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Overexpression of *NlgCycB* Isolated from Interspecific Hybrid of *N. langsdorffii* × *N. glauca* Alters Root Growth and Root Hair Development

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Abstract A cDNA clone encoding cyclin B (*NlgCycB*) was isolated from an interspecific hybrid of *N. langsdorffii* × *N. glauca*. The full-length cDNA had an open reading frame of 1,422 bp, with a deduced amino acid sequence of 473 residues. To investigate the role of *NlgCycB*, transgenic tobacco plants were constructed to overexpress *NlgCycB*. The transgenic plants showed premature root hair development in the elongation zone of the root tips. They had more and longer root hairs, which contained nuclei of increased size. Flow cytometric analysis revealed that the proportion of cells with 4C DNA content was increased in the transgenic plants. The *NlgCycB* gene in transgenic plants was coordinately enhanced with the expression of *CDKA* genes, especially in the root tips. The transgenic plants had greater growth than the wild-type in MS (1/10-strength) medium with reduced salt concentrations. Taken together, *NlgCycB* may play an important role in premature root hair development in the elongation zone of root tips and can aid in adaptation to low nutrient salt environments.

Keywords Elongation zone · Interspecific hybrid of *N. langsdorffii* × *N. glauca* · *NlgCycB* · Plant cell cycle · Root hair premature · Root hair development

Introduction

Plant root hairs are the projection of epidermal cells, increasing the root surface area, improving the plant's ability to take up water and nutrients, and playing an important role in helping to anchor the plant to the soil (Bibikova and Gilroy 2003; Ringli and others 2005). They are subcellular structures resulting from tubular outgrowths of single-root epidermal cells and are produced in the differentiating zone of the root (Carol and Dolan 2002). Root hair development is defined by four different stages: root hair specification, initiation, tip growth, and maturation (Gilroy and Jones 2000; Carol and Dolan 2002). Root hairs emerge from a subset of specialized epidermal cells called trichoblasts (Bibikova and Gilroy 2003). Root hair specification is important for determining root hair cell fate in epidermal cells. The fate of an epidermal cell may be determined by the relative abundance of *CAPRICE* (*CPC*), a negative regulator of *GL2* (Wada and others 1997), and *WEREWOLF* (*WER*), a positive regulator of *GL2* transcription (Schiefelbein 2000). In the process of root hair initiation, the trichoblast begins to form a highly localized expansion on one side to form a bulge in the cell wall, it is extended by tip growth, and leads to the formation of fully extended root hair (Gilroy and Jones 2000; Shi and Zhu 2002; Ringli and others 2005). Some mutants indicate that root hair elongation is regulated by hormones such as auxin

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and ethylene (Ringli and others 2005). The cell wall of the trichoblast is required for local acidification during root hair initiation (Bibikova and others 1998). Xyloglucan endotransglycosylase (XET) activity, which cleaves and rejoins xyloglucan chains of the cell wall, is detected in root hair initiation but is absent in *rhl1*, *rhd6-1*, and *axr2-1* mutants, which lack any root hair initiation (Vissenberg and others 2001). Several genes are involved in root hair tip growth, including *COW1*, *RHD3*, *RHD4*, *TIP1*, *WAVY*, *LRX1*, and *SOS4* (Shi and Zhu 2002). The maturation process plays an important role in determining the lengths of root hairs and is influenced by auxin, ethylene, and environmental factors (Pitts and others 1998; Schiefelbein 2000). Mutations in *AXR1*, *AUX1*, *ETR1*, and *EIN2* cause hairs to be shorter than normal but they retain their normal morphology (Pitts and others 1998).

Plant growth is normally coordinated with cell division in the meristem and the subsequent elongation of cells (Polymenis and Schmidt 1999). Both of these processes are under strict developmental control and respond to environmental stimuli. Final size and morphology of cells is determined by postmitotic cell division. The transcription factor *RHD6* has been reported to control the early stage of root hair cell differentiation. The *RSL4* (*ROOT HAIR DEFECTIVE 6-LIKE 4*, *RSL4*) gene is involved as a transcriptional regulator, integrates endogenous developmental and exogenous environmental signals, and promotes elongation of root hair cells in *Arabidopsis* (Yi and others 2010).

The cell division cycle in plants is controlled by cyclins and cyclin-dependent kinases (CDKs) that are conserved in eukaryotic organisms (Berckmans and De Veylder 2009; Marcel and Murray 2001). Cyclin-dependent kinases play a central role in mediating cell cycle progression, whereas CDK activity is regulated in association with a partner cyclin (Andersen and others 2008; Potuschak and Doerner 2001). The activity of the CDK–cyclin complexes is tightly controlled during the cell cycle and organ development. This process is regulated by phosphorylation and dephosphorylation events at conserved sites of the CDK, binding of inhibitors, and subcellular localization of the CDK–cyclin complex (Ohi and Gould 1999). Elevated *CDKA1* expression was reported in the mitotic cycle in plants (Verkest and others 2005), and the activation of cyclin B/CDK1 kinase complex in cells triggers entry into the mitosis phase (Weingartner and others 2004). The expression of the B-type cyclin is tightly controlled and restricted to late G2 and M phases (Francis 2007; Qiu and others 2007). It was reported that CDKA1 activity also peaks at the G1/S and G2/M phases in *Arabidopsis* (Joubes and others 2000). Promoter activity of three B-type cyclin genes, *Catro;CycB1;1* (*CYM*) from *Catharanthus roseus* (Ito and others 1997), *Nicsy;CycB1;1* from *Nicotiana sylvestris* (Tréhin and others 1999), and *Arath;CycB1;1* (*cyc1At*) from *Arabidopsis* (Shaul and others 1996), had similar expression patterns during the cell cycle, with a peak in GUS mRNA levels in the G2/M phase. Overexpression of *Arath;CycB1;1* caused acceleration of the root growth rate through enhanced G2-to-M progression via increased cell production and elongation of the root (Doerner and others 1996). Moreover, *CycB1;1* gene expression was localized to the root apical meristem, lateral root primordia, and shoot apical meristem (Ferreira and others 1994). However, the B-type cyclin family is large and the underlying mechanisms in plant development that are controlled by B-type cyclin are still poorly understood.

In this study we isolated the B-type cyclin gene (*NlgCycB*, accession No. AY776171) from an interspecific hybrid of *N. langsdorffii* × *N. glauca* and introduced it into *Nicotiana tabacum*, where it modified growth and surprisingly, root hair development. To investigate the role of the *NlgCycB* gene in root hair development, we examined the morphological characterization and expression profiles of overexpressed *NlgCycB* in transgenic tobacco plants. These overexpressing transgenic tobacco plants had salient morphological features: premature root hair development in the initiation zone of root tips and long root hairs. We also investigated the adaptability of transgenic plants with long root hairs in a low mineral medium and found that these plants could tolerate a limited nutrient level with enhanced growth.

Materials and Methods

Plant Materials

All plants were grown at 25°C in a growth room with a 16-h/8-h light/dark cycle. The *NlgCycB* gene was isolated from an interspecific hybrid of *N. langsdorffii* × *N. glauca*. For overexpression of the *NlgCycB* gene, the tobacco plant used for transformation was a *N. tabacum* cv. Xanti.

Gene Cloning and Sequence Analysis

Total RNA was prepared from young leaves of the interspecific hybrid of *N. langsdorffii* × *N. glauca* using a Trizol reagent kit (Invitrogen, Carlsbad, CA, USA). Reverse transcriptase polymerase chain reaction (RT-PCR) was performed with a RT-PCR kit (Takara, Japan). The specific primer of the *NlgCycB* gene was as follows: forward, 5′-ATGGCTTCAAGAATCGTTCTTC-3′ and reverse, 5′-TTAAGTCATCTTCCTAGTAGTAG-3′. The PCR product was cloned into a pGEM-T easy vector (Promega, Madison, WI, USA) and sequenced with T7 and SP6 primers. The sequence data of the *NlgCycB* gene from the interspecific

hybrid of *N. langsdorffii* × *N. glauca* has been registered in the GenBank (accession No. AY776171). Protein sequence alignments were conducted using the Clustal X program (Thompson and others 1997) with the default settings and manually adjusted. After removing the gaps from the initial alignments, a phylogenetic tree was generated using the neighbor-joining (NJ) method in the PHYLIP ver. 3.65 package (Felsenstein 1989). Bootstrap analysis was performed by repeating the procedure on 1,000 data sets.

Vector Construction and Plant Transformation

To generate *NlgCycB* overexpression constructs, the coding region of *NlgCycB* cDNA was amplified by PCR and was cloned into the pGEM-T easy vector (Promega). The PCR fragment was then isolated by a first digestion with *Nco*I and a second digestion with *Spe*I. It was introduced into the binary pCAMBIA 1303 vector (Invitrogen) that had been opened previously by an *Nco*I and *Spe*I double digestion, resulting in *NlgCycB* under the control of the CaMV35S promoter and the *NOPALINE SYNTHASE* terminator. The recombinant binary vector pCAMBIA 1303–NlgCycB, carrying the hygromycin phosphotransferase gene (*hpt*) as a selectable marker, was transferred into *Agrobacterium tumefaciens* strain LBA4404.

Surface-sterilized tobacco leaf disks were immersed in *A. tumefaciens* suspension for 5–10 min and then cultured on MS basal medium for 2 days (Horsch and others 1988). The infected disks were transferred onto selection medium containing 20 g l^{-1} sucrose, 350 mg l^{-1} cefotaxime, 50 mg l^{-1} hygromycin, 2 mg l^{-1} BA, and 3.2 g l^{-1} gelrite. Induced shoots were transferred to MS basal medium supplemented with 30 g l^{-1} sucrose, 350 mg l^{-1} cefotaxime, 30 mg l^{-1} hygromycin, and 3.2 g l^{-1} gelrite.

For the confirmation of putative transformants, genomic DNA was isolated from leaves of putative transgenic and wild-type plants using the CTAB method (Doyle and Doyle 1987). The PCR was performed using a set of specific primers to *NlgCycB*, *HPT* (hygromycin phosphotransferase), and CaMV 35S promoter, respectively. The expected fragment was about 1.4, 0.9, and 0.7 kb, respectively. The PCR reaction program was as follows: an initial denaturation at 94°C for 5 min; 25 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.

Histological and Microscopic Analysis

For histological analysis, root tips from the 2-week-old plants were fixed with 2.5% glutaraldehyde and 4% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) and embedded in EPON resin. For longitudinal and transverse sections, the plastic blocks were cut using an ultramicrotome (MTX, RMC Products, Tucson, AZ, USA). Semi-thin (50 μm) sections were examined under a light microscope after staining with toluidine blue. For confocal laser scanning microscopy (LSM510 META LNO, Carl Zeiss Jena GmbH, Germany), samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 3 h at 4°C and then stained with 4′,6′-diamidine-2-phenylindole-dehydrochloride (DAPI) and propidium iodide (PI). Cell dimensions were estimated and statistically analyzed with Image J software from the National Institutes of Health (NIH, Bethesda, MD, USA).

Flow Cytometric Analysis

To investigate the effects of overexpression of *NlgCycB* on the plant cell cycle, the DNA content was measured by flow cytometric analysis. Roots from seedlings of wild-type and transgenic plants were cut on a cold plastic petri dish. The 15-mm region from the root tip was chopped into small pieces with a razor blade in 1 ml of LB01 lysis buffer; 15 mM Tris-base, 2 mM Na_2EDTA, 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl, 0.1% (vol/vol) Triton X-100, and 15 mM β-mercaptoethanol (Doležel and others 2007). Suspensions were filtered through a nylon mesh (pore size = 100 μm) to remove large cellular debris and added to final concentrations of 100 μg ml^{-1} PI and 10 μg ml^{-1} RNase. The solution was incubated on ice for 10 min and analyzed with excitation at 488 nm in a BD FACScalibur cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Expression of Cell Cycle-related Genes

Total RNA was isolated from different tissues (root tip, root elongation region, shoot apex, leaf, and stem) of transgenic and wild-type *N. tabacum* and wild-type of the interspecific hybrid using an RNA Plant Mini Kit (Qiagen, Valencia, CA, USA). The RNA samples were then reverse-transcribed by the ImProm-II Reverse Transcription System (Promega). The first-strand cDNAs were used as a template for semi-quantitative RT-PCR analysis. The amplification profile consisted of one cycle of 5 min at 95°C, followed by 25 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C, with a final extension of 10 min at 72°C. The following pairs of primers were used: 5′-GTATGCATCTCAAATAAGAGTTAC-3′ and 5′-GCTAGATCGCTTCGCCCCGAT-3′ for *NlgCycB* (B-type cyclin of interspecific hybrid, AY776171), 5′-TGTGTGCATCTCAGACAAAAGT-3′ and 5′-TGCTCCGACAACACACTTGA-3′ for *NtCycB1* (B-type cyclin of *N. tabacum*, D50737), 5′-ATAACGAGGGGCTTTTGTGC-3′ and 5′-CTTTTCTCCATTCCCAACACCT-3′ for *NtCycB2* (B-type cyclin of *N. tabacum*, D89635), and 5′-GAGGGAGTACCAAGCACAGC-3′ and 5′-ATCTCGGAGTCACCAGGAAA-3′ for *CDKA* (A-type cyclin-dependent

kinase of *N. tabacum*, AF289467). PCR products were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide. β-actin was used as an internal control for semiquantitative RT-PCR.

Pigment Determination

The transgenic and wild-type plants were cultured on various salt strengths of liquid MS medium (full, 1/3, 1/6, and 1/10 strength) and maintained at 25 ± 2°C and a 16-h photoperiod with white fluorescent light of 50 μmol m^{-2} s^{-1}. After 3 weeks, chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids were extracted from 1 g of fresh leaves in 80% acetone and measured spectrophotometrically according to Aron (1949) and Lichtenthaler and Wellburn (1983).

Results

Isolation of B-type Cyclin Gene in Interspecific Hybrid

A full-length cDNA fragment was isolated from an interspecific hybrid of *N. langsdorffii* × *N. glauca*, designated as *NlgCycB* (accession No. AY776171). The cDNA of the *NlgCycB* gene was 1,422 bp in length and had a complete open reading frame, with a deduced amino acid sequence of 473 residues (Fig. 1a). The *NlgCycB* gene showed 94.9 and 66.1% homology to two *N. tabacum* cyclin B genes, accession Nos. D50737 (Setiady and others 1995) and D89635 (Ito and others 1997), respectively. Also, it was 59.3% identical to *C. roseus* (accession No. D86386) and 57.6% identical to *Cicer arietinum* (accession No. D287306) cyclin B genes.

The putative NlgCycB protein had a cyclin box and a destruction motif (RXALGVIXN) and was homologous to other B-type cyclins (Fig. 1a). A 12-amino-acid-long motif with a continuous stretch of six conserved residues (LTARSK) was also present in *NlgCycB* (Fig. 1a), a hallmark of the B-type cyclin group (Renaudin and others 1996). The putative destruction box, containing the conserved sequence RXALGVIXN, is thought to be involved in the destruction of mitotic cyclins through the ubiquitin pathway (Glotzer and others 1991). To assess the relationship between NlgCycB and mitotic cyclin proteins, we conducted phylogenetic analysis using the NJ method (Felsenstein 1989). Based on multiple alignments of the deduced amino acid sequences of NlgCycB and the mitotic cyclin proteins of other plants, a phylogenetic tree is shown in Fig. 1b. These analyses suggested that *NlgCycB* should be classified as a B1-type cyclin.

A reverse transcriptase polymerase chain reaction was performed to determine the tissue-specific expression profile of the *NlgCycB* gene in the interspecific hybrid of *N. langsdorffii* × *N. glauca*. The results showed that *NlgCycB* expression occurred in an organ-specific manner, with the strongest expression in the roots and shoot apices, comparatively lower expression in the leaves, and faintly detectable expression in the stem (Supplementary Fig. 1a). Three B-type cyclins from *Arabidopsis*, *Arath;CycB1;2*, *Arath;CycB1;3*, and *Arath;CycB1;4*, showed high expression in the root, callus, and flower, respectively (Day and Reddy 1998).

Overexpression of *NlgCycB* in Transgenic Tobacco Plants

To characterize putative transformants, PCR was performed using a set of specific primers for *NlgCycB*, *HPT* (hygromycin phosphotransferase), and the CaMV 35S promoter. The expected fragments were about 1.4, 0.9, and 0.7 kb in size, respectively. These PCR products for the *NlgCycB*, *HPT*, and CaMV 35S promoter genes had clear bands in all of the putative transgenic plants, but no band was observed in wild-type plants (Supplementary Fig. 1b).

The overall morphology of the transgenic plants was similar to that of the wild-type. However, root hair development in the elongation zone of the root tips showed a striking difference (Fig. 2). The interspecific hybrid of *N. langsdorffii* × *N. glauca* exhibited the shortest distance from the root tip to the first root hair and the longest root hair in the root tip (Fig. 2c) when compared with other plants. The length of the root hair in the transgenic plant was significantly longer than that observed in wild-type *N. tabacum* plants (Fig. 2b). At least 200 root hairs from five individual plants were measured to determine the mean root hair length in the initiation and elongation region at 7 and 15 mm from the root tip. The root hairs at both 7 and 15 mm from the root tip were shortest in wild-type *N. tabacum* and longest in wild-type plants of the interspecific hybrid (Supplementary Fig. 2a).

To observe the morphology of the root tip in more detail, the roots from semithin root sections of wild-type and transgenic plants were examined by scanning electron microscopy (SEM) and light microscopy. The transgenic plants had more and longer root hairs than wild-type plants (Supplementary Fig. 2b, c). In addition, the distance from the root apical meristem to the region of root hair initiation was much shorter than in wild-type plants (Supplementary Fig. 2d, f). The density of the root hair in the region of root hair initiation was also higher than in wild-type plants (Supplementary Fig. 2e, g).

The diameter and distance from the root tip to the first root hair of the root hair initiation region were measured on

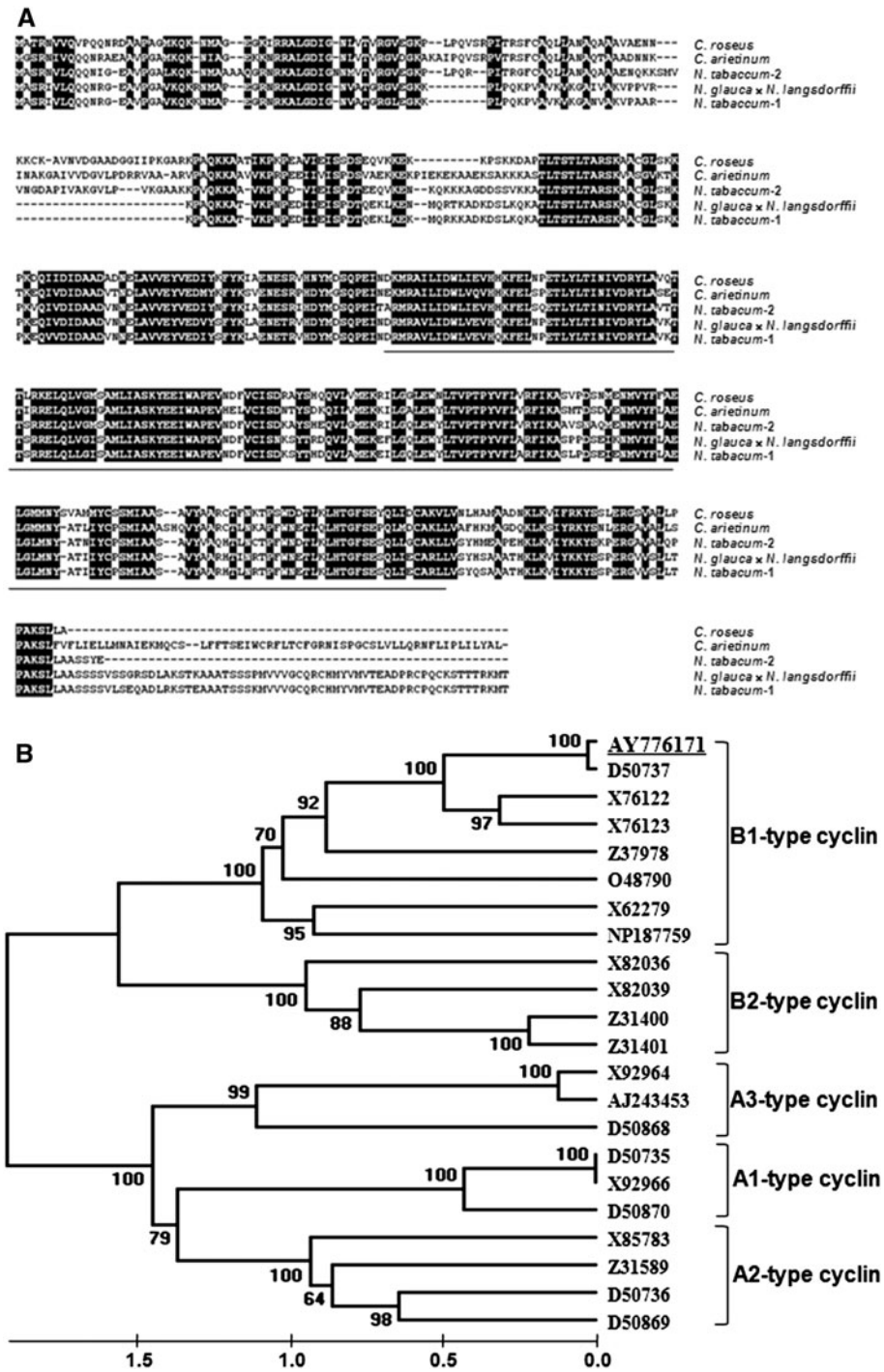

Fig. 1 Amino acid sequence comparison of cyclin B. **a** Alignment of NlgCycB in the interspecific hybrids of *N. langsdorffii* × *N. glauca* with other cyclin B proteins. The alignment was obtained using the BLAST program and the sequences of the deduced amino acids were aligned using Clustal X. Black regions indicate identical positions; dashes represent gaps introduced to maximize identity. The cyclin box regions are underlined. **b** Phylogenetic tree showing the relationship between NlgCycB and other mitotic cyclin proteins. Multiple alignments were used to construct a rooted neighbor-joining tree, based on sequence similarity. Interspecific hybrids of *N. langsdorffii* × *N. glauca* (AY776171); *N. tabacum* (D50735, D50736, D50737, D89635, X92964, X92966, Z37978); *A. majus* (X76122, X76123); *A. thaliana* (NP173083, NP173485, NP175077, NP175155, NP177758, NP177863, NP180244, NP187759, NP199122, NP564446, NP564499, NP973983, L27223, O48790, U17889, U17890, X62279, Z31400, Z31401, Z31402, Z31589); *O. sativa* (X82036); *M. sativa* (X82039, X85783); *G. max* (D50868, D50869, D50870, X62820); *D. carota* (AB091346, X62819) *P. sativum* (PSU52520)

longitudinal image sections (Fig. 2e, f, m). The root diameter of the wild-type plant was 1.34-fold greater than that of the transgenic plants in the initiation region of the first root hair (Fig. 2m). Also, the initiation zone of root hairs in transgenic plants was closer to the root apical meristem than in wild-type *N. tabacum* (Fig. 2m). To examine whether the short distance from the root tip to the first root hair in the *NlgCycB*-overexpressed plant was a result of earlier differentiation of root hair or influence of cell division, we measured the epidermal cell length in three positions: the root meristematic zone (denoted by ① in Fig. 2), the initiation zone of the root hair in the transgenic line (②), and the elongation zone of the root hair in the transgenic line (③). The average length of the epidermal cell at the root meristem was almost the same for both wild-type and transgenic plants (Fig. 2n①). However, the

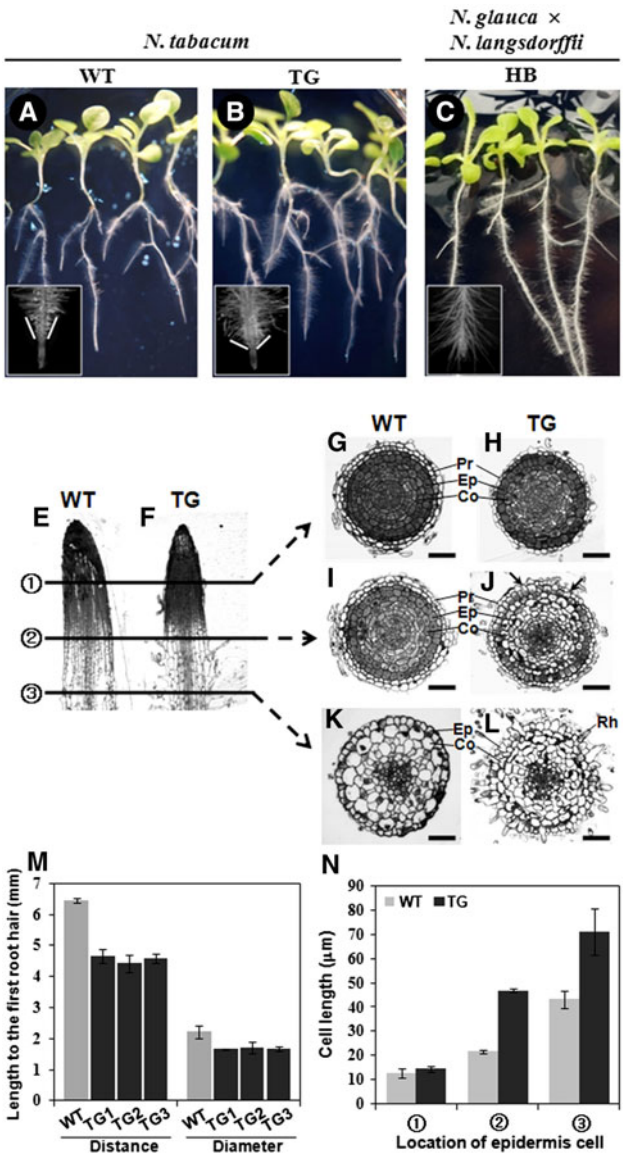

Fig. 2 Plant phenotypes of wild-type *N. tabacum*, transgenic *N. tabacum* plants, and wild-type interspecific hybrid plants. **a–c** Morphological analysis of wild-type *N. tabacum* (WT; **a**), transgenic *N. tabacum* plants (TG; **b**), and wild-type interspecific hybrid plants (HB; **c**) after 15 days of germination. **e–i** Structural characterization of the root tip cells in wild-type (**e**, **g**, **i**, **k**) and transgenic plants (**f**, **h**, **j**, **l**). Arrows indicate epidermal cells prematurely developing into root hairs. **m** The diameter and distance from the root tip to the first root hair in wild-type (WT) and transgenic plants (TG). **n** The epidermal cell length was measured in three positions: the root meristematic zone (①), the initiation zone of root hairs in the transgenic line (②), and the elongation zone of root hairs in the transgenic line (③). *Pr* protoderm, *Ep* epidermis, *Co* cortex, *Rh* root hair. *Scale bar* = 50 μm

epidermal cells of transgenic plants were significantly longer in both the initiation and the elongation region of root hairs when compared with the wild-type plants (Fig. 2n②, ③). These results indicate that the roots of transgenic plants had premature elongation of epidermal cells for root hair development.

To determine how the structural changes of the root affected cell volumes, we also measured cellular dimensions in the epidermal and cortex cells on transverse image sections (Fig. 2g–l and Supplementary Fig. 2h). The transverse section of root tips revealed that cells in the root meristem zone were approximately equal in size to those of the wild-type plant (Fig. 2g, h). There was a striking difference in the position where the root hair started on transgenic plants (Fig. 2i, j). The transgenic plants had some epidermal cells forming root hair bulges in this site (Fig. 2j, arrow), and these cells were prematurely elongated for root hair development (Fig. 2l). However, the wild-type plants had many protoderm cells and epidermal cells of almost uniform shape (Fig. 2i, k). The diameters of the epidermal cells in the elongation region of transgenic plants were significantly greater than those in wild-type plants, but the diameters of the cortex cells were shorter than those in the wild-type plants (Fig. 2k, l; Supplementary Fig. 2h).

To investigate the differentiation rate of root hairs after germination, we measured the ratio of the length of the root hair initiation region (denoted by y in Supplementary Fig. 2i) to total root length (denoted by x in Supplementary Fig. 2i). Until 3 days after germination, the differentiation rate of the root hair (y/x) was similar between wild-type and transgenic plants. At 5 days after germination, however, y/x was low in transgenic plants compared with that in the wild-type, suggesting that the transgenic plants prematurely started root hair differentiation in the initiation zone of the root tip (Fig. 2f; Supplementary Fig. 2i). These results suggest that elongation of the root hair in transgenic plants was significantly faster than in wild-type plants.

Analysis of DNA Contents

As the epidermal cells were significantly larger in transgenic plants, we investigated the nuclear size and DNA content in root epidermal cells. The roots of wild-type and transgenic plants were stained with DAPI and PI. Confocal laser microscopy showed that the nuclei of the epidermal cells in transgenic plants were larger than those in wild-type plants (Fig. 3a–d). Specifically, the nuclei in the root hair cells of transgenic plants were larger and more ellipsoidal than those seen in wild-type plants (Fig. 3d).

To confirm this finding, root cells (up to 15 mm from the tip) were stained with PI and then the DNA content was measured by flow cytometry. The DNA content distribution was 43.3 and 45.7% in 2C and 4C in wild-type plants (Fig. 3e), and 32.9 and 65.1% in 2C and 4C in transgenic plants (Fig. 3f), respectively. The ratio of 4C to 2C DNA content in the transgenic plant (1.98) was significantly greater than that observed in the wild-type plant (1.06; Fig. 3g).

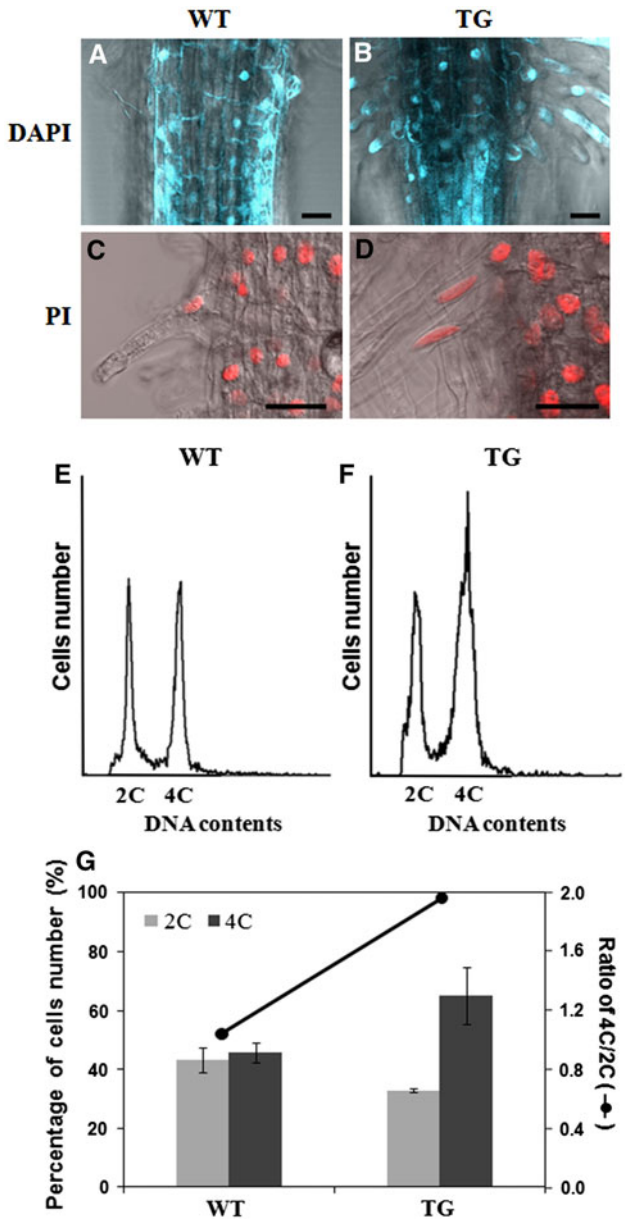

Fig. 3 Confocal fluorescence imaging of the root tip from wild-type and transgenic plants. The nucleus was stained with DAPI (**a**, **b**) and PI (**c**, **d**). Comparison of the distribution of DNA content in wild-type (WT; **e**) and transgenic plants (TG; **f**) by flow cytometry. **g** The ratio of 4C to 2C DNA content (*solid line*) and the percentage of cell number to 2C and 4C, respectively. *Scale bar* = 50 μm

Expression Pattern of Cell Cycle-related Genes

To investigate whether the ectopic overexpression of *NlgCycB* had an effect on the expression of other cell cycle-related genes, semiquantitative RT-PCR analysis was performed in various tissues from transgenic and wild-type *N. tabacum* and from wild-type interspecific hybrids. The expression of the *NlgCycB* gene, driven by the CaMV 35S promoter, showed a largely constant expression level regardless of tissue type, but was particularly strong in the root (Fig. 4). The expression level of *NlgCycB* in the root tip (RT) was higher than that seen in the root elongation (RE) region (Fig. 4a). The overexpression of the *NlgCycB* gene strongly elevated expressions of two other B-type cyclins (*NtCycB1* and *NtCycB2*) in transgenic plants (Fig. 4b, c). The *CDKA* transcript was also significantly upregulated in the RT of transgenic plants (Fig. 4d). Due to complex formation between CDKA and other cyclins, it was considered that the expression of *CDKA* might have a high level in different organs with low or no expression of B-type cyclins. The results showed that *NlgCycB* was preferentially expressed in the RT and enhanced the expression of *CDKA*.

Growth Response of *NlgCycB*-overexpressing Transgenic Plants

To investigate the effects of overexpression of *NlgCycB* on plant growth, transgenic and wild-type plants were cultured in liquid MS media with various salt strengths (full, 1/3, 1/6, and 1/10). A striking difference in growth was observed between the wild-type and transgenic plants cultured on 1/10-strength MS medium (Fig. 5a, b). The transgenic plants cultured on 1/10-strength MS medium had many more root hairs and were taller than the wild-type plants. The stem length of the transgenic plants was 1.47-fold greater than that of wild-type plants (Fig. 5c). Because the transgenic plants had more green and large leaves than the wild-type plants, we measured the chlorophyll *a*, chlorophyll *b*, and carotenoid content. The chlorophyll *b* and carotenoid contents of the transgenic plants were similar to those of the wild-type, regardless of the salt strength of the MS medium (Fig. 5d). The chlorophyll *a* content was similar in both the wild-type and transgenic plants cultured in full and 1/3-salt strength MS medium. However, the chlorophyll *a* content was significantly higher in the transgenic plants than in the wild-type plants when cultured on reduced salt-strength (1/6 and 1/10) MS medium (Fig. 5d).

Discussion

In the present study, a B-type cyclin gene (*NlgCycB*) was isolated from an interspecific hybrid of *N. langsdorffii* × *N. glauca*, and it was shown that its expression can prematurely activate root hair initiation and promote enhanced growth under low nutrient conditions. Sequence comparison showed that the deduced amino acid sequence of *NlgCycB* shared 70–95% homology with known B-type cyclins from other plants. On the basis of primary structure

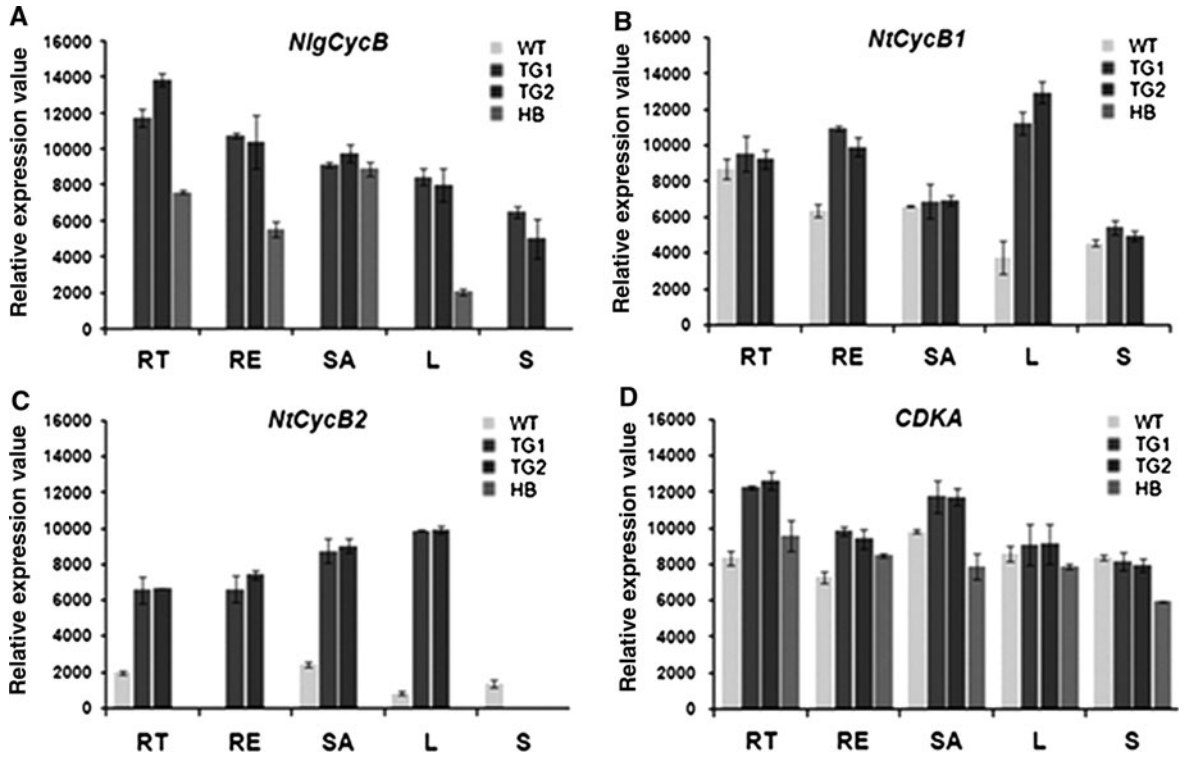

Fig. 4 Expression pattern analysis of several genes involved in the regulation of the cell cycle. Total RNA was isolated from several different tissues of wild-type *N. tabacum* (WT), transgenic plants (TG), and wild-type interspecific hybrids (HB). The *y* axis represents the relative mRNA expression value of the *NlgCycB* (**a**), *NtCycB1* (**b**), *NtCycB2* (**c**), and *CDKA* (**d**) gene, respectively. The relative quantification of expression of cell cycle-related genes was normalized against the actin gene in each isolate. *RT* root tip, *RE* root elongation region, *SA* shoot apex, *L* leaf, *S* stem. *Error bars* indicate the standard deviation; $n = 3$

comparison and the phylogenetic tree, we conclude that *NlgCycB* is a typical B-type cyclin (Fig. 1).

In our study, overexpression of *NlgCycB* in transgenic tobacco plants resulted in striking changes in morphological features in root hair development. The transgenic plants had more and longer root hairs than wild-type plants (Fig. 2). During the process of root development after germination, the timing of root hair differentiation in transgenic plants began early in the initiation zone of the root tip and accelerated the elongation of root hairs (Fig. 2f; Supplementary Fig. 2i). Due to rapid differentiation into root hairs in the initiation zone, there was a very short distance from the root apical meristem to the first root hair in transgenic plants. Other reported results were similar to our data on the morphology of root development. The *shy2-2* root had longer root hairs and a reduced distance between root tip and first root hair, and its phenotype was caused by early initiation of root hair growth (Knox and others 2003). Treatment of primary root meristems of *Allium cepa* with hydroxyurea (to stop DNA replication and to increase the length of the cell) gave rise to highly elongated cells and an enhanced concentration of cyclin B-like proteins (Zabka and others 2010). Based on these data, our results suggest that *NlgCycB* might be part of the regulatory mechanisms linked to premature root hair differentiation in the root meristem.

Because the transgenic plants had a large number of elongated root hairs in the elongation region (denoted by ③ in Fig. 2), the epidermal cells at this site were more differentiated into root hairs and larger than those of wild-type plants (Supplementary Fig. 2h). Cell differentiation in plants is often reached by a rapid increase in cell size by cell expansion (De Veylder and others 2007; Dello Ioio and others 2008). Plants during the developmental process can achieve an increase in cell size by increasing ploidy levels through endoreduplication, although they are still growing (John and Qi 2008; Sugimoto-Shirasu and Roberts 2003). In particular, an endoreduplication is also important for the development of root hairs that are significantly elongated (Sugimoto-Shirasu and others 2005). There are numerous experimental data reported for ploidy-dependent cell expansion (Sugimoto-Shirasu and others 2005; Breuer and Sugimoto-Shirasu 2007). Like root hairs, in the leaf trichome, which is developed through rapid cell growth, the trichome cell size in *gtl1* was positively accompanied by an increase in trichome ploidy level (Breuer and others 2009). Tomato fruit development comprises a cell division phase and a consecutive phase of cell expansion in which tomato

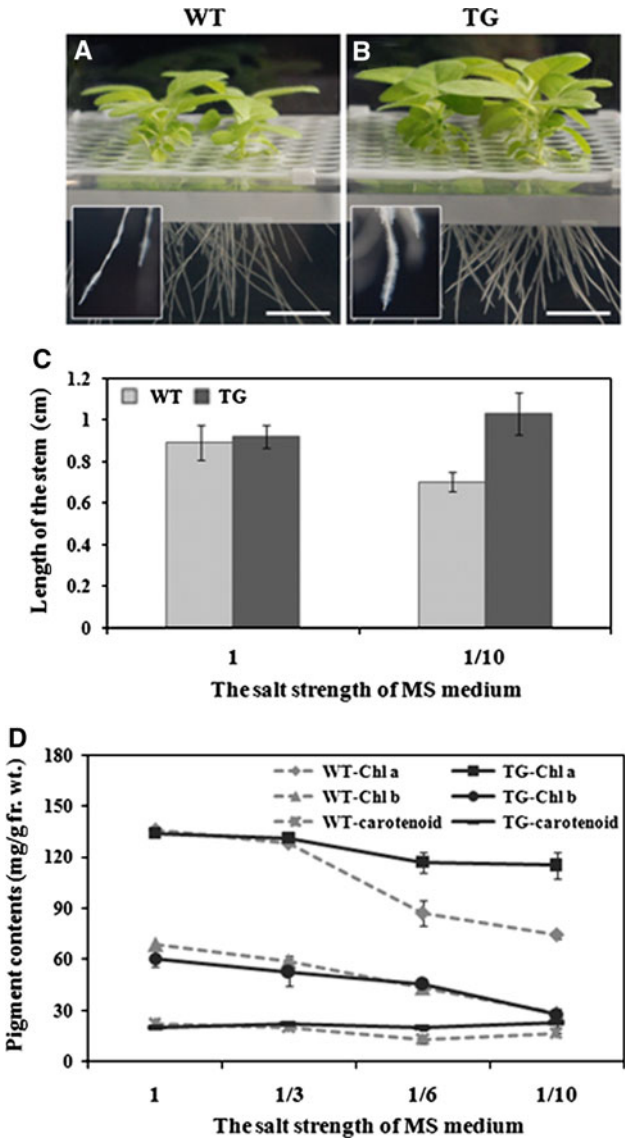

Fig. 5 Growth response of *NlgCycB* transgenic plants. Wild-type (WT; **a**) and transgenic plants (TG; **b**) were cultured on 1/10-strength liquid MS medium. **c** The length of the stem was measured in wild-type and transgenic plants cultured on full and 1/10-strength MS media. **d** The chlorophyll and carotenoid content of wild-type and transgenic plants cultured on various salt strengths of MS medium. *Scale bar* = 2 cm

fruit cells are dramatically increased in nuclear ploidy level (Joubes and others 1999; Teyssier and others 2008). In transgenic tobacco plants, both the nuclear size and the ratio of 4C to 2C DNA content in the root hair cells were significantly increased compared to those in wild-type plants (Fig. 3). As nuclei were isolated from all cells in the elongation region as well as from the root hairs, differences in the DNA content between wild-type and transgenic plants might appear to be less than the actual difference in the nucleus size of the root hairs. It has been suggested that the abundance of B-type cyclins in plants may be a limiting factor that determines the rate of cell division during developmental processes (Doerner and others 1996; Ferreira and others 1994).

The cell cycle progression, hormonal signaling, and various transcriptional regulators were shown to be important for initiation and maintenance of root hair growth (Guimil and Dunand 2006). In our study we observed elevated expression of *NlgCycB* and *CDKA* as well as two other B-type cyclins in transgenic tobacco plants (Fig. 4). A semiquantitative RT-PCR analysis showed that *CDKA* was expressed irrespective of organ, but *NlgCycB* was predominantly expressed in the RT and enhanced the expression of *CDKA* in the RT, suggesting that these genes are coregulated in *NlgCycB*-overexpressed plants (Fig. 4). The *CyclinB1:1*-overexpressing plant under the control of a *CDKA* promoter resulted in enhanced growth, which might be due to enhanced entry into mitosis leading to the increase in cell production rate and subsequent organ growth (Doerner and others 1996; Beemster and others 2003). In *Arabidopsis*, the *CycB1;1-gus* and *CDK* (*Cdc2a-gus*) were strongly expressed in the meristem of the root tip (Beeckman and others 2001). *CDKA::CYCB1;1* affects increases in root length in transgenic plants (Doerner and others 1996). Ectopic expression of *Rice;CycB2;2* also accelerates root growth in rice (Lee and others 2003). These results correspond with our observation of coordinately enhanced expression of both *NlgCycB* and *CDKA* genes and increased root hair length in the root tips of transgenic plants. However, although the *NlgCycB* sequence is most similar to B-type cyclins, the *NlgCycB*-overexpressing transgenic plant had different morphologies that rapidly promoted differentiation of epidermal cells into root hairs and increased the size of epidermal cells and the nuclei of root hair cells. From these results, we conclude that *NlgCycB* may be involved in new functions in developmental processes such as root hair differentiation and root hair elongation.

Transgenic plants showed better growth than wild-type during culture on reduced salt-strength (1/10-strength) MS medium (Fig. 5a–c). Also, the length and density of the root hairs in transgenic plants dependently increased according to the reduced salt concentration of MS medium (data not shown). In *Arabidopsis*, increased root hair length and density are synergistic in increasing phosphorus acquisition and decreasing the distance between the tip and first root hair, resulting in increased soil exploration and phosphorus acquisition (Zhong and others 2001). With decreasing salt strength of the MS medium, chlorophyll *a* and *b* content decreased in both wild-type and transgenic plants, but the chlorophyll *a* content in transgenic plants was significantly higher than in wild-type (Fig. 5d). Chlorophyll content in leaves decreased with decreasing ionic strength of the nutrient solution (Lee and others 1993). These results suggest that the *NlgCycB*-overexpressing transgenic plants are

adaptable in limited nutrient media, through efficient uptake of nutrients by more and longer root hairs.

In summary, we isolated a B-type cyclin gene (*NlgCycB*, accession No. AY776171) from an interspecific hybrid of *N. langsdorffii* × *N. glauca* and introduced it into *N. tabacum*. These *NlgCycB*-overexpressing transgenic plants showed premature root hair development in the meristematic zone and had more and longer root hairs than the wild-type. These results suggest that the *NlgCycB* gene, together with the *CDKA* gene, may be involved in the rapid conversion of meristematic cell activity into differentiating cell activity. Thus, transcriptional regulation of the *NlgCycB* gene likely contributes to the regulation of developmental control of the cell cycle in the root meristem, suggesting that overexpression of *NlgCycB* is flexibly adjustable to control growth in response to changes in the environment, such as nutrient availability. Further characterization of the *NlgCycB* promoter region may be helpful in revealing mechanisms that coordinate the rate of cell division and developmental controls.

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References

Andersen SU, Buechel S, Zhao Z, Ljung K, Novak O, Busch W, Schuster C, Lohmann JU (2008) Requirement of B2-type cyclin-dependent kinases for meristem integrity in *Arabidopsis thaliana*. Plant Cell 20:88–100

Aron DI (1949) Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24:1–15

Beeckman T, Burssens S, Inzé D (2001) The peri-cell-cycle in *Arabidopsis*. J Exp Bot 52:403–411

Beemster GT, Fiorani F, Inzé D (2003) Cell cycle: the key to plant growth control? Trends Plant Sci 8:154–158

Berckmans B, De Veylder L (2009) Transcriptional control of the cell cycle. Curr Opin Plant Biol 12:599–605

Bibikova TN, Gilroy S (2003) Root hair development. J Plant Growth Regul 21:383–415

Bibikova TN, Jacob T, Dahse I, Gilroy S (1998) Localized changes in apoplastic and cytoplasmatic pH are associated with root hair development in *Arabidopsis thaliana*. Development 125: 2925–2934

Breuer C, Sugimoto-Shirasu K (2007) Endoreduplication and cell size control in plants. In: Roberts K, Raff M (eds) Encyclopedia of Life Sciences, John Wiley & Sons, Ltd, UK

Breuer C, Kawamura A, Ichikawa T, Tominaga-Wada R, Wada T, Kondou Y, Muto S, Matsui M, Sugimoto K (2009) The trihelix transcription factor GTL1 regulates ploidy-dependent cell growth in the *Arabidopsis* trichome. Plant Cell 21:2307–2322

Carol RJ, Dolan L (2002) Building a hair: tip growth in *Arabidopsis thaliana* root hairs. Philos Trans R Soc B Biol Sci 357:815–821

Day IS, Reddy ASN (1998) Isolation and characterization of two cyclin-like cDNAs from *Arabidopsis*. Plant Mol Biol 36: 451–461

De Veylder L, Beeckman T, Inze D (2007) The ins and outs of the plant cell cycle. Nat Rev Mol Cell Biol 8:655–665

Dello Ioio R, Linhares FS, Sabatini S (2008) Emerging role of cytokinin as a regulator of cellular differentiation. Curr Opin Plant Biol 11:23–27

Doerner P, Jorgensen JE, You R, Steppuhn J, Lamb C (1996) Control of root growth and development by cyclin expression. Nature 38:520–523

Doležel J, Greilhuber J, Suda J (2007) Estimation of nuclear DNA content in plants using flow cytometry. Nat Protoc 2:2233–2244

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Felsenstein J (1989) PHYLIP (phylogeny inference package), version 3.2. Cladistics 5:164–166

Ferreira PC, Hemerly AS, Engler JDA, Bergounioux C, Burssens S, Van Montagu M, Engler G, Inzé D (1994) Developmental expression of the *Arabidopsis* cyclin gene *cyc1At*. Plant Cell 6:1763–1774

Francis D (2007) The plant cell cycle—15 years on. New Phytol 174:261–278

Gilroy S, Jones DL (2000) Through form to function: root hair development and nutrient uptake. Trends Plant Sci 5:56–60

Glotzer M, Murray AW, Kirschner MW (1991) Cyclin is degraded by the ubiquitin pathway. Nature 349:132–138

Guimil S, Dunand C (2006) Patterning of *Arabidopsis* epidermal cells : epigenetic factors regulate the complex epidermal cell fate pathway. Trends Plant Sci 11:601–609

Horsch R, Fry J, Hoffman N, Neidermeyer J, Rogers S, Fraley R (1988) Leaf disc transformation. In: Gelvin SB, Dordrecht RAS (eds) Plant Molecular Biology Manual, Kluwer Academic Publishers, Belgium, pp 1–9

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

John PC, Qi R (2008) Cell division and endoreduplication: doubtful engines of vegetative growth. Trends Plant Sci 13:121–127

Joubes J, Phan TH, Just D, Rothan C, Bergounioux C, Raymond P, Chevalier C (1999) Molecular and biochemical characterization of the involvement of cyclin-dependent kinase A during the early development of tomato fruit. Plant Physiol 121:857–869

Knox K, Grierson CS, Leyser O (2003) *AXR3* and *SHY2* interact to regulate root hair development. Development 130:5769–5777

Lee BS, Seo BS, Chung SJ (1993) Changes in growth and essential oil content as affected by the different ionic strength of nutrient solution in sweet basil (*Ocimum basilicum* L.). J Kor Soc Hort Sci 34:330–338

Lee J, Das A, Yamaguchi M, Hashimoto J, Tsutsumi N, Uchimiya H, Umeda M (2003) Cell cycle function of a rice B2-type cyclin interacting with a B-type cyclin-dependent kinase. Plant J 34:417–425

Lichtenthaler HK, Wellburn AR (1983) Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochem Soc Trans 11:591–592

Marcel M, Murray JA (2001) Cell cycle controls and the development of plant form. Curr Opin Plant Biol 4:44–49

Ohi R, Gould KL (1999) Regulation the onset of mitosis. Curr Opin Cell Biol 11:267–273

Pitts RJ, Cernac A, Estelle M (1998) Auxin and ethylene promote root hair elongation in *Arabidopsis*. Plant J 16:553–560

Polymenis M, Schmidt EV (1999) Coordination of cell growth with cell division. Curr Opin Genet Dev 9:76–80

Potuschak T, Doerner P (2001) Cell cycle controls: genome-wide analysis in *Arabidopsis*. Curr Opin Plant Biol 4:501–506

Qiu L, Jiang S, Zhou F, Huang J, Guo Y (2007) Molecular cloning and characterization of a cyclin B gene on the ovarian maturation

stage of black tiger shrimp (*Penaeus monodon*). Mol Biol Rep 36(1):127–134

Renaudin JP, Doonan JH, Freeman D, Hashimoto J, Hirt H, Inzé D, Jacobs T, Kouchi H, Sauter M, Rouse P, Savoure A, Sorrell DA, Sundaresan V, Murray JAH (1996) Plant cyclins: a unified nomenclature for plant A-, B- and D-type cyclins based on sequence organization. Plant Mol Biol 33:1003–1018

Ringli C, Baumberger N, Keller B (2005) The *Arabidopsis* root hair mutants der2–der9 are affected at different stages of root hair development. Plant Cell Physiol 46:1046–1053

Schiefelbein JW (2000) Constructing a plant cell: The genetic control of root hair development. Plant Physiol 124:1525–1531

Setiady YY, Sekine M, Hariguchi N, Yamamoto T, Kouchi H, Shinmyo A (1995) Tobacco mitotic cyclins: cloning, characterization, gene expression and functional assay. Plant J 8:949–957

Shaul O, Montagu MV, Inzé D (1996) Cell cycle control in Arabidopsis. Ann Bot 78:283–288

Shi H, Zhu JK (2002) A pyridoxal kinase gene, is required for root hair development in *Arabidopsis*. Plant Physiol 129:585–593

Sugimoto-Shirasu K, Roberts K (2003) "Big it up": endoreduplication and cell-size control in plants. Curr Opin Plant Biol 6:544–553

Sugimoto-Shirasu K, Roberts GR, Stacey N, McCann MC, Maxwell A, Roberts K (2005) RHL1 is an essential component of the plant DNA topoisomerase VI complex and is required for ploidy-dependent cell growth. Proc Natl Acad Sci USA 102:18736–18741

Teyssier E, Bernacchia G, Maury S, How Kit A, Stammitti-Bert L, Rolin D, Gallusci P (2008) Tissue development variations of DNA methylation and endoreduplication levels during tomato fruit development and ripening. Planta 228:391–399

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Tréhin C, Glab N, Perennes C, Planchais S, Bergounioux C (1999) M phase-specific activation of the *Nicotiana sylvestris* Cyclin B1 promoter involves multiple regulatory elements. Plant J 17:263–273

Verkest A, Manes CL, Vercruysse S, Maes S, Schueren EV, Beeckman T, Genschik P, Kuiper M, Inzé D, Veyldera LD (2005) The cyclin-dependent kinase ınhibitor KRP2 controls the onset of the endoreduplication cycle during *Arabidopsis* leaf development through ınhibition of mitotic CDKA;1 kinase complexes. Plant Cell 17:1723–1736

Vissenberg K, Fry SC, Verbelen JP (2001) Root hair initiation is coupled to a highly localized increase of xyloglucan endotransglycosylase action in *Arabidopsis* roots. Plant Physiol 127:1125–1135

Wada T, Tachibana T, Shimura Y, Okada K (1997) Epidermal cell differentiation in *Arabidopsis* determined by a Myb homolog, CPC. Science 277:1113–1116

Weingartner M, Criqui MC, Meszaros T, Binarova P, Schmit AC, Helfer A, Derevier A, Erhardt M, Bogre L, Genschikb P (2004) Expression of a nondegradable cyclin B1 affects plant development and leads to endomitosis by inhibiting the formation of a phragmoplast. Plant Cell 16:643–657

Yi K, Menand B, Bell E, Dolan L (2010) A basic helix-loop-helix transcription factor controls cell growth and size in root hairs. Nat Genet 42:264–267

Zabka A, Polit JT, Maszewski J (2010) Inter- and intrachromosomal asynchrony of cell division cycle events in root meristem cells of *Allium cepa*: possible connection with gradient of cyclin B-like proteins. Plant Cell Rep 29:845–856

Zhong MA, Thomas CW, Andrew M, Jonathan PL (2001) Morphological synergism in root hair length, density, initiation and geometry for phosphorus acquisition in *Arabidopsis thaliana*: a modeling approach. Plant Soil 236:221–235