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Transgenic tobacco plants overexpressing the *Nicta; CycD3; 4* gene demonstrate accelerated growth rates

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D-type cyclins control the onset of cell division and the response to extracellular signals during the G1 phase. In this study, we transformed a D-type cyclin gene, *Nicta;CycD3;4*, from *Nicotiana tabacum* using an *Agrobacterium*-mediated method. A predicted 1.1 kb cyclin gene was present in all of the transgenic plants, but not in wild-type. Northern analyses showed that the expression level of the *Nicta;CycD3;4* gene in all of the transgenic plants was strong when compared to the wild-type plants, suggesting that *Nicta;CycD3;4* gene driven by the CaMV 35S promoter was being overexpressed. Our results revealed that transgenic plants overexpressing *Nicta;CycD3;4* had an accelerated growth rate when compared to wild-type plants, and that the transgenic plants exhibited a smaller cell size and a decreased cell population in young leaves when compared to wild-type plants. [BMB reports 2008; 41(7): 542-547]

INTRODUCTION

Cyclin-dependent kinases (CDKs) play a central role in mediating cell-cycle progression and are regulated by their association with cyclin subunits, reversible phosphorylation, and association with other regulatory factors (1). Different groups of cyclins possess different expression characteristics. In comparison to animals, a unique feature of plant cell-cycle control is that quiescent meristematic and differentiated cells are capable of reentering the cell cycle (2). The cyclins are well conserved, so the naming of plant cyclin classes indicates their homology to their closest animal class (3). A number of A-type, B-type, and D-type cyclins in different plant species have been characterized. These plant cyclins, control cell-cycle progression by activating CDKs and can be suppressed by inhibitors of CDKs (ICKs) similar to the animal cyclins, Transgenic plants overexpressing ICKs grow to smaller sizes relative to wild-type plants (4-6).

There is a substantial evidence of a relationship between the rates of cell division and growth in response to environmental and physiological treatments (7, 8) However, D-type cyclins play an important role in the cell cycle's response to external signals by forming the regulatory subunit of cyclin-dependent kinase complexes in both animals and plants (9).

D-type cyclins have a prominent role in the G1-to-S transition and are referred to as G-specific cyclins (10). The overall homology between mammalian cyclin D and plant CycD is only 9-14% across the region corresponding to the cyclin core, but they share the retinoblastoma protein (Rb)-binding motif LxCxE (x is any amino acid). CycDs were first isolated from *Arabidopsis* by complementing a yeast strain deficient in endogenous G1 cyclin activity (11). D-type cyclins might have a substantial effect on plant growth. To study the role of CycD in plant growth and development, CycDs have been introduced into plants in order to observe the effect of ectopic expression. *CycD2;At* in transgenic tobacco plants has the function of increasing the rate of leaf generation and accelerating overall plant growth and development (12). Constitutive expression of CycD3 in transgenic plants allows induction and maintenance of cell division in the absence of exogenous cytokinin (13). Constitutive *CycD3;1* overexpression retards plant development in *Arabidopsis* (14).

Here, we characterized a D-type cyclin gene (GenBank accession No. AY776172) from *Nicotiana tabacum*, called *Nicta; CycD3;4*, in order to determine the effect of D-type gene overexpression on cell-cycle-related plant growth and development. In present study, the expression profile of this gene was assayed and its role in plant growth and development was discussed.

RESULTS AND DISCUSSION

Sequence analysis of D-type cyclin

A 1104 bp D-type cyclin including the stop codon TGA was obtained (GenBank accession no. AY776172). It is 48.8% identical to *Arabidopsis thaliana* cyclin D (AY063729) and 49.1% identical to *Arabidopsis thaliana* cyclin D (AY052665). Like other cyclins, including the mammalian cyclins, it has the Rb-binding motif LxCxE (17-21aa) at the N-terminus of the protein. It shares most of the conserved motifs of WxLxV (97-101aa) (single-letter code, x being any amino acid) and DR (120-121aa) with other plant CycDs. It shows a 96% identity

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with the CycD cloned from Tobacco Bright Yellow-2 cells by Sorrell (15). It was given the name *Nicta;CycD3;4* according to the conventions of plant cyclin nomenclature (3). A BLASTP search was conducted against proteins in the NR database for matching proteins. CLUSTALX1.81 was employed to generate multiple sequence alignments using the deduced amino acid sequence of the D-type cyclin and other cyclin D proteins from *Arabidopsis thaliana*.

Vector construction and molecular analysis of transgenic tobacco

The *Nicta;CycD3;4* gene was inserted into the plant expression vector under the control of the constitutive CaMV 35S promoter. Tobacco explants infected with *Agrobacterium* containing the *Nicta;CycD3;4* gene were selectively cultured on hygromycin medium. Finally, independent hygromycin-resistant plant lines were obtained and transplanted into soil. Four putative transgenic lines were randomly selected, and seeds (T1) resulting from those lines (T_0) were planted in a greenhouse under standard conditions. Four plants were assayed for each group. PCR was performed to confirm the transformation events. A predicted 1.1 kb PCR product was present in all of the independent transgenic plants, but not in the wild-type plants (Fig. 1b). Northern analyses showed that the expression level of the *Nicta;CycD3;4* gene was very strong in all of the transgenic plants, but the expression level in the wild-type plants was too weak to be detected (Fig. 1c). This observation suggested that in transgenic plants the exogenous *Nicta;CycD3;4* gene, driven by the CaMV 35S promoter, was overexpressed.

Phenotype of wild-type and transgenic tobacco plants at different stages

The growth and development of transgenic and wild-type plants under standard soil conditions were investigated. The results indicated that *Nicta;CycD3;4* overexpression in the transgenic line enhanced tobacco growth relative to that of wild type (Figs. 2a and 2b). Other than this accelerated growth and development of the transgenic lines relative to wild type, there was no visible morphological variation between them.

The growth patterns of wild-type and transgenic plants are shown in Fig. 2d. The aerial height was calculated every 10 days during plant development, and these measurements revealed an enhanced growth pattern. A 2-fold increase in aerial height were recorded at 30 d and *Nicta;CycD3;4* transgenic plants showed an accelerated growth rate especially from 50 d, while wild-type plant increased growth rate until 60 d, resulting in a further increase in the aerial height was observed at 60 d. Transgenic and wild-type plants initiated flowering at the same height and reached the same terminal height. However, because of the increased growth rate of *Nicta;CycD3;4* transgenic plants, flowering started approximately 10 days earlier (Fig. 2c)

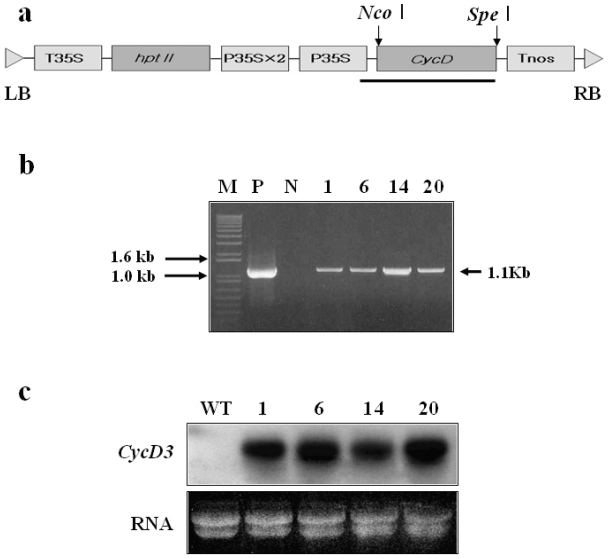

Fig. 1. Vector construction and molecular analysis of transgenic tobacco. (a) T-DNA map of binary pCAMBIA 1303 vector used for plant transformation. Bar indicates PCR-amplified regions. (b) PCR was performed using a forward primer specific to the CaMV 35S promoter and a reverse primer specific to the *Nicta;CycD3;4* cDNA. The expected fragment was 1.1 kb in size. M, 1 kb ladder marker; P, recombinant p1303 vector was used as positive control; N, DNA from a wild-type strain was used as a negative control; 1, 6, 14, and 20 DNA were from independent transgenic lines. (c) Northern analysis of *Nicta;CycD3;4* expression level in wild-type and transgenic plants. Total RNA was isolated from wild-type and transgenic plants, then hybridized with a *Nicta;CycD3;4* cDNA probe. WT represents wild-type plants; 1, 6, 14, and 20 represent independent transgenic lines.

SEM observation of leaf epidermal cells

In order to determine the effects of *Nicta;CycD3;4* expression, the size of cells taken from the surfaces of young leaves was measured for wild-type and transgenic plants. SEM analysis revealed that there was no significant morphological variation between the cells; merely, the cell size in the transgenic lines was slightly smaller relative to wild type (Fig. 3). The cell size of wild-type and transgenic plant was quantified as follows: wild-type plants, length: 108.3 $\pm$ 10.4 μm, width: 75.0 $\pm$ 5.0 μm (n=10); transgenic plants, length: 78.3 $\pm$ 16.1 μm, width: 60.0 $\pm$ 15.0 μm (n=15). These results suggest that the accelerated growth at the young stage of the transgenic tobacco is due to the enhanced number of cells and not the cell size, indicating an increased rate of cell division.

In plants, the expression level and activity of CycD cyclins in response to signals such as hormones and carbohydrate levels are important factors influencing the decision by plant cells to divide (16). D-type cyclins respond to signals such as cytokinin and sucrose at the G1/S boundary (13). These extracellular signals must be integrated with the cell-cycle control machinery during the G1/S transition in plants, and may utilize a similar mechanism as mammalian cells (17-19). In tobacco, *Nicta;*

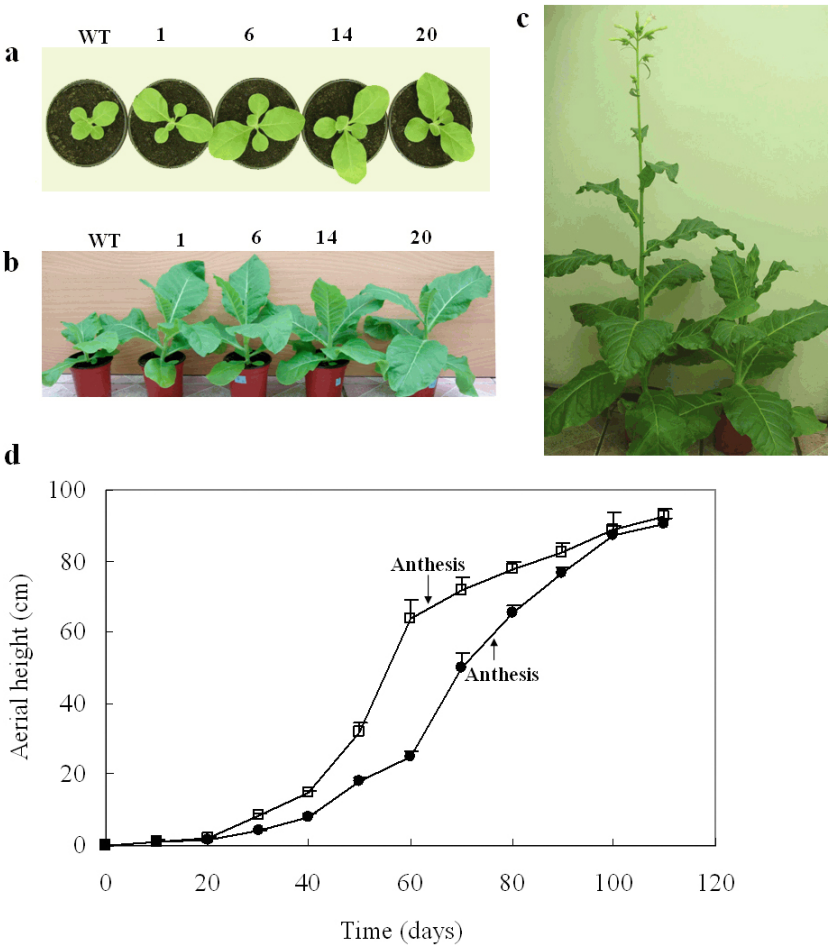

Fig. 2. Phenotype and growth curves of wild-type and transgenic tobacco plants at different stages. Transgenic plants from independent transgenic lines (1, 6, 14, and 20) and wild-type (WT) plants were cultured in a greenhouse. Photos were taken for 4-week-old (a) and 6- week-old (b) tobacco plants. (c) Transgenic plants flowered earlier (left) than wild-type (right). (d) Growth curves of wild-type (filled dot) and transgenic plants (open square) as established by measuring aerial height. Data are the average of 4 distinct measurements.

CycD3;3-associated kinases phosphorylate Rb-related proteins during the transition from middle-G1 phase to early-S phase (20). *Nicta; CycD3;1* and *Nicta; CycD2;1* transcripts accumulate between G2/M- to M-phases (15). Plant D-type cyclins are involved in controlling the commitment to cell division and the response of plant cells to extracellular signals during G1 (11, 13).

To date, CycD cyclin cDNAs have been isolated from various plant species. In the present study, the *Nicta;CycD3;4* gene was isolated from *Nicotiana tabacum* and shown to share the essential characteristics of CycD cyclins including the presence of the LxCxE motif. Northern analysis revealed that the expression level of the *Nicta;CycD3;4* gene was sharply enhanced in transgenic plants compared to wild-type plants. As a result, the transgenic plants exhibited an enhanced growth rate compared to wild-type plants, which was manifested as a faster growth and earlier flowering. However, the transgenic plants showed an increased growth rate only in the early vegetative stage, their final leaf sizes and aerial heights were not altered when compared to wild-type plants (Data not shown). Considering these results, we concluded that the

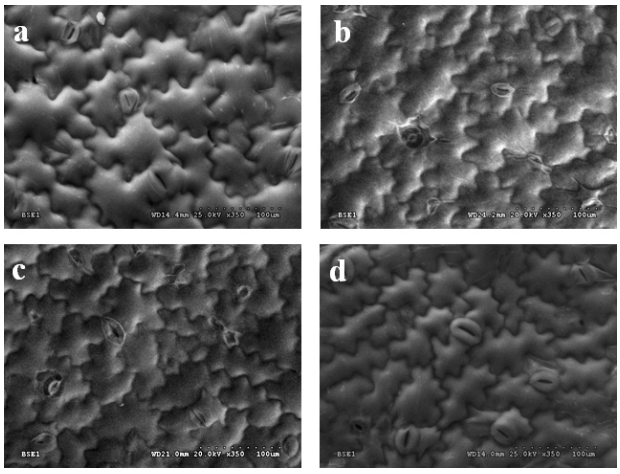

Fig. 3. SEM observations of leaf epidermal cells. Transgenic tobacco (b-d) showed smaller cell size relative to wild-type plants (a) by SEM assay. Bar = 100 μm. Leaves b, c and d were from lines 1, 6 and 20, respectively.

Nicta;CycD3;4 gene's ectopic expression accelerated the plant growth rate. The exogenous *CycD2;At* gene, driven by the CaMV 35S promoter, accelerated the cell division in the meristems by shortening the G1 phase and, as a consequence, the overall plant growth was accelerated (12). The shorter cell cycle resulted in faster growth- and leaf-generation rates, but the meristem size was unaffected at juvenile stages (9). The cell cycle was affected by CYCD2 and CYCD3 overexpression, as demonstrated by the enlargement of the peripheral zones and the accompanied increase in the leaf generation rate (21). Brassinosteroid functions have been reported to be involved in the promotion of plant cell division through CycD3 induction (22). The improvement in growth rate was induced by ectopic expression of CycD (6).

Ectopic expression of some regulators can alter not only the cell cycle but also cell morphology. CYCD2 and CYCD3 expression induced by hormones and sucrose might play a major role in plant response to external signals by controlling cell division and differentiation. 35S-CYCD3 plants show another type of SAM reorganization. The SAM is enlarged as in the 35S-CYCD2 plants and the cytophysiological zonation is maintained (21). *Arabidopsis* expressing very high levels of *CYCD3;1* demonstrate a reduced cell size and an enlarged proliferation zone (14). Transgenic *Arabidopsis* overexpressing ICK1 have larger cells than wild-type plants (5, 23), and they also show a smaller young-leaf-surface cell size relative to wild type (5). In our study, the cell size of young leaves was assayed. Transgenic plants showed a smaller cell size relative to wild type, however, no significant difference in the size of mature cells and leaves was observed between wild-type and transgenic plants. One possible explanation is that the overexpression of *Nicta;CycD3;4* accelerated cell division and therefore reduced the time required to reach the final cell number in one leaf. The reduced cell size in CycD transgenic plant was also supported by Zhou (24). The reduced cell population in G0-G1 and the increased population in G2-M in transgenic plants relative to wild-type plants has been explained as being a consequence of the shortened G1 phase due to the effect of CycD overexpression. ICK overexpression inhibits endoreduplication in *Arabidopsis* (25), however, an FCM assay showed that CycD overexpression, as a counterpart of ICK overexpression, did not induce exceptional endoreduplication in the cells of the transgenic plants.

Two mechanisms may be proposed to explain the *Nicta;CycD3;4* promotion of plant growth. First, CycD could specifically accelerate G1 and the increased rate of cell division would therefore be due to the direct action of CycD on cell-cycle progression. Alternatively, CycD kinase activity might function in plants by stimulating cellular growth within the meristem because of its effect on general biosynthesis, which could then lead to an increase in cell division perhaps as a consequence of the reduced time required to reach a critical cell size during G1 (26).

In the present study, the results showed *Nicta;CycD3;4* overexpression in tobacco increased the overall growth rate without affecting morphology. In animals, some CycD cyclins are overexpressed in cancer cells (27, 28) and ectopic expression in transgenic mice promotes adenocarcinomas (29). However, our results demonstrated that overexpression of *Nicta;CycD3;4* accelerated the growth rate of transgenic plants without affecting the morphology, and no neoplasia was observed in transgenic plants overexpressing this CycD gene. Considering the traits of the CycD, it suggests CycD may be used for enhancement of crop growth rates.

MATERIALS AND METHODS

Plant materials

Seeds of *Nicotiana tabacum* cv. 'Havana SRI' were obtained from the National Crop Experiment Station (Korea). For tissue culture, seeds were sterilized by dipping them in 70% ethanol for 1 min, 5% NaClO for 5 min, and then rinsed 3 times in sterile distilled water. The seeds were germinated on half-strength MS medium (30) with 20 g/L sucrose and 8 g/L agar. For subculture, the adventitious shoots were induced from axenic leaf disks on MS medium containing 20 g/L sucrose, 0.1 mg/L 1-Naphthylacetic acid (NAA), 0.5 mg/L 6-Benzylaminopurine (BA), and 8 g/L agar.

CycD gene cloning and plasmid construction

The CycD gene (GenBank accession no. AY776172) was obtained by RT-PCR amplification from total RNA of *Nicotiana tabacum* using a TaKaRa PCR Kit (TaKaRa, Japan). Forward (5'-GGGCCATGGGGCCATGGATGGTTTTCCCTTTAGATACTC-3') and reverse (5'-GGGACTAGTTCAACGAGGACTACTGCCCAC-3') primers were designed to amplify the complete ORF of the *Nicta;CycD3;4* gene from *Nicotiana tabacum* cDNA by RT-PCR technique. *Nco*I and *Spe*I endonuclease sites were added to the forward and reverse primers, respectively. The ORF finder program (http://www.ncbi.nlm.nih.gov) was used to locate the *Nicta;CycD3;4* cDNA sequence (31). Total RNA was isolated from young leaves of *Nicotiana tabacum* (or from genetic tumors derived from a hybrid of *N. langsdorffii* and *N. glauca*) using TRI-reagent® according to the manufacturer's instructions (MRC, USA). Total RNA was treated with 1 U DNase for 10 min at 37°C and then subjected to a second round of TRI-reagenet purification. From the DNase-treated total RNA (1 μg), first-strand cDNA was synthesized using the AccuPower® PCR PreMix (Bioneer, Korea) containing oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Invitrogen, USA). pCAMBIA 1303 was used to construct the plant expression vector, which carries hygromycin phosphotransferase gene (*HPT* II) driven by a double-enhancer version of the CaMV35S promoter (Fig. 1a). The *Nicta;CycD3;4* gene was introduced into pCAMBIA 1303 under the control of CaMV35S promoter. The recombinant binary vector was then transformed into *Agrobacterium tumefaciens* strain LBA4404.

Plant transformation and regeneration

A. tumefaciens cultures were grown at 28°C in YEP medium with shaking (200 rpm) to an OD_{600} of 0.6. The bacterium suspension was centrifuged and resuspended in 1/3 volume of MS medium. Tobacco leaf disks were immersed in *A. tumefaciens* suspension for 3-5 min (32). Two days after co-cultivation, the infected disks were transferred onto selection medium containing 20 g/L sucrose, 350 mg/L cefotaxime, 50 mg/L hygromycin, 0.1 mg/L NAA, 0.5 mg/L BA, and 8 g/L agar. Induced shoots were transferred to rooting medium supplemented with 20 g/L sucrose, 350 mg/L cefotaxime, 30 mg/L hygromycin, 0.2 mg/L NAA, and 8 g/L agar. The culture was maintained at 24 ± 2°C under a 16/8 h (light/dark) photoperiod.

PCR and Northern analysis of transgenic plants

For the confirmation of putative transformants, genomic DNA was isolated from leaves of putative transgenic and wild-type plants using the CTAB method (33). PCR was performed using a set of primers with forward: 5'-TGACGCACAATCCCACTATCC-3' and reverse: 5' TCAACGAGGACTACTGCCCAC-3'. Forward and reverse primers were specific to CaMV 35S promoter and CycD cDNA respectively. PCR conditions were as follows: an initial denaturation at 94°C for 2 min; 30 cycles of 30 s at 94°C, 30 s at 60°C and 2 min at 72°C; a final extension at 72°C for 10 min. PCR products were separated electrophoretically on a 1% agarose gel. To compare the expression level of *Nicta;CycD3;4* in transgenic and wild-type plants, total RNA was isolated from leaves of transformants (34). For Northern blot analysis, equal quantities of the total RNA (10 μg) were loaded into 1% agarose gel containing 7.4% formaldehyde. Membranes were hybridized overnight with a ^{32}P-labeled fragment of *Nicta;CycD3;4* cDNA in buffer consisting of 1% BSA, 1 mM EDTA, 0.5 M NaHPO4 (pH 7.2), and 7% SDS at 65°C, then washed in 0.5% BSA, 1 mM EDTA, 40 mM NaHPO4 (pH 7.2), and 7% SDS at room temperature for 5 min. The blot was then washed three times with high-stringency wash buffer (1 mM EDTA, 40 mM NaHPO4 (pH 7.2), and 5% SDS) at 65°C, and the dried blots were placed on X-ray film at -80°C for one week and then developed. To detect the effect of overexpression of *Nicta; CycD3;4* on plant growth, transgenic lines were selected randomly for analysis.

Effect of overexpression of CycD on plant growth

To detect the effect of overexpression of CycD on plant growth, 4 independent transgenic lines were selected randomly for analysis. Transgenic T_1 and wild-type plants were cultured with soil in a greenhouse under standard conditions. For determination of plant growth height, height of the aboveground portion was measured. The morphology of transgenic and wild-type plants were observed for the presence of variations.

SEM observation on leaf epidermal cells

The cell structure was observed by scanning electron microscopic (SEM) assay. Young leaves from transgenic and wild-type plants were cut and treated in liquid nitrogen, then the cells were surveyed using S-3500N scanning electron microscope (Hitachi, Japan).

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