Short Communication Plant Biotechnology, **21** (4), 289-293 (2004)

An E2F-Regulated Reporter Construct is Transcriptionally Activated Following the Transient Expression of Cyclin D in Plants.

Kazue KAWAMURA[1], Kenji UEMUKAI[1], Shunichi KOSUGI[2], Ko KATO[1], Masaki ITO[3], Atsuhiko SHINMYO[1] and Masami SEKINE[1]*

[1]*Graduate School of Biological Sciences, Nara Institute of Science and Technology (NAIST), Takayama 8916-5, Ikoma, Nara 630-0101, Japan*
[2]*Institute of Advanced Biosciences, Keio University, Tsuruoka, Yamagata 997-0035, Japan*
[3]*Department of Regulation of Biological Signals, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Aichi 464-8601, Japan*
**Corresponding author* E-mail address: sekine@ bs.aist-nara.ac.jp

Received 6 July 2004 ; accepted 7 July 2004 (Edited by H. Sano)

Abstract

The E2F transcription factors play important roles in the regulation of gene expression at the G1/S transition in plants. Here, we show that the rice proliferating cell nuclear antigen (*PCNA*) promoter is activated by transient expression of tobacco NtE2F and *Arabidopsis* AtDPa in tobacco cells. This transcriptional activation is repressed by co-transfection with a plasmid encoding the tobacco Rb-related protein (NtRBR1), whereas further co-expression of cyclin D overcomes this repression. Importantly, the rice *PCNA* promoter is activated when cells are transfected with cyclin D alone, and this activation is enhanced by co-transfection with plasmids encoding NtE2F and AtDPa. These results suggest that the effect of cyclin D expression is mediated not only by its associated kinase, which allows it to phosphorylate NtRBR1 thereby releasing the NtE2F/NtDP complex to activate transcription, but also by a mechanism which does not involve transfected NtRBR1.

Key words: cyclin D, E2F, Rb, transcriptional repression, PCNA.

Abbreviations

CDK, cyclin-dependent serine/threonine protein kinase; Rb, retinoblastoma.

Progression through the cell cycle is regulated by cyclin-dependent kinases (CDKs) activated at key transition points in association with various cyclins (Pines, 1995). In plants, two major CDKs, CDKA and CDKB, are thought to control the cell cycle (Mironov *et al.,* 1999). CDKA contains a canonical PSTAIRE motif in its cyclin-binding domain, and functionally complements yeast *cdc2/cdc28* mutations. Expression and protein levels of CDKA are constant during the cell cycle, but its kinase activity is tightly regulated. In contrast, expression of CDKB is strictly regulated during the cell cycle, and complementation of yeast *cdc2/cdc28* mutations by CDKB has been unsuccessful, suggesting that CDKB is a plant-specific CDK.

Plants also contain many types of cyclins. A-type cyclins (CycAs) are involved throughout the S to M phase transition, and B-type cyclins (CycBs) control the G2/M transition, most likely in association with CDKA and CDKB. D-type cyclins (CycDs) are thought to act as regulators of the G1 control point in conjunction with CDKA (Meijer and Murray, 2000; Oakenfull *et al.,* 2002).

The *Arabidopsis* D-type cyclin CycD3 is rate-limiting at the G1/S transition (Dewitte *et al.,* 2003), and is transcriptionally upregulated at this point in response to plant hormones and sucrose (Riou-Khamlichi *et al.,* 2000). *Arabidopsis* CycD3; 1 is not only regulated at the transcriptional level, but also subjected to proteasome-dependent degradation at the protein level (Planchais *et al.,* 2004). *Arabidopsis* CycD2 and CycD3 interact with CDKA (Healey *et al.,* 2001), and the tobacco CycD3/CDKA complex prepared from insect cells phosphorylates the tobacco retinoblastoma-related protein (NtRBR1) (Nakagami *et al.,* 1999). CycD3 immunoprecipitates prepared from synchronized tobacco BY-2 cells exhibit kinase activity against NtRBR1 during middle G1-phase to early S-phase

(Nakagami *et al.,* 2002).

A number of plant cDNAs encoding E2F and DP family members have recently been identified (Gutierrez *et al.,* 2002). Plant E2F proteins bind to the same E2F binding site (TTTC/GC/GCGC) as animals, and their DNA binding activities are stimulated by heterodimerization with plant DP proteins (Albani *et al.,* 2000; Magyar *et al.,* 2000; Ramirez-Parra and Gutierrez, 2000; Kosugi and Ohashi, 2002). Carrot (Albani *et al.,* 2000), rice (Kosugi and Ohashi, 2002) and *Arabidopsis* E2Fs exhibit transcriptional activities in plant protoplasts which depend on the presence of DP proteins (Magyar *et al.,* 2000; Ramirez-Parra and Gutierrez, 2000; Mariconti *et al.,* 2002). It has also been shown that plant E2F proteins can bind to plant RBRs through their C-terminal transactivation domain (Ramirez-Parra *et al.,* 1999). Recently, we showed that E2F-regulated genes were effectively activated in tobacco protoplasts following co-transfection with plasmids encoding tobacco NtE2F and NtDP (Uemukai *et al.,* in press). This transcriptional activation was repressed by co-expression with NtRBR1; however, this repression was abrogated when cells were further co-transfected with a plasmid encoding CycD but not with those encoding CycA or CycB. Therefore, as in animals, plant RBR appears to govern the G1/S transition through its interaction with the E2F transcription factors, and CycD-associated kinase-mediated phosphorylation of RBR allows the release of the E2F/DP complex to activate transcription of its regulated genes (Gutierrez *et al.,* 2002).

In this study, we have further examined the effect of CycD expression on the transcriptional regulation of the rice *PCNA* gene. During the course of this experiment, we observed that the rice *PCNA* promoter was activated to a greater extent than the tobacco *PCNA* promoter. It has been reported that the E2F-binding sites in the rice and tobacco *PCNA* promoters play a significant role in activation of these promoters in proliferating tobacco tissues (Kosugi and Ohashi, 2002). Mutation of the E2F-binding sites reduced the activity of both promoters in transgenic tobacco plants. Although the rice *PCNA* promoter does not share a high degree of similarity with the tobacco *PCNA* promoter, it was capable of transcriptional activation in tobacco. Additionally, we found that the tobacco *PCNA* promoter in transient assays of tobacco protoplasts was higher activity than that of the rice *PCNA* promoter (data not shown), suggesting that the tobacco *PCNA* promoter is more strongly activated by endogenous tobacco E2F/DP complexes. This may explain our observation that the rice *PCNA* promoter is more strongly activated than the tobacco *PCNA* promoter when co-transfected with plasmids encoding E2F and DP.

To examine whether the rice *PCNA* promoter used for the experiment was sufficient to direct the S-phase-specific expression in tobacco as seen with the tobacco *PCNA* promoter (Sekine *et al.,* 1999), the rice *PCNA* promoter was used to drive the β-glucuronidase (GUS) reporter gene. These constructs were introduced into tobacco BY-2 cells by *Agrobacterium*-mediated transformation. Stably transformed cell lines were synchronized by treatment with aphidicolin (Nagata *et al.,* 1992), which caused complete arrest of the cell cycle in early S phase. As shown in Fig. 1A, the removal of aphidicolin resulted in synchronous progression through the cell cycle, with a peak in the mitotic index (35%) after 7 h. Cell cycle-dependent changes in the activity of the rice PCNA promoter were examined by measuring GUS transcript levels by RNA gel blot analysis. As shown in Fig. 1C and 1D, *GUS* transcript levels increased rapidly from 6 h, about 2 h prior to the start of DNA synthesis which was measured by monitoring the incorporation of [^{3}H] thymidine (**Fig. 1B**), and a peak was observed at 11 h. Subsequently, *GUS* transcript levels gradually declined to a level higher than that at the G2/M phase (between 2 and 6 h). On RNA blots, we have consistently observed two bands for *GUS* transcripts, which seems to reflect incomplete function of the nos terminator located downstream from the *GUS* gene. Similar results were observed in tobacco BY-2 cells expressing the *CYM* promoter-GUS construct (Ito *et al.,* 1997). The pattern of expression of *GUS* transcripts closely resembled that of the tobacco *PCNA* transcripts (Sekine *et al.,* 1999). Thus, activation of the rice *PCNA* promoter leads predominantly to S-phase-specific expression in tobacco BY-2 cells.

Next, we evaluated the ability of NtRBR1 to repress rice *PCNA* promoter activity in transient assays (**Fig. 2**). Tobacco BY-2 protoplasts were transfected with plasmids encoding tobacco NtE2F and *Arabidopsis* AtDPa, with or without the addition of plasmid encoding NtRBR1, when a reporter gene consisting of the rice *PCNA* promoter fused to a firefly luciferase (*LUC*) gene was used. To account for variation in transfection efficiency, the GUS reporter construct was included in all the experiments, and LUC activity was normalized against GUS activity. Each experiment was performed at least three times, with consistent results. Transient expression of NtE2F and AtDPa strongly increased LUC/GUS activity, whereas co-expression of NtRBR1 reduced this activity in a dose-

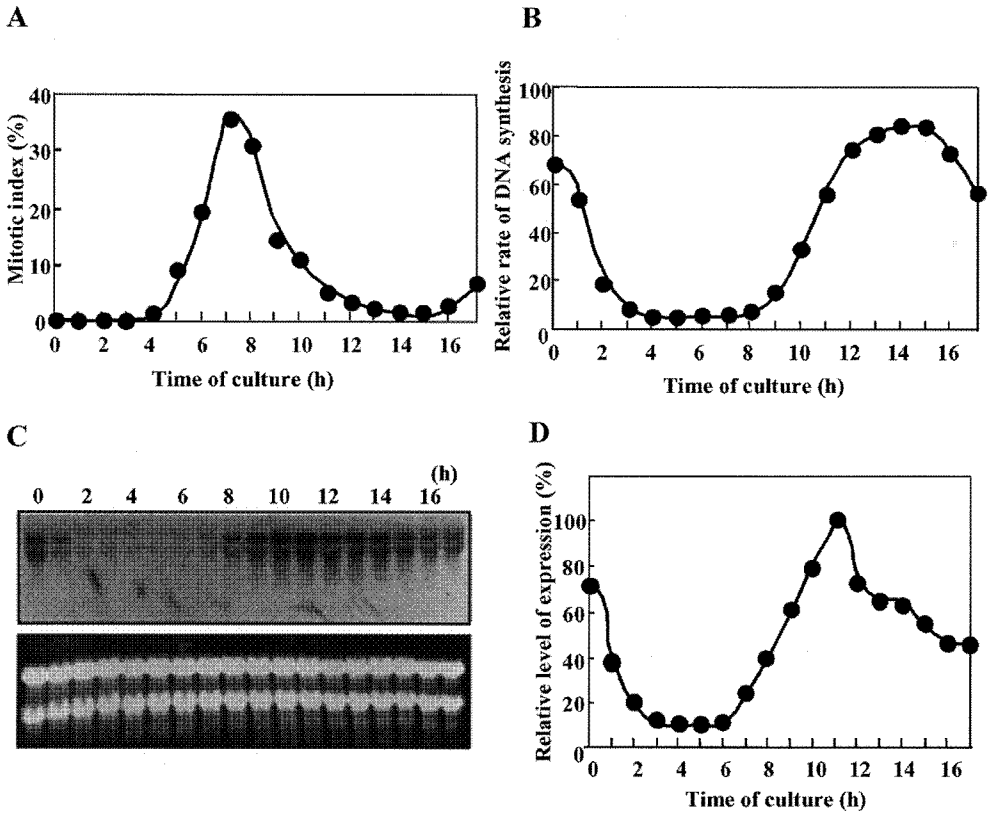


Fig. 1 S-phase-specific expression of the rice *PCNA* promoter. (A) Changes in mitotic index during synchronous culture of BY-2 cells. The efficiency of cell synchronization was examined by measuring the mitotic index at 1-h intervals. (B) The rate of DNA synthesis was monitored by measuring incorporation of [^{3}H] thymidine into DNA. (C) S-phase-specific transcription of the rice *PCNA* promoter-GUS transgene in tobacco BY-2 cells. Tobacco BY-2 cells transformed with the rice *PCNA* promoter-GUS construct were arrested by aphidicolin treatment. Cells were collected at 1-h intervals after release from aphidicolin block (0 h). Total RNA was isolated and hybridized with a probe consisting of the *GUS* coding region. Ethidium bromide staining of rRNA is shown below. (D) The relative level of *GUS* transcript was determined by quantifying the radioactivity in each signal on the RNA gel blot using a phosphorimager.

dependent manner (**Fig. 2**). It was previously shown that putative E2F-binding sites in the rice *PCNA* promoter mediate activation in actively dividing tobacco cells (Kosugi and Ohashi, 2002). Additionally, electrophoretic mobility shift assays (EMSAs) revealed that the rice E2Fs OsE2F1 and OsE2F2 bind the E2F-binding sites in rice and tobacco *PCNA* promoters. The DNA binding activity of both OsE2F1 and OsE2F2 was significantly stimulated by heterodimerization with *Arabidopsis* DP proteins (Kosugi and Ohashi, 2002). These observations suggest functional conservation of the regulatory factors involved in expression of the E2F-regulated genes between monocotyledonous and dicotyledonous plants. Therefore, we used the regulatory factors and the E2F-regulated genes arisen from the heterogeneous species. We observed, by EMSA with *in vitro* translated products, that the DNA binding ability of tobacco NtE2F was stimulated by the presence of *Arabidopsis* AtDPa (data not shown). This suggests that NtRBR1 is capable of binding an NtE2F/AtDPa-DNA complex derived from two different species through a direct interaction with NtE2F, furthermore, that NtRBR1 forms a ternary complex to repress the transactivation of the NtE2F/AtDPa complex.

In mammalian cells, two classes of cyclins, cyclin D and cyclin E, act during the G1 phase, and the sequential phosphorylation of retinoblastoma protein (pRb) by these G1 cyclins relieves the repression of E2F-controlled gene expression (Trimarchi and Lees, 2002). We previously showed that the repressor activity of NtRBR1 is blocked when it is further transiently co-transfected with a plasmid encoding CycD (Uemukai *et al.*, in press). Here, we evaluated the effect of CycD on the repressive activity of NtRBR1 towards the rice *PCNA* promoter when co-expressed with NtE2F/ AtDPa. To do this, cells were transfected with combinations of various amounts of plasmids encoding CycD and

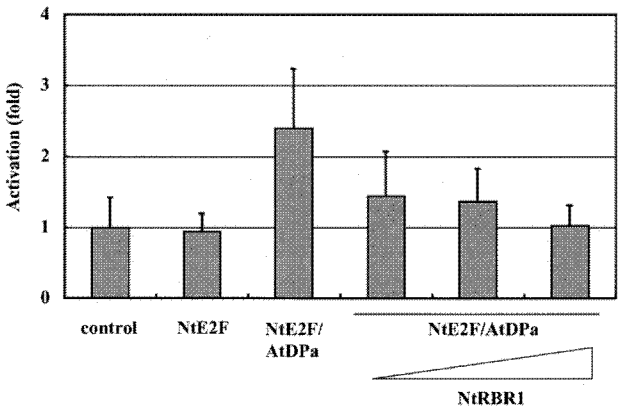
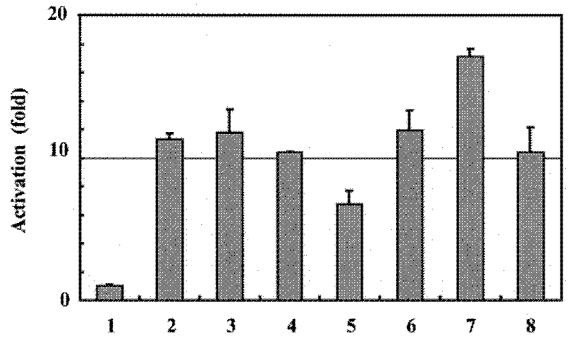


Fig. 2 Transactivation of NtE2F/AtDPa is repressed by co-expression with NtRBR1. Transfection of plasmid DNA into the tobacco BY-2 protoplasts was performed by electroporation. Tobacco BY-2 protoplasts were prepared from four-day-old cells as previously described (Dansako *et al.*, 2003). Protoplasts (1.5 x 10^6) were suspended in 0.5 ml electroporation buffer (5 μmM MES (pH 5.8), 70 mM KCl, 0.3 M mannitol), and electroporated at 250 μF and 200 V using a Gene Pulser (Bio-Rad Laboratories, Hercules, CA). Typically, protoplasts were transfected with different combinations of the rice *PCNA* reporter construct (10 μg), effector constructs [NtE2F (10 μg) and AtDPa (10 μg)] and internal control, CaMV35S-GUS (5 μg), with or without the addition of plasmid DNA encoding NtRBR1 (2, 5 and 10 μg). The CaMV35S-GUS construct, in which the *GUS* gene is placed under the control of CaMV35S promoter, was used as an internal control in each transfection. After incubation at 25℃ for 16 h, the LUC activity of the lysate from the transfected protoplasts was divided by the GUS activity to normalize the data for variation in transformation efficiency and cell viability. The results presented are the means of triplicate samples ± SD.



PCNA-LUC	10	10	10	10	10	10	10	10
NtE2F	–	10	10	10	10	10	10	–
AtDPa	–	5	5	5	5	5	5	–
NtRBR1	–	2	5	10	5	5	5	–
CycD	–	5	5	5	2	5	10	5

Fig. 3 Effect of cyclin D expression on transcriptional activation of the rice *PCNA* promoter. Tobacco protoplasts were transfected with plasmid DNA containing the rice PCNA reporter constructs [PCNA-LUC (10 μg)], effector constructs [NtE2F (10 μg) and AtDPa (10 μg)] and internal control [CaMV35S-GUS (5 μg)], along with the indicated amounts (in μg) of NtRBR1 and tobacco CycD3; 3 (CycD) (Nakagami *et al.*, 2002). For each transfected protoplast sample, the LUC activity was divided by GUS activity and expressed relative to the reporter construct alone (value set to 1). Error bars indicate ± SD.

NtRBR1 (**Fig. 3**). Remarkably, further co-expression of CycD overcomes the repressive activity of NtRBR1, suggesting that CycD-associated kinases probably phosphorylate NtRBR1 and allow the subsequent release of the NtE2F/AtDPa complex and activation of transcription (**Fig. 3** bars 2-7). However, the level of activation was significantly higher than that seen with transfection of only plasmids encoding NtE2F and AtDPa. Surprisingly, we found that strong activation of the rice *PCNA* promoter was observed by transfection with a plasmid encoding CycD alone (**Fig. 3** bar 8). This activation was enhanced by co-transfection with plasmids encoding NtE2F and AtDPa (**Fig. 3** bar 7), suggesting that the effect of CycD is synergistically regulated by NtE2F/AtDPa. However, NtRBR1 had no influence on the effect of CycD (**Fig. 3** bar 6). We therefore conclude that the effect of CycD is independent of the transfected NtRBR1, but our results do not rule out the possibility that CycD would affect the repressive activity of endogenous NtRBR1.

Recently, mammalian cyclin D has been reported to function in transcription, given that it interacts with several transcription factors to regulate their activity (Coqueret, 2002). Cyclin D1, but not D2 or D3, can activate estrogen receptor (ER)-mediated transcription. Importantly, a mutant form of cyclin D1 carrying a mutation in the cyclin box which abolishes CDK binding, potentiates ER activity to the same extent as wild-type cyclin D1 (Zwijsen *et al.*, 1998). This suggests that the kinase activity of the CDK partner is not involved in the effects of cyclin D1, and that free cyclin D1 activates ER transcription in a cell cycle-independent manner. It will be of particular interest to determine whether the effect of tobacco CycD expression on transcriptional activation is likewise mediated by a CDK-independent process.

Acknowledgements

The authors wish to thank Drs. Hiroshi Kouchi

and Kazuya Yoshida for their helpful discussions and suggestions throughout this work. We also thank Dr. Ian Smith for critically reading the manuscript. This research was supported by a Grant-in Aid for Scientific Research from the Ministry of Education, Sports, Science, Culture and sports, Japan.

References

Albani, D., Mariconti, L., Ricagno, S., Pitto, L., Moroni, C., Helin, K., Cella, R., 2000. DcE2F, a functional plant E2F-like transcriptional activator from *Daucus carota*. J. Biol. Chem., **275**: 19258-19267.

Coqueret, O., 2002. Linking cyclins to transcriptional control. Gene, **299**: 35-55.

Dansako, T., Kato, K., Satoh, J., Sekine, M., Yoshida, K., Shinmyo, A., 2003. Untranslated region of the *HSP18.2* gene contributes to efficient translation in plant cells. J. Biosci. Bioengi., **95**: 52-58.

Dewitte, W., Riou-Khamlichi, C., Scofield, S., Healy, J.M. S., Jacqmard, A., Kilby, N. J., Murray, J.A.H., 2003. Altered cell cycle distribution, hyperplasia, and inhibited differentiation in *Arabidopsis* caused by the D-type cyclin CYCD3. Plant Cell, **15**: 79-92.

Gutierrez, C., Ramirez-Parra, E., Castellano, M.M., del Pozo, J.C., 2002. G1 to S transition: more than a cell cycle engine switch. Curr. Opi. Plant Biol., **5**: 480-486.

Healey, J.M.S., Menges, M., Doonan, J.H., Murray, J.A. H., 2001. The *Arabidopsis* D-type cyclins, CycD2 and CycD3, both interact in vivo with the PSTAIRE cyclin dependent kinase, Cdc2a, but are differentially controlled. J. Biol. Chem., **276**: 7041-7047.

Ito, M., Marie-Claire, C., Sakabe, M., Ohno, T., Hata, S., Kouchi, H., Hashimoto, J., Fukuda, H., Komamine, A., Watanabe, A., 1997. Cell-cycle-regulated transcription of A- and B-type plant cyclin genes in synchronous cultures. Plant J., **11**: 983-992.

Kosugi, S., Ohashi, Y., 2002. E2F sites that can interact with E2F proteins cloned from rice are required for meristematic tissue-specific expression of rice and tobacco proliferating cell nuclear antigen promoters. Plant J., **29**: 45-59.

Magyar, Z., Atanassova, A., De Veylder, L., Rombauts, S., Inze, D., 2000. Characterization of two distinct DP-related genes from *Arabidopsis thaliana*. FEBS Lett., **486**: 79-87.

Mariconti, L., Pellegrini, B., Cantoni, R., Stevens, R., Bergounioux, C., Cella, R., Albani, D., 2002. The E2F family of transcription factors from *Arabidopsis thaliana*: novel and conserved components of the retinoblastoma/E2F pathway in plants. J. Biol. Chem., **277**: 9911-9919.

Meijer, M., Murray, J.A. H., 2000. The role and regulation of D-type cyclins in the plant cell cycle. Plant Mol. Biol., **43**: 621-633.

Mironov, V., De Veylder, L., Van Montagu, M., Inze, D., 1999. Cyclin-dependent kinases and cell division in plants-the nexus. Plant Cell, **3**: 29-41.

Nagata, T., Nemoto, Y., Hasezawa, S., 1992. Tobacco BY-2 cell line as the "Hela" cell in the cell biology of higher plants. Int. Rev. Cytol., **132**: 1-30.

Nakagami, H., Kawamura, K., Sugisaka, K., Sekine, M., Shinmyo, A., 2002. Phosphorylation of retinoblastoma-related protein by the cyclin D/cyclin-dependent kinase complex is activated at the G1/S-phase transition in tobacco. Plant Cell, **14**: 1847-1857.

Nakagami, H., Sekine, M., Murakami, H., Shinmyo, A., 1999. Tobacco retinoblastoma-related protein phosphorylated by a distinct cyclin-dependent kinase complex with Cdc2/cyclin D *in vitro*. Plant J., **18**: 243-252.

Oakenfull, E. A., Riou-Khamlichi, C., Murray, J.A.H., 2002. Plant D-type cyclins and the control of G1 progression. Philos. Trans. R. Soc. Lond. B. Biol. Sci., **357**: 749-760.

Pines, J., 1995. Cyclins and cyclin-dependent kinases: a biochemical review. Biochem. J., **308**: 697-711.

Planchais, S., Samland, A.K., Murray, J.A.H., 2004. Differential stability of *Arabidopsis* D-type cyclins: CYCD3; 1 is a highly unstable protein degraded by a proteasome-dependent mechanism. Plant J., **38**: 616-625.

Ramirez-Parra, E., Xie, Q., Boniotti, M.B,, Gutierrez, C., 1999. The cloning of plant E2F, a retinoblastoma-binding protein, reveals unique and conserved features with animal G (1)/S regulators. Nucl. Acids Res., **27**: 3527-3533.

Ramirez-Parra, E., Gutierrez, C., 2000. Characterization of wheat DP, a heterodimerization partner of the plant E2F transcription factor which stimulates E2F-DNA binding. FEBS Lett., **486**: 73-78.

Riou-Khamlichi, C., Menges, M., Healy, J.M., Murray, J. A.H., 2000. Sugar control of the plant cell cycle: differential regulation of *Arabidopsis* D-type cyclin gene expression. Mol. Cell. Biol., **20**: 4513-4521.

Sekine, M., Ito, M., Uemukai, K., Maeda, Y., Nakagami, H., Shinmyo, A., 1999. Isolation and characterization of the E2F-like gene in plants. FEBS Lett., **460**: 117-122.

Trimarchi, J.M., Lees, J.A., 2002. Transcription: Sibling rivalry in the E2F family. Nature Rev. Mol. Cell Biol., **3**: 11-20.

Uemukai, K., Iwakawa, H., Kosugi, S., de Jager, S., Kato, K., Kondorosi, E., Murray, J.A.H., Ito, M., Shinmyo, A., Sekine, M., Transcriptional activation of tobacco E2F is repressed by co-transfection with the retinoblastoma-related protein; cyclin D expression overcomes this repressor activity. Plant Mol. Biol. in press.

Zwijsen, R.M., Buckle, R.S., Hijmans, E.M., Loomans, C. J., Bernards, R., 1998. Ligand-independent recruitment of steroid receptor coactivators to estrogen receptor by cyclin D1. Genes Dev., **12**: 3488-3498.