Isolation and characterization of the E2F-like gene in plants[1]

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Abstract The transcription factor E2F regulates the expression of genes involved in the progression of G1/S transition and DNA replication in mammalian cells. We cloned and characterized a cDNA (*NtE2F*) corresponding to a E2F homolog of tobacco (*Nicotiana tabacum*). The transcription of *NtE2F* was induced as cells progressed from G1 to the S phase and expressed much earlier than that of the proliferating cell nuclear antigen (PCNA) gene. We demonstrated that NtE2F can interact with the tobacco retinoblastoma (Rb)-related protein in a yeast two-hybrid assay. To further characterize NtE2F, the *trans*-activation activity of NtE2F was examined by using a transient assay in the tobacco Bright Yellow-2 (BY-2) cells with NtE2F fused to the DNA-binding domain of the yeast transcriptional activator GAL4. NtE2F activated the transcription of the β-glucuronidase (GUS) reporter gene driven by a cauliflower mosaic virus (CaMV) 35S core promoter containing the GAL4-binding sequence. This is the first report of the identification of a functionally equivalent E2F-like gene in plants.

Key words: E2F; Cyclin; Cell cycle; Transcription factor; *Nicotiana tabacum*

1. Introduction

The cell cycle is a highly conserved mechanism for the control of cell proliferation in eukaryotic cells, in which cyclin-dependent kinases (CDKs) and cyclins play a pivotal role [27,28]. In mammalian cells, the commitment to division normally occurs during the late G1 phase called the restriction (R) point. The retinoblastoma (Rb) tumor suppressor protein is a negative regulator of cell proliferation [33,38]. The growth-suppressive activity of Rb is regulated by CDK-mediated phosphorylation. The function of Rb to control the G1/S transition is largely through its regulation of the E2F transcription factor family, which is required for the expression of genes involved in the G1/S transition and DNA replication [7]. Underphosphorylated, active forms of Rb dominate in the G1 phase, bind to the activation domain of E2F and inhibit *trans*-activation activity of E2F [12,37]. During the mid-G1 to S phases, Rb is inactivated by sequential phosphorylation with cyclin D-CDK4, cyclin D-CDK6 complexes and cyclin E-CDK2 complex. The resulting 'free E2F' released from Rb activates the transcription of E2F-regulated genes.

E2F binds DNA as a heterodimer composed of two structurally related subunits, E2F and DP. Each of these proteins is encoded by gene families. To date, six E2F genes and two DP genes have been found in mammals [6,7]. Consensus E2F-binding sites (TTTCGCGC) are found in the promoters of growth-promoting genes, including c-Myc, B-Myb, cyclin E and cyclin A [7]. E2F-binding sites are also present in the promoters involved in DNA replication, such as DNA polymerase α, dihydrofolate reductase (DHFR) and proliferating cell nuclear antigen (PCNA) [4]. Although the carboxy-terminal regions of E2F-1–E2F-5 contain a potent *trans*-activation domain, no equivalent activity has been found in E2F-6 and DP family proteins and therefore, E2F-6 is proposed to repress E2F-regulated genes [2,8,36].

The regulatory mechanisms in the G1/S transition in yeast and mammals have been extensively studied, but much less is known about these controls in plants [23]. Recently, cDNA clones for Rb-related protein were isolated from maize [1,9,39] and we also isolated a cDNA encoding the tobacco Rb-related protein [26]. The existence of cyclin D [3,25,34,35] and Rb-related genes in plants suggests that mechanisms controlling the G1/S transition in mammals and plants are functionally conserved [5,10,21].

In this study, we isolated a clone that encodes a E2F homolog from tobacco by reverse transcription-polymerase chain reaction (RT-PCR). Our studies indicate that the E2F-like protein may function in cell cycle regulation in plants.

2. Materials and methods

2.1. Cells and cell cycle synchronization

Suspension cultures of *Nicotiana tabacum* L. cv. Bright Yellow-2 (tobacco BY-2) cells were maintained at 27°C in 300 ml Erlenmeyer flasks on a rotary shaker at 130 rpm. Every 7 days, 2 ml of culture was transferred to a new flask containing a modified Linsmaier and Skoog medium as previously described [30]. A synchronization protocol based on the method described by Nagata et al. was used [24]. Briefly, 10 ml of 7 day old cells were diluted 10 times with fresh medium containing aphidicolin (final concentration of 5 mg/l), cultured for 24 h, washed with 1 l of fresh medium and suspended in 100 ml of medium [14]. After cells were cultured at 27°C for 6 h, propyzamide (final concentration of 3 mM) was added, maintained for 4 h, washed with 1 l of fresh medium and finally suspended in 100 ml of medium. The mitotic index and the rate of DNA replication were determined as described previously [14].

2.2. PCR amplification and cDNA library screening

Total RNA was isolated from 3 day old suspension cultures of tobacco BY-2 cells as previously described [30]. Poly(A) RNA was purified by the Oligotex-dT30 kit (Takara) according to the instruc-

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[1] The sequences reported in this paper have been deposited in the GenBank database (accession numbers AB025347 for NtE2F and AB025029 for tobacco PCNA).

Abbreviations: CDK, cyclin-dependent serine/threonine protein kinase; Rb, retinoblastoma; PCNA, proliferating cell nuclear antigen; RT-PCR, reverse transcription-polymerase chain reaction; CaMV, cauliflower mosaic virus

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tion manual and used for RT-PCR. We used MuLV reverse transcriptase (Perkin-Elmer) for reverse transcription and primers (forward primer, CGATATGATAGTTCGTTAGGTC; reverse primer, TAGGTACACGTCTATTGGTCC) to amplify an E2F-like fragment from the tobacco first-strand cDNA. The DNA fragment was isolated and cloned using a pGEM-T vector system (Promega) and sequenced using the Dye-terminator sequence kit (Perkin-Elmer). The DNA fragment was labelled with [^{32}P]dCTP (ICN) and was used to screen a tobacco BY-2 cDNA library constructed as previously described [31]. Positive clones were recovered from a cDNA library constructed with a λ ZipLox kit (Gibco BRL) by in vivo excision according to the manufacturer's manual.

2.3. RNA extraction and gel blot hybridization

Total RNA was purified by the method described by Schmidt et al. [29]. For Northern blot analysis, RNA was subjected to electrophoresis on 1% agarose gels that contained 2% formaldehyde by a modified version of the procedure of Lehrach et al. [20]. RNA was then blotted onto nylon membranes (Biodyne A). The membranes were hybridized with the ^{32}P-labelled entire open reading frames of the

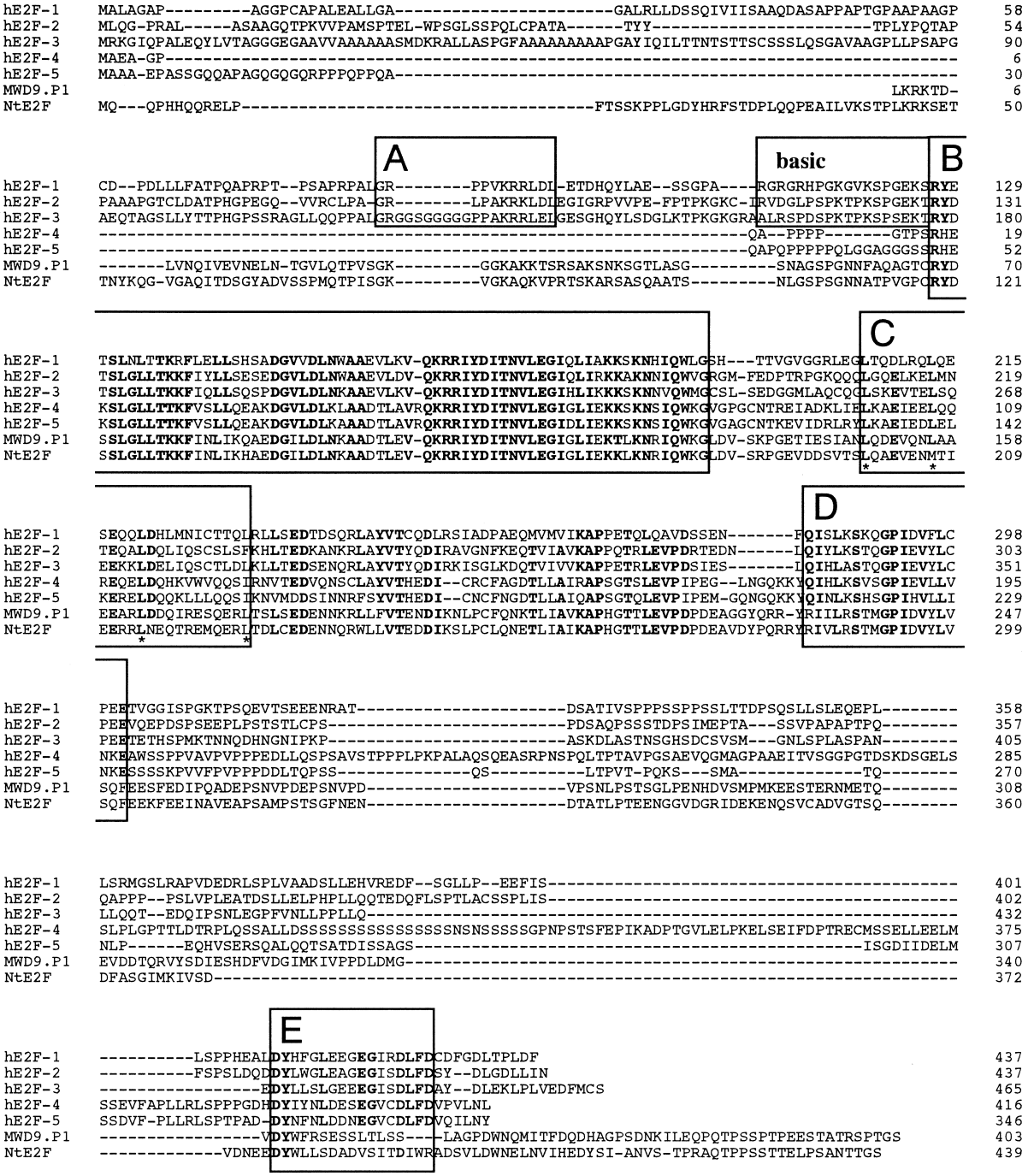

Fig. 1. Alignment of the deduced amino acid sequence of the NtE2F with *Arabidopsis* MWD9.P1 and the human E2F family. Amino acid positions are indicated on the right. Dashes indicate the gaps that are inserted in the amino acid sequence to create a maximum match among the E2F family. The domains corresponding to the cyclin-binding region (A), basic region (basic), DNA-binding domain (B), leucine zipper region (C), marked box (D) and the Rb-binding domain (E) are boxed. Asterisks indicate the hydrophobic residues in the leucine zipper region.

tobacco *NtE2F* and PCNA genes, using the Megaprime DNA labelling system (Amersham). Hybridization and washing were performed as described previously [14]. Signals were detected using the Fuji Imaging Analyzer (model BAS2000, Fuji Photofilm, Tokyo, Japan).

2.4. Yeast two-hybrid assays

The cDNA fragment encoding amino acids 375–961 of the tobacco Rb (*NtRb1*) was subcloned into the two-hybrid vector pDBLeu, which produced fusion proteins with the GAL4 DNA-binding domain [25]. A fragment encoding amino acids 74–439 of *NtE2F* was fused with the GAL4 *trans*-activation domain to construct a fusion protein in the plasmid pPC86. The *Sal*I and *Not*I sites were introduced by PCR with the following primers: for NtRb1, 5′-GTCGACAATGGCTTCCCCAGCAAAAACG-3′ and 5′- GCGGCCGCCTAAGACTCAGGCTGCTCAGT-3′; for NtE2F, 5′-GTCGACGCAGACACCAATCTCAGGGAAAG-3′ and 5′-GCGGCCGCTCAGCTCCCAGTTGTATTTGC-3′. The PCR products were digested with *Sal*I and *Not*I and ligated into the same sites of pDBLeu and pPC86 to construct pDB-NtRb1 and pPC86-NtE2F, respectively. Other plasmids were obtained from a ProQuest two-hybrid system (Gibco BRL). pPC97-Rb encodes amino acids 302–928 of human Rb, pPC86-E2F1 encodes amino acids 342–437 of human E2F-1, pPC97-dDP encodes amino acids 1–377 of *Drosophila* DP and pPC86-dE2F encodes amino acids 225–433 of *Drosophila* E2F. Yeast transformations were done with *Saccharomyces cerevisiae* strain MaV203 as previously described [31]. β-Galactosidase activity of individual transformants was quantitated in mid-log phase cultures using the standard colorimetric method [22].

2.5. Construction of effector plasmids

Effector plasmids were generated using PCR-based construction. Two *Spe*I sites were introduced into both ends of the coding region of NtE2F (amino acids 1–439) by PCR with the following primers: 5′-ACTAGTATGCAGCAGCCACATCATCAAC-3′ and 5′-ACTAGTTCAGCTCCCAGTTGTATTTGCAG-3′. The PCR fragment was digested with *Spe*I and cloned into the *Xba*I site of pG4DD(1–94)-1 [26] to construct fusion with the DNA-binding domain of GAL4 (amino acids 1–94) under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The *Bam*HI and *Xba*I sites were introduced into the human E2F-1 (amino acids 90–437) by PCR with the following primers: 5′-GGATCCGCGGAGGCTGGACCTGGAAACT-3′ and 5′-TCTAGATCAGAAATCCAGGGGGGTGAGG-3′. The PCR fragment was digested with *Bam*HI and *Xba*I and cloned into pG4DD(1–94)-1 [26].

2.6. Transient expression analysis

Protoplasts were isolated from the tobacco suspension cultured BY-2 cells [16] and the β-glucuronidase (GUS) activity was assayed by fluorometric quantification of 4-methylumbelliferone produced from the glucuronide precursor as previously described [16].

3. Results

3.1. Cloning and structural features of a tobacco E2F-like protein

Searches of the Kazusa *Arabidopsis* data Opening Site (http://www.kazusa.or.jp/arabi/) identified one clone (MWD9.P1) whose predicted products have a high similarity to the human E2F-3 transcription factor. A partial tobacco cDNA of 539 bp encoding a E2F-like protein was amplified by RT-PCR with primers based on the conserved sequences between human E2F and *Arabidopsis* E2F-like genes. Two clones were subsequently isolated by screening a tobacco BY-2 cells cDNA library with the partial cDNA at high hybridization stringency. The determination of the nucleotide sequences of the clones revealed that two clones have the same sequences with a different length of the 5′-untranslated region. One of the clones (*NtE2F*) consists of 1780 bp and encodes a 439 amino acid long peptide with a calculated molecular mass of 48.5 kDa (Fig. 1). Several in-frame stop codons were found at the 5′-untranslated region of the *NtE2F* cDNA, indicating that this clone appears to encode a full-length polypeptide.

Analysis of the deduced amino acid sequence of *NtE2F* revealed that it contains extensive sequence similarity with the domains conserved among the mammalian E2F family proteins (Fig. 1). For simplicity, we compared the deduced amino acid sequence of *NtE2F* with the most highly homologous protein E2F-3. NtE2F shows 73% identity in the DNA-binding domain to human E2F-3, which rises to 83% similarity when conservative homologies are permitted. The positions of the hydrophobic residues in the leucine zipper region are well conserved in NtE2F and a region termed the 'marked box' that is highly conserved between the human E2F family of proteins shares 50% identity with E2F-3. In addition to these domains, NtE2F shares 33% identity with E2F-3 in the Rb-binding region.

DNA probes prepared from the full-length NtE2F cDNA hybridized to multiple DNA fragments on Southern blots of genomic DNA at high stringency (data not shown). This result suggests that the E2F homologous gene may be a member of a gene family as is the case for mammalian cells.

3.2. Expression of NtE2F during the cell cycle

To examine the expression of the *NtE2F* gene, tobacco BY-2 cells were synchronized by treating them with aphidicolin and then with propyzamide (Fig. 2). After removal of propyzamide, cells immediately exited the M phase and the mitotic index dropped rapidly and remained at low levels until 12 h. A second peak of the mitotic index was observed from 12 to 16 h. In contrast, a peak in the DNA synthesis was observed at 9 h, after which the rate of DNA synthesis gradually decreased. We have chosen the PCNA gene as a control, since

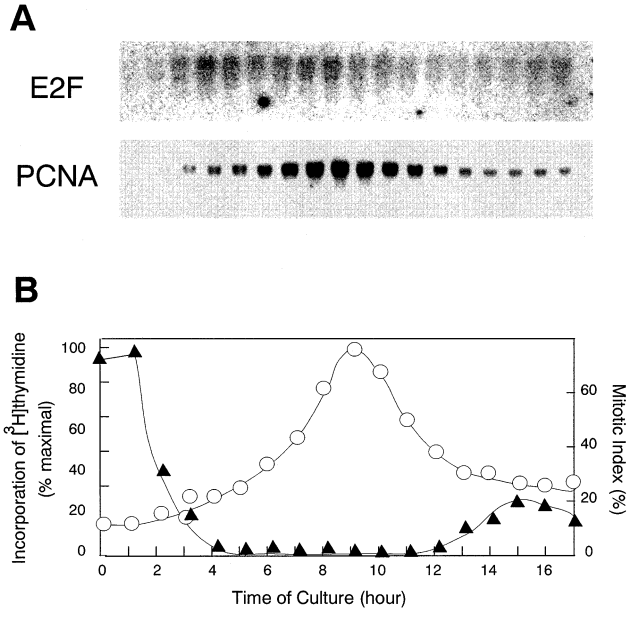

Fig. 2. Expression of *NtE2F* during the cell cycle. (A) Tobacco BY-2 cells were synchronized by the aphidicolin and propyzamide method. After propyzamide was removed (point 0), cells were harvested at 1 h intervals. Total RNA was isolated, electrophoresed on 1% agarose gels and blotted onto nylon membranes. Two separately prepared membranes were probed with the ^{32}P-labelled *NtE2F* and the tobacco PCNA cDNAs. (B) Cell cycle progression was monitored by measuring incorporation of [^{3}H]thymidine into DNA (open circle) and the mitotic index (solid triangles).

expression of the *Catharanthus roseus* PCNA gene was preferentially observed in the S phase [17]. Consistent with this observation, the tobacco PCNA gene was also accumulated predominantly during the late G1 and S phases (Fig. 2). Although the expression levels of *NtE2F* were low, we found that the *NtE2F* expression was observed predominantly during the G1 phase. These results indicate that expressions of *NtE2F* and PCNA oscillated during the cell cycle, while *NtE2F* was expressed much earlier in the cell cycle than PCNA.

3.3. NtE2F interacts with the tobacco Rb-related protein

The human E2F family proteins have been shown to interact with Rb family proteins [7,11,32]. The conservation of the Rb-binding region in NtE2F suggests that it can bind the tobacco Rb-related protein. To test this possibility, we used a yeast two-hybrid system to detect binding of NtE2F to NtRb1, which encodes the tobacco Rb-related protein (Table 1).

For two-hybrid assays, a plasmid that contains NtE2F (amino acids 74–439) was constructed with the GAL4 DNA-binding domain. Yeast strain MaV203 was transformed with the plasmid together with the plasmids expressing NtRb1 (amino acids 374–961) fused with the GAL4 *trans*-activation domain. As a control, MaV203 cells were transformed with plasmids that express various fusion proteins. Transformants were assayed for β-galactosidase activity to detect protein interaction (Table 1). This assay showed that NtE2F interacts with NtRb1 similarly to the fact that human Rb binds with human E2F-1 and *Drosophila* dE2F binds with *Drosophila* dDP. These results indicate that NtE2F-Rb-related protein interaction is conserved in plants.

3.4. NtE2F acts as a transcriptional activator

Since we have not succeeded in detecting the binding activity of NtE2F with the mammalian E2F-binding site (data not shown), the *trans*-activation activity of NtE2F was examined by using a transient assay with NtE2F fused to a heterologous GAL4 DNA-binding domain [26]. Effector plasmids were constructed with the NtE2F and human E2F-1 fused to the GAL4 DNA-binding domain driven by a CaMV 35S promoter. The reporter plasmid G4TS-50 35S/GUS was composed of the coding sequence of the GUS gene with the GAL4 target sequence just upstream of a CaMV 35S core promoter. These two plasmids were co-transfected into tobacco BY-2 protoplasts by electroporation and the GUS activity was analyzed (Fig. 3). The results were shown as relative values of the GUS activity obtained with the GAL4 DNA-binding domain alone, because this effector slightly enhanced the expression level of the GUS gene as reported previously [26] and the absolute values varied in each experiment. As shown in Fig. 3, NtE2F significantly activated the GUS gene in a dose-dependent manner. This *trans*-activation activity was comparable to the human E2F-1 but much weaker than the activation domain of the herpes simplex virus protein VP16 used as a positive control (Fig. 3).

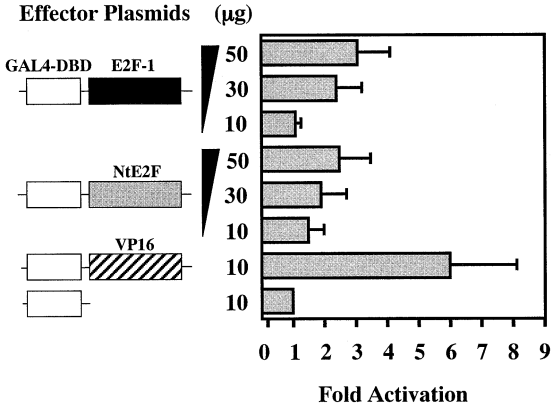

Fig. 3. *Trans*-activation of NtE2F in tobacco BY-2 protoplasts. Different amounts of effector plasmids encoding the GAL4 DNA-binding domain (pG4DD(1–94)-1) alone or the GAL4-binding domain fused to either NtE2F (amino acids 1–439), human E2F-1 (amino acids 90–437) or the acidic activation domain of the herpes simplex virus protein VP16 were co-transfected into tobacco BY-2 cells with the GUS reporters (20 μg) containing a GAL4-binding site fused to a CaMV 35S core promoter. Transfected protoplasts were incubated for 24 h and then, the GUS activity was assayed. The results were shown as relative values of the GUS activity obtained with the GAL4 DNA-binding domain alone. Mean values with S.D. were shown with six independent preparations of protoplasts from two experiments.

4. Discussion

Here, we report the isolation and characterization of a tobacco E2F homolog, *NtE2F*. Although the domains in NtE2F, such as the DNA-binding domain, the potential leucine zipper region, the marked domain and the Rb-binding domain, are conserved with the mammalian E2F family proteins [7], NtE2F shows more similarity with E2F-1, 2 and 3 rather than with E2F-4 and 5 subfamilies (Fig. 1). However, NtE2F lacks the potential cyclin-binding region along with a basic region, which are both conserved in E2F-1, 2 and 3 [7,19]. Whether functional properties of NtE2F resemble the E2F-1, 2 and 3 or E2F-4 and 5 subfamilies, along with whether there are related genes in plants, remains to be determined.

Table 1
Yeast two-hybrid assay for the interaction between NtE2F and NtRb1

Fusion with GAL4-DBD	Fusion with GAL4-AD	Reporter gene *LacZ* (U)[a]
pDB-NtE2F1 (74–439)[b]	pPC86	0.26
pDB-NtE2F1 (74–439)	pPC86-NtRb1 (375–961)	3.4
pDB-NtE2F1 (74–439)	pPC86-hDP1 (1–410)	0.72
pPC97-hRb (302–928)	pPC86-hE2F1 (342–437)	2.0
pPC97-dDP (1–377)	pPC86-dE2F (225–433)	4.0
pPC97	pPC86	0.17

[a]MaV203 yeast cells transformed with indicated plasmids were assayed for β-galactosidase activity in liquid cultures using *o*-nitrophenyl-β-D-galactopyranoside as a substrate. Data are the averages for triplicate assays. pDBLeu was derived from pPC97.
[b]Amino acids used in constructing each protein were indicated.

Although Ito et al. [15] reported that a M-specific activator is a common *cis*-acting element that controls the M phase-specific expression of cell cycle-related genes, the control of G1 and S phase-specific expression is poorly understood in plants. In mammalian cells, the E2F/DP family of transcription factors is thought to play important roles in gene regulation during the S phase [7]. E2F-binding sites are found in promoters of many genes specifically expressed during the S phase and include genes involved in DNA replication such as PCNA [4].

In an attempt to determine the expression pattern of *NtE2F*, we have chosen the PCNA gene as a control to characterize mRNA expression during the cell cycle. As shown in Fig. 2, transcript of the tobacco PCNA gene accumulated predominantly during the late G1 and S phases. The expression pattern of the tobacco PCNA gene was similar to that of the periwinkle gene as well as to mammalian cells, suggesting that common mechanisms may regulate the expression of PCNA during the S phase in mammalian cells and plants. It is possible that E2F-like protein may regulate the expression of the PCNA gene in plants. This possibility was supported by the finding that *NtE2F* was expressed much earlier in the cell cycle than the PCNA gene (Fig. 2). Two transcription factors, PCF1 and PCF2, have been reported to be involved in meristematic tissue-specific expression of the rice PCNA gene [18]. However, it is unclear whether these factors are also involved in cell cycle-regulated transcription of the PCNA gene. To our knowledge, *NtE2F* is the first marker for the G1 phase in cycling plant cells.

Since NtE2F might require DP homolog from plants to precisely bind with the proper binding site, we performed a transient assay with NtE2F fused to a heterologous GAL4 DNA-binding domain. NtE2F significantly enhanced the expression level of the GUS reporter gene (Fig. 3), which indicates that NtE2F has a *trans*-activation activity in plant cells. However, the degree of activation by NtE2F was much weaker than the VP16, but was comparable to the human E2F-1. As an attractive hypothesis, the *trans*-activation activity of NtE2F and the human E2F-1 may be inhibited by the tobacco Rb-related protein in BY-2 cells. In mammalian cells, the binding of Rb to E2F family proteins inhibits transcriptional activation even when E2F family proteins are bound to E2F-binding sequences [7]. NtE2F may play an important role in the G1/S phase transition through the transcriptional activation of the G1 and S phase-specific genes. The interaction of NtE2F with the tobacco Rb-related protein might inhibit its *trans*-activation activity by analogy to the mechanism in mammalian cells (Table 1) [7]. This possibility is supported by evidence of the ability of maize Rb-related protein to bind human E2F-1 and inhibit E2F-dependent transcriptional activation in human cells [10,13].

Our results show that E2F homologs exist in plants and NtE2F can bind with the tobacco Rb-related protein in a yeast two-hybrid assay. Furthermore, and most importantly, we demonstrated that the NtE2F has a *trans*-activation activity in plant cells. This study will provide new insights to elucidate whether E2F-like protein together with Rb-related protein may regulate the cell cycle in plants.

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