 Nucleic Acids Research, 1999, Vol. 27, No. 17 **3527–3533**

The cloning of plant E2F, a retinoblastoma-binding protein, reveals unique and conserved features with animal G_1/S regulators

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Received May 6, 1999; Revised and Accepted July 12, 1999 DDBJ/EMBL/GenBank accession no. AJ238590

ABSTRACT

Association of the retinoblastoma (Rb) protein with E2F transcription factors is central to cell cycle-specific gene expression and growth in animal cells. Whether Rb–E2F complexes are also involved in plant cell growth and differentiation is still unknown since E2F proteins have not yet been identified in plants. Here we report the isolation and characterisation of a wheat E2F (TmE2F) cDNA clone. Interestingly, the overall domain organisation of plant E2F is related to the human E2F-1/2/3 subset but its primary sequence is slightly more related to the E2F-4/5 subset. TmE2F–Rb binding depends on residues, located at the C-terminus, which are different from those of animal E2Fs. However, the acidic or hydrophobic nature of certain residues is maintained, strongly suggesting that they may have a crucial role in E2F activities. Plant E2F is expressed in proliferating cultured cells and in differentiated tissues and is up-regulated early in S phase. Our studies reinforce the idea that G_1/S regulators in plants are unrelated to those of yeast cells but similar to those of animal cells and provide new tools to analyse the links between cell cycle regulators, plant growth and developmental signals.

INTRODUCTION

Cell cycle progression is the result of a highly regulated network. Strict regulation of the transcriptional activity of certain genes, e.g. S phase-specific genes (1,2), is crucial for correct passage of the cell through the different cell cycle stages. In animal cells, the E2F family of transcription factors plays this pivotal role in transcriptional regulation at the G_1/S transition. Their concerted action is thought to modulate the expression of genes involved in cell cycle regulation and in DNA metabolism (1,2). E2F activity is modulated by the retinoblastoma (Rb) tumor suppressor protein as well as by the related p107 and p130 proteins (3). In this way, for example, Rb is targeted to E2F-responsive gene promoters and inhibits transcription through interaction with adjacent factors, as recently shown for histone deacetylase (4,5).

Plants have unique properties in terms of cell growth and plasticity, body organisation and development. The factors involved in cell cycle regulation, in particular at the G_1/S transition, and their mechanism of action are poorly understood. A strict control of gene expression, linked to and responsible for cell cycle progression, exists in plant cells whereby some genes are expressed at specific stages throughout the cell cycle (6). For example, ribonucleotide reductase, histone, proliferating cell nuclear antigen (PCNA) and cyclin D gene expression has been reported to increase at the G_1/S transit and in S phase (7–10). However, the molecular nature of G_1/S phase-specific plant transcription factors is poorly understood and, in particular, the question as to whether they have structural and/or functional similarities to animal E2F family members still needs to be answered.

The first direct indications that a Rb-like pathway could regulate the G_1/S transition in plant cells came after the isolation of three different D-type cyclins in plants (11,12) and the observation that a protein from a plant DNA virus, whose replication depends on host functions, can associate with human Rb-related proteins (13). Plant cDNAs encoding Rb-related (RBR) proteins with a conserved so-called 'A/B pocket' domain were later identified (14–18). Plant RBR proteins interact with plant D-type cyclins in a LXCXE-dependent manner (16,19) and, interestingly, when a maize RBR protein (ZmRb1; 15) is expressed in human cells, it is able to repress an E2F-responsive promoter (19). These studies are consistent with the prediction that S phase-specific transcription factors exist in plant cells (7,13). However, those identified so far, e.g. PCF1 and PCF2, which control PCNA gene expression (20), are not related to members of the E2F family of G_1/S cell cycle regulators.

Here, we describe the isolation, cloning and characterisation of a wheat cDNA encoding a protein (TmE2F) which interacts with a plant RBR protein (ZmRb1; 15). Our studies have allowed us to establish that this cDNA clone encodes a plant E2F family member. Interestingly, plant E2F contains a unique Rb-binding motif with residues different from those found in animal E2Fs. However, the hydrophobic or acidic properties of certain residues is maintained, thus allowing the identification

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of putative residues which may play crucial roles in both animal and plant E2Fs. Although the overall similarity at the amino acid level is slightly higher with the animal E2F-4/5 subset, the size of TmE2F as well as its domain organisation and increased expression early in S phase makes it more related to the E2F-1/2/3 subset.

MATERIALS AND METHODS

DNA manipulations and plasmid constructions

Standard DNA manipulation techniques were applied as described (21). DNA sequencing was carried out using an Applied Biosystem 373A device. Oligonucleotides were from Isogen Bioscience BV (Maarsen, The Netherlands). ZmRb1 (15) plasmid derivatives were constructed as follows: pGBT-ZmRb1 by cloning the ZmRb1 cDNA (15) in-frame to the Gal4 DNA-binding domain (Gal4^{BD}) of pGBT8; pGBT-ZmRb1ΔC2(1–558) by deleting a *Msc*I–*Xho*I fragment of pGBT-ZmRb1; pGBT-ZmRb1ΔNΔC2(69–558) by deleting a *Msc*I–*Xho*I fragment of pGBT-ZmRb1ΔN. Plasmid pGBT-ZmRb1ΔN(69–683) contains an N-terminal deletion of ZmRb1. Plasmid pGAD-TmE2F(236–458) is a partial clone isolated in the screening and pGAD-TmE2F(236–373) was made by deleting a *Ssp*I–*Xho*I fragment. The C-terminal region (residues 391–458) of TmE2F was generated by PCR amplification of the TmE2F cDNA with the 5' primer GGGGATCCATGGAGG GATGACAAGGATAAT and the 3' primer GTAATACGA-CTCACTATAGGG. The PCR product was digested with *Bam*HI and *Xho*I and ligated in-frame to the Gal4 activation domain (Gal4^{AD}). Plasmids pGAD-E2F-1 and pGAD-E2F-5, containing human E2F-1 and E2F-5, respectively, were provided by N. LaThangue and S. de la Luna and plasmids p130Rbr2 (22) and pGT-RB (23) by M. Serrano. Plasmid GST-ZmRb1 was constructed by cloning the ZmRb1 cDNA (15) in-frame into the pGEX-KG vector and pGST-ZmRb1ΔC2 by deleting a *Msc*I–*Xho*I fragment of pGST-ZmRb1. Plasmid pGST-TmE2F(236–458) was constructed by cloning the partial clone of TmE2F isolated in the two-hybrid screening in-frame into the pGEX-KG vector. For *in vitro* transcription-translation, the full-length TmE2F cDNA was cloned into pBluescriptKS+.

Construction of wheat cDNA library

Five micrograms of poly(A)$^+$ mRNA isolated from wheat suspension cultured cells were used with the cDNA Synthesis kit (Stratagene). The cDNA was ~1.3 kb in length, on average. An aliquot (500 ng) was ligated to *Eco*RI/*Xho*I-digested pGAD-GH vector (750 ng; Clontech) for 48 h at 8°C. The library was dialysed against distilled water and electroporated into *Escherichia coli* DH10B. Total DNA was obtained by plating primary transformants on 50 150-mm LB plates (+Amp). Colonies were scraped off into LB (+Amp) medium and plasmid DNA was prepared as described (21).

Yeast two-hybrid screening

Yeast growth conditions and two-hybrid analysis have been described (13,24). Yeasts were first transformed with plasmid pGBT-ZmRb1 and then with the wheat cDNA library. The transformation mixture was plated on yeast drop-out selection medium lacking tryptophan, leucine and histidine, supplemented with 3-amino-1,2,4-triazole (3-AT). Transformants, recovered during a 3–8 day period, were checked for growth in the presence of ≥20–30 mM 3-AT. The interaction was corroborated by a β-galactosidase assay (25).

Purification of GST fusion proteins and *in vitro* transcription and translation

Escherichia coli BL21(DE3) transformed with plasmids expressing the GST fusion proteins were grown to an OD_{600} of 0.6–0.9 and induced with 1 mM IPTG. GST fusion proteins were purified using glutathione–Sepharose beads (Pharmacia). [^{35}S]Methionine-labelled TmE2F protein was obtained using the TNT kit (Promega). To obtain a polyclonal serum against TmE2F, purified GST–TmE2F(236–458) was used to immunise rats according to standard protocols.

Wheat cell cultures

The *Triticum monococcum* suspension culture (P. M. Mullineaux, John Innes Centre, UK), was maintained as described (13). For synchronisation, wheat cells were treated with 10 mM hydroxyurea (HU) for 48 h and then washed and released into HU-free medium.

Northern and western analysis

Ten micrograms of total wheat cell RNA were denatured, fractionated in a 1.2% agarose gel plus 2.2 M formaldehyde and transferred to a Zeta-Probe membrane (Bio-Rad). The TmE2F (nt 935–1635) and wheat histone H4 (Q.Xie and C.Gutierrez, unpublished results) probes were labelled by random priming with [α-^{32}P]dCTP and mixed for hybridisation. For western analysis, total wheat cell extracts (30 μg) were fractionated by SDS–10% PAGE and blotted onto Immobilon-P membranes (Millipore). The blots were probed with anti-TmE2F polyclonal serum and developed with ECL enhanced chemiluminiscence detection reagents (Amersham).

RESULTS

Isolation of the plant E2F cDNA clone

To identify proteins which interact with a plant RBR protein, we carried out a yeast two-hybrid screening of a wheat (*T.monococcum*) cDNA library made from suspension cultured cells. 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 and partially sequenced from their 5'-ends. Two of them, containing ~1.1 kb cDNA inserts, had a sequence which, when used as a query in a BLAST search, retrieved several animal E2F members. The deduced amino acid sequence of the plant cDNA clones showed the highest homology to the heterodimerisation domain of human E2F-5. Further screening of a wheat cDNA library by colony hybridisation allowed us to recover four clones containing ~2.0 kb inserts with identical restriction patterns and sequences at their 5'-ends. The sequence of the longest cDNA (TmE2F) contains a single ORF of 1374 bp (GenBank accession no. AJ238590), with the potential to encode a protein of 458 amino acids (predicted M_r 49 948). This ORF is flanked by 170 and 439 bp of 5'- and 3'-untranslated regions, respectively. The 5'-untranslated region contains a stop codon in-frame with the putative initiator methionine.

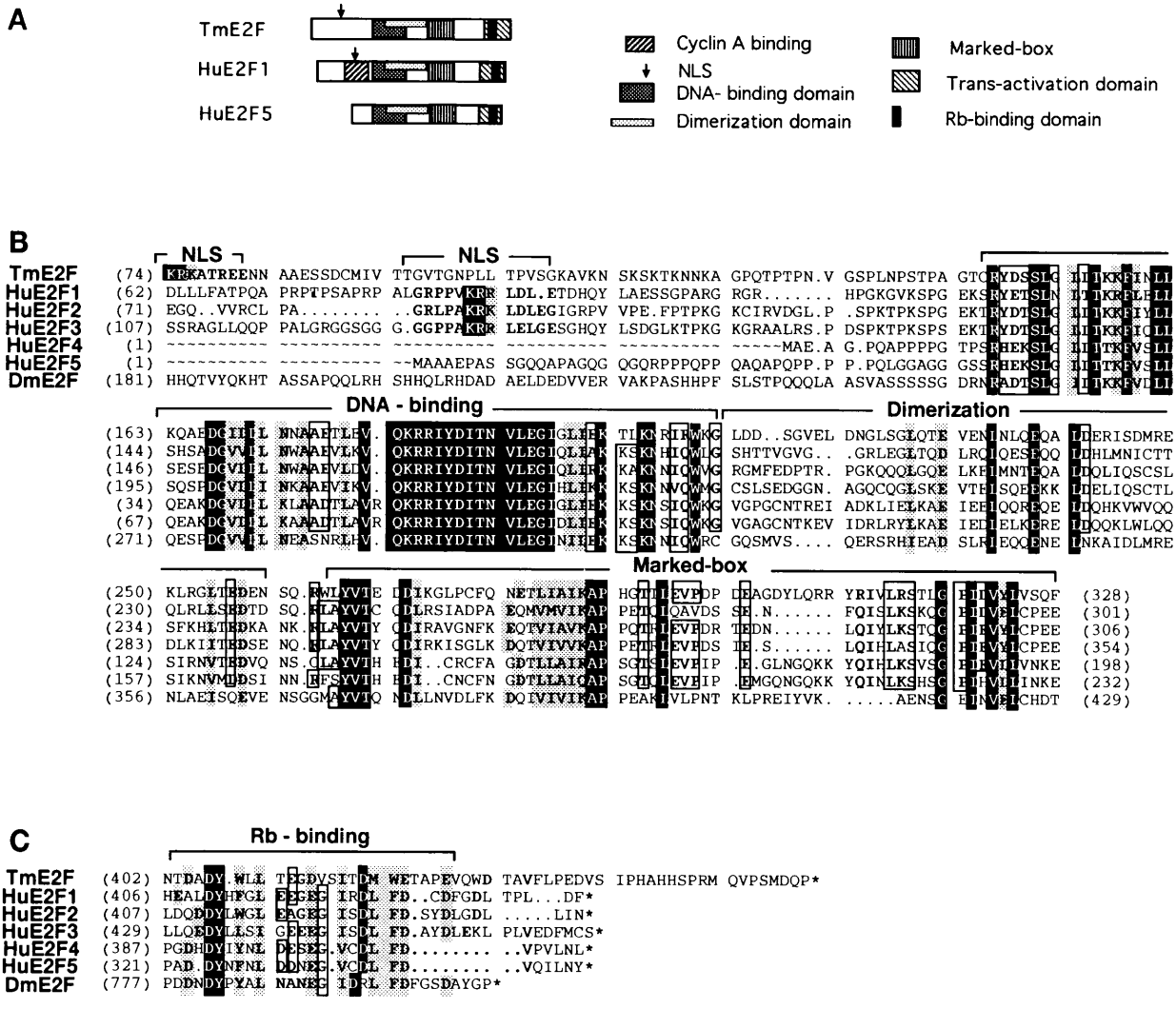

Figure 1. (**A**) Domain organisation of TmE2F compared with that of human E2F-1 and E2F-5, representative of the two subfamilies of human E2F with an Rb-binding domain. The key to identify the different domains appears on the right. Note that plant E2F contains an N-terminal extension similar to that of human E2F-1. (**B**) Alignment of the deduced amino acid sequence of the putative Rb-binding motif of TmE2F with that of human (HuE2F) and *Drosophila* (DmE2F) E2F members. Conserved and similar residues appear with a black and gray background, respectively. Residues conserved only in several members are boxed. Further details are discussed in the text. (**C**) Alignment of the region containing the NLS, the DNA-binding and the dimerisation domain and the 'marked box'. The highlighting of residues is as in (B).

The idea that the TmE2F cDNA clone encodes a plant member of the E2F family was reinforced by analysis of the amino acid homology and domain organisation. A pairwise distance analysis indicated that plant E2F exhibits an overall ~24–27% amino acid similarity with the subset formed by human E2F-1 (26–28), E2F-2 (29,30) and E2F-3 (30), a slightly greater homology (~25–30%) with E2F-4 (31–33) and E2F-5 (33) and a much lower similarity (~19%) with *Drosophila* E2F (34,35). Amino acid alignment of plant and animal E2F proteins revealed an overall domain organisation similar to that of the human E2F-1 subset (Fig. 1A), as well as some specific characteristics of plant E2F.

The DNA-binding domain (Fig. 1B) includes a stretch of 15 amino acids (residues 182–196) fully conserved with that of human E2Fs (27,36). A significant degree of conservation was also found within the homo- and heterodimerisation domains (Fig. 1B), including a leucine zipper motif (residues 219–240). A region with homology to the 'marked box', which in human E2Fs mediates the interaction with adenovirus E4 protein (37), also occurs in TmE2F (residues 264–328; Fig. 1B).

The homology within the C-terminal third of the plant E2F protein, which contains both the transactivation and the Rb-binding domains in human E2F members, is very reduced at the level of primary sequence (Fig. 1C). In fact, when the C-terminal amino acid sequence of plant E2F is used as a query, members of the human E2F family are not retrieved, since the sequence of the C-terminal residues required for Rb-binding in human E2Fs are not present in plant E2F. However, a manual adjustment of the alignment output allows the identification of a 17 amino acid motif in plant E2F (**DY**X_6**D**X_4**DMWE**, positions

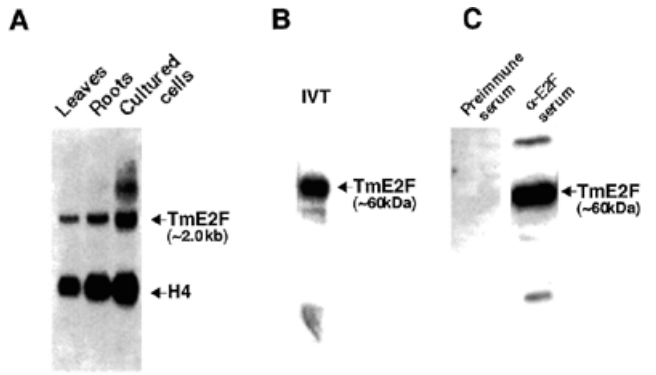

Figure 2. (**A**) Detection of TmE2F mRNA by northern analysis (10 µg of total RNA loaded per lane) relative to wheat histone H4 mRNA. Details are described in Materials and Methods. (**B**) Detection of the product *in vitro* transcribed and translated from the TmE2F cDNA. (**C**) Western analysis of total wheat cell extracts with the rat preimmune serum and a polyclonal anti-E2F serum.

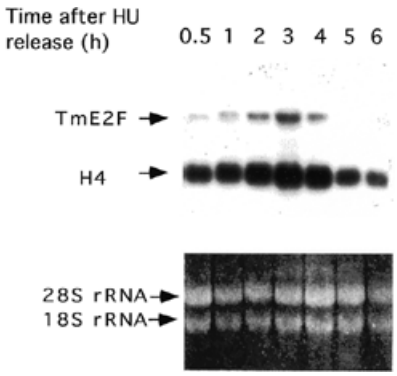

Figure 3. Expression of wheat E2F mRNA after release from a hydroxyurea block. Cultured wheat cells were partially synchronised at the G_1/S transition by treatment with HU. Detection of E2F mRNA was carried out using the same probes as in Figure 2A. The lower panel shows an ethidium bromide staining of the gel to identify the rRNA species.

406–422) which may behave as a functional equivalent of the fully-conserved Rb-binding motif of animal E2Fs. Interestingly, a similar spacing between critical amino acids and a conservation of the acidic and hydrophobic nature of certain residues strongly supports the idea that they may play crucial roles in E2F activities (Fig. 1B).

Plant E2F also contains, at its N-terminus, a putative cyclin A box (38) and a potential nuclear localisation signal (NLS; residues 74–76), typical of the human E2F-1 subset (39,40).

Characterisation of the TmE2F cDNA clone

Northern analysis indicated that a major ~2.0 kb message, with the capacity to encode the entire TmE2F ORF, is present in wheat leaves, root and cultured cells (Fig. 2A). An extra band of larger size was also apparent, in particular in the sample prepared from cultured cells, which may correspond to partially processed RNA species or to other mRNA species related to the TmE2F cDNA clone described here. *In vitro* transcription-translation reactions using a plasmid containing the entire TmE2F cDNA yielded a product with a mobility corresponding to an apparent molecular mass of ~56–60 kDa (Fig. 2B). This is slightly larger than predicted, as is also the case with human E2F-1 (26,27). A genomic DNA blot analysis under low stringency revealed a pattern consistent with only one E2F gene, but we cannot rule out the possibility that other, less related, E2F genes may exist in wheat (not shown). Finally, a western analysis of total wheat cell extracts with a rat anti-TmE2F serum revealed the presence of a band with a mobility similar to that of the protein translated *in vitro* from the isolated TmE2F cDNA clone (Fig 2C). Altogether, these data lead us to conclude that the cDNA isolated in this study, which is expressed ubiquitously in both proliferating and non-proliferating plant cells, encodes a full-length plant E2F protein.

Plant E2F is expressed at higher levels early after release from a HU block

A characteristic feature of human E2F proteins is that they are expressed with different tissue and/or cell cycle specificity (41). To determine whether expression of plant E2F is cell cycle regulated, we partially synchronised cultured wheat cells at the G_1/S transition by treatment with HU. Northern analysis of total RNA revealed that the number of E2F messages increased several-fold a few hours after HU release, following a pattern similar to that of histone H4 mRNA (Fig. 3).

Identification of protein domains required for plant E2F–Rb interaction

To determine the regions of plant E2F and Rb involved in binding, we used a yeast two-hybrid assay. Yeast cells were co-transformed with plasmids expressing the $Gal4^{AD}$–E2F fusion protein and plasmids expressing the $Gal4^{BD}$ alone or fused to several truncated versions of ZmRb1 (15). Deletion of the 125 C-terminal residues of plant Rb (ZmRb1-ΔC2) did not markedly reduce protein interaction, as was also the case with a truncated Rb protein containing the A/B pocket and the C-terminal domain (ZmRb1-ΔN). The A/B pocket alone (ZmRb1-ΔNΔC2) was able to support interaction, although with a slightly reduced efficiency (Fig. 4A).

A parallel study was carried out to determine the region in plant E2F required for Rb binding. Yeast co-transformants expressing a truncated plant E2F containing C-terminal residues [TmE2F(236–458)] bound to plant Rb as efficiently as the full-length plant E2F (Fig. 4B). However, deletion of the 85 C-terminal residues [TmE2F(236–373)] abolished interaction while the C-terminal 68 residues [TmE2F(391–458)] were sufficient for Rb binding (Fig. 4B), indicating that this region, which includes the 17 amino acid motif identified in this study by sequence homology (Fig. 1), contains the Rb-binding motif of plant E2F.

We also wanted to assess plant E2F–Rb interaction *in vitro* using pull-down experiments with purified proteins. *In vitro* translated, ^{35}S-labelled plant E2F bound to purified plant Rb, although weakly (Fig. 4C). A likely explanation for the low recovery of labelled E2F is that E2F–Rb interaction could be stimulated by other proteins absent in this assay, e.g. a DP-like protein which forms heterodimers with E2F and contributes to increased E2F transactivation (2). The E2F–Rb interaction was even weaker when a truncated ZmRb1 protein, lacking its C-terminal domain,

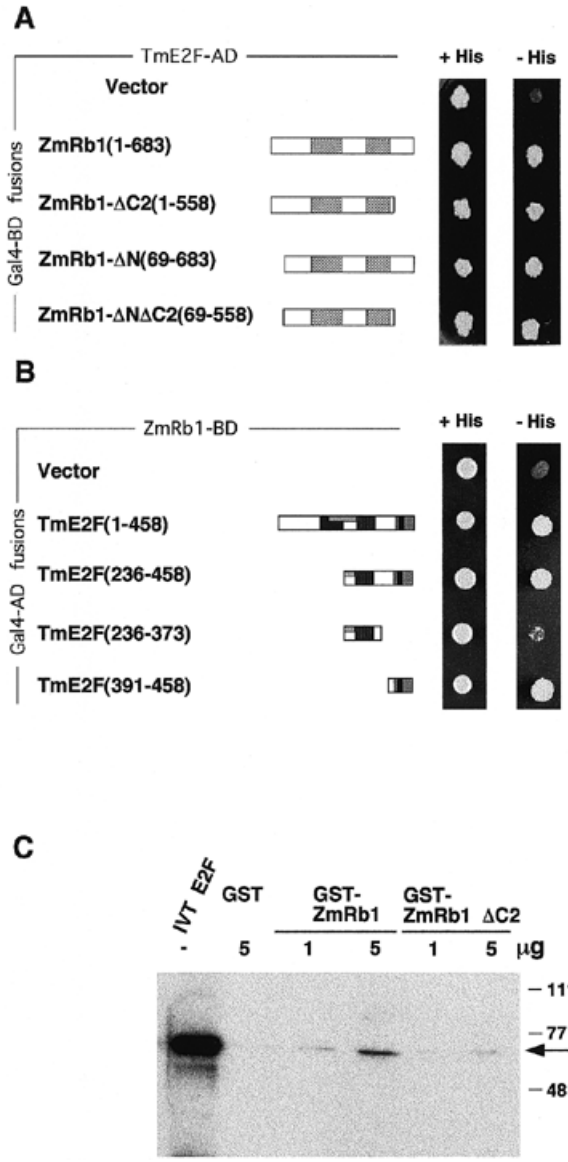

Figure 4. (**A**) Identification of the protein domain in plant Rb required for interaction with TmE2F. Yeast HF7c cells were co-transformed with a plasmid expressing full-length TmE2F fused to the Gal4^{AD} and the indicated plasmids expressing different versions of plant ZmRb1 protein (15) fused to the Gal4^{BD}. Yeast cells were plated with and without histidine, as indicated. (**B**) Identification of the protein domain in TmE2F required for interaction with ZmRb1. Yeast cells were co-transformed with a plasmid expressing the ZmRb1–Gal4^{BD} fusion protein and plasmids expressing truncated TmE2F proteins, as indicated. As in (A), yeast cells were plated with and without histidine, as indicated. (**C**) Interaction between plant E2F and Rb detected by pull-down experiments. *In vitro* transcribed-translated (IVT) ^{35}S-labelled E2F was incubated with purified GST–ZmRb1 or a C-terminal deleted (GST–ZmRb1ΔC2) protein (1 and 5 µg, as indicated). The material bound to glutathione–agarose beads was fractionated in SDS–PAGE gels. The arrow points to the ^{35}S-labelled TmE2F protein.

was used (Fig. 4C). To evaluate whether plant RBR and E2F proteins associated in plant cells, we carried out pull-down experiments by mixing purified bacterially expressed GST–ZmRb1 with total wheat cell extracts. Analysis of the bound proteins by western analysis with the anti-TmE2F serum indicated that the amount of free TmE2F in the extracts that could be recovered

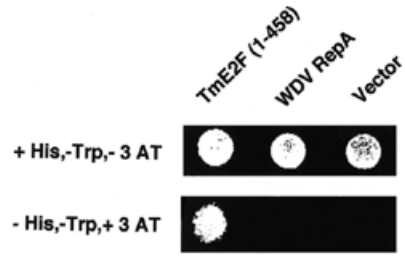

Figure 5. TmE2F acts as a transactivator in yeast. HF7c cells were transformed with a plasmid expressing the Gal4^{BD} alone (vector) or fused to full-length TmE2F (1–458) or wheat dwarf virus (WDV) RepA and plated in selective medium (±His, –Trp) with or without 3-AT (30 mM), as indicated.

by this technique was undetectable under our conditions (not shown). We also tried to co-immunoprecipitate TmE2F with the anti-ZmRb1 serum (and vice versa). Unfortunately, the anti-ZmRb1 serum does not immunoprecipitate any specific protein and the anti-TmE2F serum does so very inefficiently, thus precluding at the present time the use of these approaches to analyse Rb–E2F association in plant cells (not shown).

The TmE2F protein identified in this study can act as a transactivator in yeasts, as indicated by the ability of yeast cells bearing a plasmid expressing TmE2F fused to the Gal4^{BD} to grow in the absence of histidine and tryptophan (Fig. 5). Under similar stringent conditions (–His, –Trp, +30 mM 3-AT), other proteins such as the wheat dwarf geminivirus RepA or the Gal4^{BD} alone (vector) did not support yeast cell growth (Fig. 5).

Homologous and heterologous interactions between plant and human E2Fs and RBR proteins

The five human E2F Rb-binding proteins are grouped in two subsets depending on their binding affinities for the three human A/B pocket proteins (3). Plant E2F showed the highest overall amino acid similarity to human E2F-5. However, its size and domain organisation point to a structural similarity with the E2F-1/2/3 subset. Regarding plant RBR protein (ZmRb1), its domain B has a slightly greater amino acid similarity to human p130 than to human Rb. Thus, to investigate whether we can establish a similarity between plant E2F and one of the subsets of human E2Fs, we determined the binding properties of plant E2F and Rb and compared them with those of human E2Fs and pocket proteins.

Yeast cells were co-transformed with plasmids expressing the Gal4^{BD} alone or fused to plant ZmRb1 or human Rb or p130 and plasmids expressing the Gal4^{AD} fused to plant E2F or human E2F-1 or E2F-5. Co-transformants carrying human Rb and E2F-1, human p130 and E2F-5 and plant E2F and ZmRb1 were able to grow in the absence of histidine at high 3-AT concentrations, while heterologous combinations did not allow efficient growth (Fig. 6). However, interaction between human E2Fs and plant Rb was slightly more efficient than interaction between plant E2F and human pocket proteins, which was barely detectable. This effect was less pronounced under less stringent conditions (not shown). Based on the specificity of the E2F–Rb interactions, we conclude that plant E2F cannot be considered a typical member of either the human E2F-1/2/3 or E2F-4/5 subsets, but, rather, as a distinct member of the E2F family.

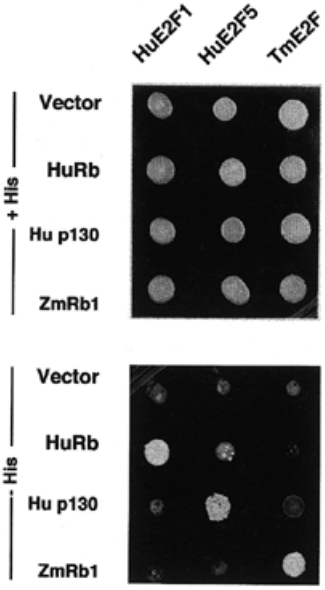

Figure 6. Specificity of the interaction between plant and human E2F and Rb family members. Yeast HF7c cells were co-transformed with the indicated plasmids expressing human Rb or p130 or plant Rb (ZmRb1) proteins fused to the $Gal4^{BD}$ (left) and human E2F-1 or E2F-5 or wheat E2F proteins fused to the $Gal4^{AD}$ (top), as indicated. Yeast cells were allowed to grow under stringent conditions (30 mM 3-AT).

DISCUSSION

The Rb/E2F growth regulatory pathway is crucial to cellular events related to the G_1/S transition as well as to certain differentiated states. With the aim of identifying the components of this pathway in plants we recently cloned a cDNA encoding a plant Rb family member (15,42). Here, we report the isolation and molecular characterisation of a cDNA encoding the first E2F member identified in plant cells as well as its expression pattern and Rb-binding properties.

Plant E2F: domain organisation and properties

Plant E2F shares properties of different animal E2F members. Its primary sequence is slightly more similar to the human E2F-4/5 subset while it resembles members of the E2F-1/2/3 subset in its size and domain organisation.

One of the most striking features of plant E2F is the relatively low amino acid similarity in the C-terminal region, which contains the Rb-binding motif. The C-terminal domain of human E2F-1, which contains a high proportion of acidic residues (**DY**X_7**E**X_3**DLFD**), is sufficient for Rb binding (36,43,44). TmE2F does not contain a full match with this Rb-binding motif. In contrast, plant E2F contains a 17 amino acid stretch (**DY**X_6**D**X_4**DMWE**, positions 406–422) in which, interestingly, the spacing between certain amino acids and their acidic or hydrophobic nature are conserved. Thus, we propose that one or more of them are crucial in mediating interaction between plant E2F and Rb. In spite of the structural conservation of the A/B pocket domain within the Rb family members (45), heterologous interactions between plant and human Rb and E2F proteins are very weak in the two-hybrid assay. The stimulation of such heterologous interactions by other proteins, absent in yeast, could explain the ability of ZmRb1 to partially repress an E2F-responsive promoter when it is expressed in human cells (19). It should also be kept in mind that the two assays (yeast two-hybrid and expression in human cells) are quite different in terms of the efficiency of protein interaction required.

The DNA-binding domain of plant E2F is the most conserved region among all E2F family members, but totally unrelated to other plant S phase-specific transcription factors (20). Recently, the crystal structure of a human E2F-4–DP-2 heterodimer has revealed that the DNA-binding domain of E2F-4 has a structure related to the winged-helix DNA-binding motif (46) instead to the basic helix–loop–helix motif suggested previously (27,36). The high conservation of residues in the DNA-binding domain of plant E2F strongly suggests that it may also adopt a similar folded structure. In addition, one could predict that plant E2F should bind to DNA sequences similar to the consensus E2F-binding site (38). Interestingly, in the promoter region of some plant genes up-regulated at the G_1/S transition, e.g. ribonucleotide reductase (C.Gigot, personal communication) and cyclin D3 (J.Murray, personal communication), matches to the E2F-binding consensus site occur. The availability of plant E2F will facilitate the investigation of the molecular mechanisms governing gene expression during the plant cell cycle. Previous studies in human cells have shown the need of a DP protein partner for efficient DNA binding (2,47). The lack, so far, of a DP-like protein identified in plant cells precludes more detailed DNA-binding studies of plant E2F. However, the conservation of a heterodimerisation domain in plant E2F predicts the existence of such a DP-like protein in plants as a major E2F partner.

The human E2F-1/2/3 subset of proteins contains a typical monopartite NLS, absent in the E2F-4/5 subset. Transcriptional activity of human E2F is finely regulated by changes in the subcellular localisation (39,40). One possibility is that plant E2F is translocated to the nucleus by other partner proteins. Alternatively, a different amino acid motif, such as that formed by residues 74–76, may act as an NLS in plant E2F, an aspect which remains to be analysed in the future.

G_1/S regulators in yeast, animal and plant cells

The existence of a member of the E2F family of transcription factors in plants as well as its domain organisation, similar to that of animal counterparts, together with the conserved features in other components of the G_1/S regulatory pathway, have implications for the evolution of cell cycle regulatory strategies in eukaryotes. The general strategy based on the activity of cell cycle-regulated kinases which act on selected substrates seems to have been maintained in eukaryotes. The accumulating evidence indicates that, in general terms, the molecular mechanisms regulating cell cycle transitions seem to have been largely conserved in plant and animal cells, while cell cycle transitions in yeast are controlled by a set of totally unrelated factors (19,42). Thus, several CDKs (6,48), kinase inhibitors (49), cyclins D (11,12), RBR proteins (14–18) and E2F (this work) have been identified to date. Plants encode an abundant family of cdc2-related proteins of which some members have a typical PSTAIRE motif while others contain unusual CDKs with a variant PSTAIRE motif (6,48). The typical PSTAIRE-containing plant cdc2 can complement CDC28 mutants of budding yeast and cdc2 mutants of fission yeast, whereas the cdc2-related proteins with a variant PSTAIRE motif do not (50,51). Furthermore, both plant and animal cyclins D can functionally substitute for yeast G1 cyclins (11). Therefore, it

will be important to identify the targets of plant CDKs, e.g. RBR, and the CDK–cyclin complexes, functionally equivalent to animal CDK4,6–cyclin D, whose activity inactivates Rb and allows the release of transcriptionally active E2F complexes (3). Our results are also relevant to the question of whether appearance of a Rb/E2F growth regulatory pathway and development of multicellular organisms were concomitant processes (42). In this context, it is worth mentioning that proteins antigenically related to human Rb have been detected in fungi such as *Physarum polycephalum* (52), which develop multicellular structures. Whether acquisition of a Rb/E2F pathway was a requirement for multicellularity or whether it was acquired afterwards remains to be demonstrated.

Genetic experiments indicate that in addition to their participation in cell cycle progression, Rb and E2F family members also have a role in differentiation and development (36,53,54). These activities depend on changes in the type of complexes formed by different Rb and E2F family members as well as on their tissue-specific expression pattern (53). Expression of TmE2F can be detected in differentiated tissues, as is the case with Rb mRNA and protein (15,19). Thus, it is conceivable that Rb–E2F complexes have roles not only in cell cycle regulation but also during differentiation and development. These and other related questions should be addressed in the future.

ACKNOWLEDGEMENTS

The authors are indebted to P. M. Mullineaux for the wheat cell culture, to M. Serrano, E. Lam, N. LaThangue and S. de la Luna for plasmids, to E. Madueño for help with automatic sequencing and to E. Martínez-Salas and M. Serrano for discussions. E.R.-P was the recipient of a fellowship from Comunidad de Madrid, Q.X. from the European Union and M.B.B. from the Spanish Ministry of Education and Culture. This work has been partially supported by grants PB96-0919 (DGES), CI1*-CT94-0079 (European Union) and 06G/046/96 and 07B/0020/98 (Comunidad de Madrid) and by an institutional grant from Fundación Ramón Areces.

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