is stimulated by DP (this work). The E2F/DP proteins can be rendered functionally active after RBR phosphorylation by one or more CDKs [9,35]. Consequently, one of our current interests is to establish which genes regulated at the G1/S transition and/or in response to a number of different signals are actually dependent of the E2F/DP activity. Further studies on the molecular components of this pathway should be of help for such a challenging endeavor.

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