Plant Biotechnology 22, 261–270 (2005)

Original Paper

Cell cycle specific isopentenyl transferase expression led to coordinated enhancement of cell division, cell growth and plant development in transgenic *Arabidopsis*

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Received August 8, 2005; accepted September 5, 2005 (Edited by Y. Hotta)

Abstract Cytokinin is an essential plant developmental regulator that requires precise temporal and spatial control for synergistic action on morphogenesis. To identify the cellular target for proper cytokinin function, the bacterial gene *IPT*, encoding an isopentenyl transferase for *de novo* cytokinin biosynthesis, was expressed in transgenic plants using promoters with different specificities. Analysis of the transgenic plants revealed that ectopic *IPT* expression was detrimental to plant development, whereas exclusive expression of *IPT* in cycling cells led to normal plant development with increased growth and final organ size. The enlarged organ size was a result of increase in both cell number and cell size, which was accompanied by increased expression of *CycD3* and *CycB1*, indicating that cytokinin controls organ size by regulating cyclin expression. The cell cycle-specific cyclin promoters were active in multiple organs, including root, leaf and flower, suggesting the biological significance of the locally produced cytokinin on morphogenesis, and the amplified cytokinin biosynthesis in the pre-existing dividing cells of these organs is necessary and sufficient for coordinated cell division, cell growth, pattern formation and organ development. To our knowledge, this is the first case that cytokinin was genetically manipulated in transgenic plants to produce dramatically enhanced phenotypes without noticeable negative effect, providing a promising opportunity for crop improvement.

Key words: Amplified expression, cyclin promoter, cytokinin, organogenesis, plant vigor.

The phytohormone cytokinin represents an essential class of developmental cues controlling plant morphogenesis. Extensive physiological, mutational and transgenic studies have shown that change in plant cytokinin homeostasis causes drastic alteration in growth and development, including cell division, shoot meristem activity, apical dominance, chloroplast development, leaf morphogenesis, leaf senescence, and stress tolerance (Mok 1994; Chaudhury et al. 1993; Beinsbergeret al. 1991; Medford et al. 1989; Werner et al. 2003). Among these developmental effects, cell cycle control is believed to be the primary target for cytokinin action (Werner et al. 2001).

Cytokinin exerts its effect on cell division at the two checkpoints of cell cycle. First, cytokinin signals the G1/S transition. Exogenous addition of cytokinins or mutants overproducing cytokinins led to increased expression of *CycD3*, a cyclin controlling G1/S transition in cell cycle, resulting in ectopic cell division (Riou-Khamlichi et al. 1999; Helliwell et al. 2001). Secondly, cytokinin is required for G2/M transition by stimulating dephosphorylation of a mitotic kinase (Zhang et al. 1996; Zhang et al. 2005). Consistent with its role in G2/M transition, endogenous cytokinins in cultured BY-2 cells showed phase-specific fluctuation, peaking at G2/M for cell cycle progression (Redig et al. 1996).

Traditionally, cytokinin is believed to be produced in the roots and become translocated to the upper parts of the plant to regulate growth and development (Letham et al. 1994). However, both physiological and genetic examination of cytokinin biosynthesis in recent years suggests that cytokinin is also produced in aerial organs, and such locally produced cytokinin determines much of the cytokinin-dependent morphogenesis (Faiss et al. 1997). Consistently, multiple members of a cytokinin biosynthetic gene, *IPT*, have been identified in *Arabidopsis*, with each exhibiting distinct expression patterns among various tissues (Miyawaki et al. 2004). Furthermore, direct measurement on endogenous cytokinins in shoot apical meristem tissues and cultured cells demonstrated that cytokinins were present in the actively dividing cells within these tissues, and they were predominantly in the nucleus (Dewitte et al. 1999; Moncalean et al. 2001). Deuterium labeling and mass spectrometry of tobacco leaf tissues also demonstrated that the highest *de novo* synthesis of cytokinin occurred in young leaves that were still undergoing cell division

(Nordstrom et al. 2004). These findings suggest that the actively dividing cells are the primary target for cytokinin action, and likely the *de novo* site of cytokinin biosynthesis.

Genetic manipulation of cytokinins in plants to enhance plant performance through transgenics has largely been unsuccessful due to the cell autonomous action in cytokinin signaling. Misexpression of cytokinin in transgenic plants often enhances cell division, but the increased cell division is not followed by proper pattern formation and organogenesis, resulting in negative pleiotropic morphogenesis. The *IPT* gene from the *T-DNA* of *Agrobacterium tumefaciens* encodes the enzyme isopentenyl transferase, a rate-limiting step in *de novo* cytokinin biosynthesis (McGaw and Burch 1995). Expression of *IPT* under the control of a number of promoters with a range of specificities typically led to elevated cytokinin accumulation and cell division that was not accompanied by appropriate morphogenesis, resulting in uncontrolled axillary bud growth, curling leaves, growth retardation and abnormal tissue formation in stem (Beinsberger et al. 1991; Medford et al.1989; Faiss et al. 1997; Klee et al. 1987; Smigocki et al. 1988; Schmülling et al. 1989; Hewelt et al. 1994; Smart et al. 1991; Van Loven et al. 1993; Hedden et al. 2000). Remarkably, such deleterious pleiotropic phenotypes occurred even when some tissue or organ specific promoters were used to drive the *IPT* expression in plants. For example, when an auxin-inducible bidirectional promoter from the soybean *SAUR* gene was used to drive the expression of *IPT* in transgenic tobacco plants, the transgene-produced cytokinin was present in a tissue- and organ-specific manner. Yet the localized overproduction of cytokinins still resulted in similar morphological and physiological abnormalities (Li et al. 1992). These results demonstrate that, although the ectopically produced cytokinin is capable of dedifferentiating cells to re-enter cell cycle, it typically fails to signal coordinated cell growth and organ formation, resulting in abnormal morphogenesis.

In this study, we took a different transgenic strategy, in which, instead of exploring ectopic transgene expression, the expression of the transgene, *IPT*, was restricted to the native target cells of cytokinin action, that is, the actively dividing cells that, by default development program, have committed to pattern formation and organogenesis. Such amplified expression was achieved by using promoters directing cell cycle-specific gene expression. Specifically, *IPT* was expressed under the control of four different promoters with clearly defined expression patterns, from cell phase specific to constitutive. The first promoter was from *Arabidopsis CycB1*, which encodes a mitotic cyclin and is transcribed only in the G2/M transition of dividing cells (Hemerly et al. 1992; Ferreira et al. 1994; Fuerst et al. 1996; Shaul et al. 1996; Menges et al. 2002). The second promoter was from the *Arabidopsis* G1/S cyclin, *CycD3*, which expresses only in cycling cells, but throughout the cell cycle (Fuerst et al. 1996; Menges et al. 2002; Soni et al. 1995; Sorrell et al. 1999; Mironov et al. 1999). The third promoter was from the *Arabidopsis* cyclin dependent kinase gene, *CDC2a*, which not only directs low level expression in cycling cells, but also exhibits scattered expression in certain non-dividing and differentiated cells (Martinez et al. 1992; Hemerly et al. 1993; Segers, et al. 1996). Finally, the *CaMV 35S* promoter was used as a control to direct strong constitutive expression (Klee et al. 1987).

Analysis of the transgenic plants expressing these transgenes demonstrated that over-production of cytokinin within the native dividing cells increased plant organ size and organ number as a result of coordinated increase in cell size and number, followed by proper organ formation and normal plant development. Our results suggest that plant can avoid cytokinin mis-localization and subsequent abnormal organogenesis by restricting its production in cells that are committed to division, and thus provide an effective method for manipulating cytokinin signal transduction for plant improvement.

Materials and methods

Cloning and vector construction

The *IPT* gene (GenBank No. 4586309) of 720 bp from *A. tumefaciens* was amplified out by PCR from an in house plasmid (*pMON38210*), using two primers that introduced a *Nco*I site at the 5′ end and an *Eco*RI site at the 3′ end of the amplified gene. The two primer sequences are CGCGGCCCATGGATCTGCGTCTAA-TTTTCGGTCCAAC and CGGCGCGAATTCTAATAC-ATTCCGAATGGATGACCTT. To clone the promoter of *Arabidopsis CycB1* (*X62279*), the upstream sequence of *CycB1* was determined from the GenBank genomic clone *AL035601*, from which two primers were designed to amplify from the total genomic DNA the 1550 bp fragment (designated as P_{CycB1}) immediately upstream from the ATG start codon, introducing a *Pac*I site at the 5′ end and a *Nco*I site at the 3′ end. The two primer sequences are CGCGGCTTAATTAAGAGCAATAAC-AGTGTGTGAGGCATT and CGGCGCCCATGGTCT-TAGTGTTCTCTTCTCTTTCTCTC. To clone the promoter of *Arabidopsis CycD3* (*X83371*, also known as *CycD3;1*), the upstream sequence of 1500 bp (designated as P_{CycD3}) was determined from the genomic contig *AL161585*, which was then cloned by PCR using two primers that introduced a *Pac*I site at the 5′ end and a *Nco*I site at the 3′ end. The primer sequences are CGCGGCTTAATTAAGCATTATGCGGGAGCAAAGG TAAGT and CGGCGCCCATGGTGGGGGACTAAA-CTCAAGAATGAGAA. Similarly, to clone the promoter

of *CDC2a* (*D10850*), the corresponding 1400 bp (designated as P_{CDC2a}) upstream sequence was determined from *AL132963*, and amplified from genomic DNA using two primers, introducing a *Asc*I site at the 5′ end and a *Nco*I site at the 3′ end. The two primer sequences are CGCGGCGGCGCGCCATATA-TATTATTATATAAATATAAC and CGCGGCCCATGG-TTTCCTGAATAATAAAGCTGAAG.

To construct the binary vector of *IPT* driven by the constitutive *35S* promoter (P_{35S}), the binary vector *pMON23450* was linearized with *Nco*I and *Eco*RI, followed by ligation with the *Nco*I-*EcoR*I *IPT* fragment. The resulting vector was named *pMON66507*, in which the *IPT* gene is under the control of P_{35S}. To put the *IPT* gene under the control of the P_{CycB1}, *pMON66507* was digested with *Pac*I and *Nco*I to release P_{35S}, which was then replace by the *Pac*I-*Nco*I P_{CycB1} fragment. The resulting vector was named as *pMON66508*. Similarly, the *Pac*I-*Nco*I *PCycD3* fragment was used to replace P_{35S} in *pMON66507* to generate the plasmid *pMON66509*, in which the *IPT* gene is under the control of P_{CycD3}. To construct the *IPT* gene under the control of P_{CDC2a}, the amplified P_{CDC2a} fragment was digested with *Asc*I and *Nco*I and ligated to *pMON66507* that had the P_{35S} promoter released by *Asc*I and *Nco*I. The resulting plasmid was named as *pMON66510*. To construct the vector of *CycD3* driven by the native *CycD3* promoter, P_{CycD3}, *pMON23450* was linearized by *Pac*I and *Sal*I, followed by a three-way ligation to the *Pac*I-*Spe*I *PCycD3* fragment and the *Spe*I-*Sal*I *CycD3* fragment. The resulting plasmid was named as *pMON71757*. For each construct, multiple clones were sequenced and only error-free vectors were used for plant transformation.

Plant transformation and growth conditions

The constructed binary expression vectors were used to transform *Arabidopsis thaliana*, ecotype *Columbia*, using *Agrobacterium*-mediated vacuum infiltration procedure described by Bechtold and Pelletier (1998). Transformed seeds were selected on kanamycin MS plates, thirty two independent transformation events were transferred to potted soil in growth chamber and grown to maturity for seed harvest. Expression of the transgene was determined at the transcript level using quantitative real-time RT-PCR analysis (see below). Seeds of the transgenic plants were sown in potted soil along with wild-type control, and were vernalized at 4°C for three days before moving to a growth chamber. The plants were grown under the following conditions: at 22°C, 24 hours constant light with light intensity of 170–200 μm Einstein $m^{-2} s^{-1}$, and a humidity of 70%. Plants were also grown under short day conditions, with 10 hours of light period. Plants were fertilized twice a week using Peters 20-20-20 fertilizer (in half strength) from Hummert International (Earth City, MO).

RT-PCR and quantitative real-time PCR (Taqman) analysis

Total RNA was prepared from roots, rosette leaves, flowers and young seedlings of *Arabidopsis* plants, and used for semi-quantitative RT-PCR analysis, as previously described (He et al. 2004). Quantitative genomic DNA PCR and RT-PCR were performed as previously described (He et al. 2004). Briefly, yong leaf tissues were homogenized in nucleic acid purification lysis solution (ABI, Foster City, CA). Genomic DNA was prepared using the Automated Nucleic Acid Workstation (ABI6700) according to the manufacturer's procedure (ABI). Total RNA was isolated using a Trizol based procedure, and the first strand cDNA was synthesized by reverse transcriptase. PCR reactions were prepared using the ABI two-fold SYBR-Green PCR Master Mix, followed by real-time thermocycling on ABI 7700 with the following standard cycling conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 20 sec, 60°C for 1 min. Standard data analysis procedures were performed for baseline adjustment and the data were screened for two-fold standard deviation of variance. Fluorescence-labeled gene specific probes were also used for RNA expression quantitation using same PCR reaction conditions.

Microscopy

A low voltage, low temperature Scanning Electron Microscopy (SEM) procedure was used as previously described (He et al. 2004). Briefly, frozen leaf or petal samples were transferred into the cryo-chamber connected to SEM column for microscopy. Low electron beam voltages (1–3 KV) and a special electron detection system were used to reduce the effect of charging during the SEM observation. The sample temperature was kept at lower than −140°C in the entire process. Digital images were acquired from the same or similar regions on the plant leaves or flower petals of the corresponding transgenic and wild type *Arabidopsis* plants.

Seed size analysis

To determine the seed size of different transgenic plants, about 50 transgenic seeds (T2 and T3) were dispersed evenly in an area of about 0.5 cm in diameter on a flat surface, along with wild-type controls. Magnified Images were then taken and the image size in pixels was determined using the Image-Plus™ software. Four samples for each seed packages were used for the analysis, and statistical significance of the seed size variation was determined by *t*-test.

A high throughput screen developed internally was also used to determine the *Arabidopsis* seed size. The software is a UNIX command line image processing program that automatically segments images containing touching seeds, screens out non-seed subjects, and then

measures the areas of seeds on TIF images.

Canopy size measurement

Transgenic plants, along with wild-type controls, were arranged in Latin Square, with 12 plants for each genotype, and grown under uniform conditions. At days 25, 32 and 42, images were taken for each plants from the top, and the canopy size of the one-dimensional image was determined with the Image-Plus™ software. Statistical significance of size variation was determined using *t*-test. The growth rate for a given genotype was determined by comparing the percentage of canopy size variation of that genotype to wild-type among different growth stages.

Results

Developmental abnormalities associated with ectopic IPT expression

Four binary vectors with the bacterial gene *IPT* under the control of P_{e35S}, P_{CDC2a}, P_{CycB1} and P_{CycD3}, respectively, were constructed and used to transform *Arabidopsis thaliana* (ecotype *Columbia*), using *Agrobacterium*-mediated vacuum infiltration. An empty vector without the *IPT* gene was also included as a control. About 10,000 infected seeds for each construct were plated on kanamycin selection plates. The total number of seedlings generated for each construct was scored, and the seedling development was examined.

As is shown in Table 1, seeds from the empty vector control had a shoot formation frequency of 1.23%. However, only 0.11% of the seeds infected with P_{e35S}:*IPT* and 0.40% of those with P_{CDC2a}:*IPT* germinated and developed to seedlings on plates; all but one seedling containing P_{e35S}:*IPT* and 68.7% of the seedlings expressing P_{CDC2a}:*IPT* failed to develop a healthy root system. When the seedlings on selection plates were transplanted to soil pots and grown in a growth chamber, as is shown in Figure 1A and 1B, the rootless seedlings expressing P_{e35S}:*IPT* and P_{CDC2a}:*IPT* continued to show poor root development and eventually died before reaching maturity; for the plants that managed to survive to maturity, a range of negative phenotypes typical of cytokinin overexpression was observed, including serrated leaves, leaf curling, retarded shoot growth and aberrant seed development (Figure 2I). These data suggest that ectopic biosynthesis of cytokinin as a result of *IPT* misexpression from the *e35S* promoter or *CDC2a* promoter led to deleterious pleiotropic morphogenesis.

Normal plant development associated with cell cycle specific IPT expression

In contrast to those expressing P_{e35S}:*IPT* and P_{CDC2a}:*IPT*, transformants expressing P_{CycB1}:*IPT* and P_{CycD3}:*IPT* exhibited normal development. The shoot formation frequencies for seeds infected with P_{CycB1}:*IPT* and P_{CycD3}:*IPT* were 1.96% and 2.20%, respectively, which were even higher than the empty vector control (1.23%) (Table 1), suggesting that *IPT* expression driven by the cell cycle specific promoters not only had no deleterious effect on seed germination and subsequent seedling development, but also stimulated the seedling formation. All the seedlings harboring P_{CycB1}:*IPT* and P_{CycD3}:*IPT* developed normal roots. When these seedlings were transplanted to soil, they continued to develop normally, with no noticeable negative pleiotropic phenotypes (Figure 1C and 1D). These results suggest that *IPT* expression driven by cell cycle specific promoters can effectively eliminate uncontrolled cell division and cell growth that typically couples ectopic cytokinin overproduction. Because the plants of P_{CycB1}:*IPT* and P_{CycD3}:*IPT* were morphologically similar, in most cases, only the P_{CycD3}:*IPT* plants were chosen for detailed analysis.

Enhanced growth of IPT transgenic plants driven by cyclin promoters

Plants expressing *IPT* from the cell cycle specific promoters not only displayed normal organogenesis and plant development, but, more importantly, exhibited enhanced growth and overall productivity. Table 2 and Figure 2 summarize the improved phenotypes of the transgenic plants when compared to wild-type control.

The transgenic plants expressing P_{CycD3}:*IPT* had significantly ($p<0.01$) accelerated growth rate, as

Table 1. Seedling development.

Transgene	Shoot formation		Root formation	
	Number of shoots[a]	Frequency (%)	Number of rooted seedlings	Frequency (%)[b]
Vector control	123	1.23	123	100
P_{e35S}:*IPT*	14	0.11	3	21.4
P_{CDC2a}:*IPT*	56	0.40	17	31.3
P_{CycB1}: *IPT*	258	1.96	258	100
P_{CycD3}: *IPT*	325	2.20	325	100

[a] From a total of about 10,000 seeds.
[b] Percentage of shoots that developed roots.

determined by canopy size measurement at different developmental stages (Table 2). Because the life cycle and flowering time of the transgenic plants were not changed when compared to wild-type controls (data not shown), the accelerated plant growth rate led to the final size increase of all the organs examined, including increased root length (Figure 2A), seedling size (Figure 2B), leaf size (Figure 2C), flower size (Figure 2D), flower petal size (Figure 2E), plant height and branching (Figure 2F), and seed size (Figure 2G and 2H).

Expressing *IPT* from the cyclin promoter in transgenic plants also led to increased organ number, including the number of leaves and the number of flowers (Table 2). At maturity, there was on average four more leaves in transgenic plants than that of wild-type control. At day 48 after planting under standard long day growth conditions, the total number of flowers of the transgenic plants was increased by 50% when compared to wild-type plants growing under the same conditions. Significantly, the floral structure was not affected, with no floral organ number alteration (Figure 2D), suggesting the cell cycle specific cytokinin overproduction was subjective to normal higher order meristem differentiation and organ development.

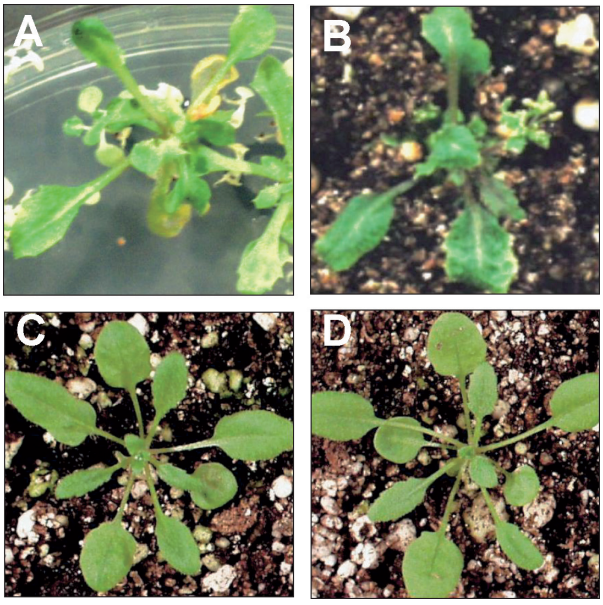

Figure 1. Morphogenesis of seedlings carrying different *IPT* expression cassettes. (A) A seedling expressing P_{e35S}:*IPT* on *kan* selection plate, showing abnormal leaf development and lack of roots. (B) A seedling expressing P_{CDC2a}:*IPT* (grown in soil), showing abnormal leaf development. (C) A seedling expressing P_{CycB1}:*IPT* (grown in soil). (D) A seedling expressing *PCycD3:IPT* (grown in soil).

Penetrance of different transgenes

The frequencies with which the transgenes manifest their

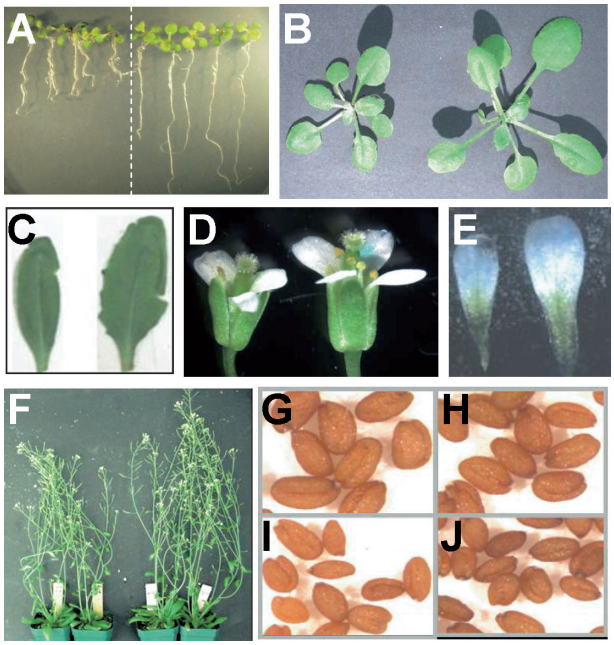

Figure 2. Increased growth and organ size of transgenic plants expressing *IPT* from the cyclin promoters. (A)–(F) Each panel shows the organ size comparison between wild-type (left) and transgenic plants expressing P_{CycD3}:*IPT* (right). A: roots of 11-day old seedlings grown on MS plate supplemented with sucrose under constant light at 21C (see Methods), B: seedlings from a statistically designed growth experiment (see Methods), C: the largest leaves of 32-day old plants of the respective genotypes, D: fully opened flowers, E: petals of fully opened flowers, and F: adult plants. (G)–(J) Seed size comparison of plants expressing different transgenes, including P_{CycB1}:*IPT* (G), P_{CycD3}:*IPT* (H), P_{CDC2a}:*IPT* (I), and wild-type control (J).

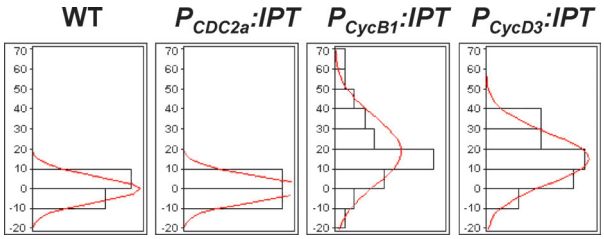

Figure 3. Seed size distributions for different transgenic plants. The X axis is the requency of events, the Y axis is the percentage of size increase compared to the mean of wild-type control.

Table 2. Increased growth rate and organ number of transgenic plants.[a]

Transgene	Canopy size[b]			Leaf number	Flower number
	Day 25	Day 32	Day 42		
WT	8514±430	33589±1354	42451±2247	15.7±1.5	186±38
P_{CycD3}:*IPT*	11048±513	45436±2355	49980±3079	19.8±1.1	284±23

[a] All the values were the means plus/minus standard errors.
[b] Measured as the green area of the top-view images in pixel units.

phenotypic effects were examined quantitatively. Since seed is a major sink organ and therefore its size could reflect the effectiveness of different transgene expression in altering the corresponding biological processes, we examined the seed size of all the transgenic events for all the four transgenes. Using an automated assay, we measured the seed size of 70 independent transgenic lines that had been advanced, including 29 transgenic lines of P_{CycB1}:*IPT*, 30 lines of P_{CycD3}:*IPT*, 10 lines of P_{CDC2a}:*IPT*, and 1 line of P_{e35S}:*IPT*, along with 7 lines of wild-type controls. Power calculation determined that, at the detection power of 95%, the standard operation protocol was capable of detecting 9% seed size difference at critically significant level ($p<0.01$). Figure 3 shows the distributions of seed size increase against the mean of wild-type control. (The one P_{e35S}:*IPT* line was omitted from the table, which had similar seed size as the control.) Comparison of the distributions between the wild-type and the different transgenes shows that, while the seed size of P_{CDC2a}:*IPT* plants was essentially same as that of wild-type of control, both P_{CycB1}:*IPT* and P_{CycD3}:*IPT* resulted in significant shift toward increased seed size. Thus using 10% as the cut-off value, 72% of the P_{CycB1}:*IPT* lines and 63% of the P_{CycD3}:*IPT* lines exhibited significant seed size increase, ranging from 10% to 70% in size enlargement over wild-type controls.

These results demonstrated that cell cycle specific *IPT* expression not only ensured normal pattern formation and organogenesis, but was also highly effective in affecting the involved biological processes and morphogenesis; on the other hand, leaky *IPT* expression to non-dividing cells not only led to negative pleiotropic morphogenesis, but was also inefficient in exerting positive effect on target organ development.

Transgene expression

To understand why P_{CycD3}- and P_{CycB1}-driven *IPT* expression led to enhanced and normal plant development, whereas P_{CDC2a}-driven *IPT* expression led to abnormal morphogenesis, we examined the expression patterns and levels of genes driven by these promoters. The cell-type specificities of these three promoters were well characterized, namely, P_{CycD3} and P_{CycB1} are expressed only in dividing cells, or meristematic cells, P_{CDC2a} in meristematic cells as well as scattered expression in certain non-meristematic cells. Because each organ (such as root, leaf, or flower) of a plant contains meristematic cells, we examined in wild-type plants whether there might be organ specificity for the expression of any of the native *CDC2a*, *CycD3* and *CycB1*. As is shown in Figure 4, all three genes were expressed in root, floral and, at a lower level, in immature leaf tissues, suggesting that the abnormal development with *IPT* driven by the *CDC2a* promoter was not likely due to a higher level of transgene expression in these organs.

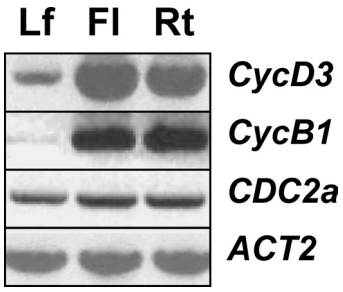

Figure 4. Expression of the native genes of the three *Arabidopsis* promoters used in this study among different organs. Total RNA was prepared from immature leaf (Lf), flower (Fl) and root (Rt) of wild-type *Arabidopsis* plants, and was subjected to semi-quantitative RT-PCR using gene-specific primers, each with same amount of RNA and same number of amplification cycles. The amplification products for each gene were then resolved by gel electrophoresis and photographed. *CycD3*: G1 cyclin; *CycB1*: mitotic cyclin; *CDC2a*: cyclin-dependent kinase; *ACT2*: an actin encoding gene used as a loading control.

Table 3. Quantification of transgene expression.

Trangene	Number of plants to soil	*IPT* RNA[a]	Number of plants survived	*IPT* tolerance level[b]
P_{e35S}:*IPT*	4	0.02–882	1	0.02
P_{CDC2a}:*IPT*	32	0.04–0.11	10	0.11
P_{CycB1}:*IPT*	32	0.01–0.48	31	0.48
P_{CycD3}:*IPT*	32	0.01–3.99	31	3.99

[a] The range of leaf *IPT* RNA level among all the seedlings.
[b] The highest leaf *IPT* RNA level of plants with no negative phenotypes.

To confirm, quantitative real-time PCR (Taqman) analysis was performed to determine *IPT* expression levels in young leaves, where the morphological alterations were clearly seen among plants expressing *IPT* from different promoters (Figure 1). A total of 120 transgenic plants of the four transgenes were analyzed. Table 3 shows the range of *IPT* expression levels for all the plants of each construct, (column 3), as well as the RNA level of the highest expressor of each construct that was able to grow normally to maturity (column 5). It showed that transgenic plants expressing P_{CDC2a}:*IPT* had in general lower level of *IPT* expression when compared to those expressing P_{CycB1}:*IPT* and P_{CycD3}:*IPT*, and plants expressing P_{e35S}:*IPT* and P_{CDC2a}:*IPT* tolerated only low level of *IPT* expression, whereas plants expressing *IPT* from the cyclin promoters had much higher levels of *IPT* while still displaying normal development. Although high *IPT* expression was observed in two P_{e35S}:*IPT* seedlings, they died at very early stage.

Taken together, these data suggest that *IPT* expression by the P_{CDC2a} promoter resulted in leaky cytokinin biosynthesis in non-dividing cells and subsequently cell-autonomous action of the ectopically produced cytokinin in the residing cells, leading to abnormal organogenesis; on the other hand, plants expressing *IPT* from the cyclin

promoters were able to tolerate much higher level of cytokinin production within the actively dividing cells, maintaining normal organ formation and plant development.

Cell size and cell number

To determine the cellular basis of the observed organ size increase, we examined and compared the cell size and cell number of transgenic plants expressing P_{CycD3}*:IPT* and those of wild-type plants. Both leaves and flower petals were sampled for scanning electron microscopic analysis. Because of the variation in cell size and number at different positions of a leaf blade or among leaves of different developmental stages, care was taken to ensure the comparison was made at specific position of specific leaf or petal. The leaf size was determined (see Methods), and based on the cell size and leaf size, cell number per leaf was calculated.

The leaf epidermal cells were highly irregular in shape, especially in older leaves, so younger expanding leaves were used for cell size and cell number calculation. Figure 5A and 5B show the upper part of adaxial epidermal surface of the V6 leaf from 20 day old plants growing under the standard long day conditions. A comparison showed that the cell size of the transgenic leaf was increased by 25% over the wild-type control, and the leaf cell number of the transgenic plants was increased by 165% over wild-type control (Table 4). The floral petal adaxial epidermal cells varied dramatically in size from the base to the tip, and therefore the cell number per leaf was not calculated. However, a comparison between the transgenic (Figure 5C) and the wild-type (Figure 5D) at similar position showed that the cell size of the transgenic plants appeared similar or slightly increased, and because the floral petal size is drastically larger in transgenic plants (Figure 2E), we conclude that the petals of the transgenic plants expressing P_{CycD3}*:IPT* also had increased cell number.

Taken together, these results demonstrated that cell cycle specific *IPT* expression led to increased organ size primarily by increasing cell number. Cell enlargement also contributed to the final organ size increase, though to a lesser degree, and likely an indirect transgene effect in response to accelerated cell division. The requirement of precise *IPT* expression in dividing cells in order to enlarge the organ size also implied that cytokinin-promoted organ growth was mediated by cell cycle.

Gene regulation by transgene

Although exogenous cytokinins have been shown able to up-regulate the expression of certain cyclin genes, such as *CycD3*, we were interested to determine the effect of the elevated production of cytokinin within the dividing cells on cyclin expression. To this end, we examined the expression of *CycB1* and *CycD3* in the transgenic plants expressing *IPT* from the P_{CycD3} promoter. As is shown in Figure 6, both *CycD3*, a G1/S cyclin, and *CycB1*, a mitotic cyclin, were up-regulated in the transgenic plants when compared to that of wild-type control. The results demonstrated that cytokinins endogenous to dividing cells controls cell division by regulating the expression of cyclins within these cells that have been programmed for subsequent patterning, leading to normal plant growth and development.

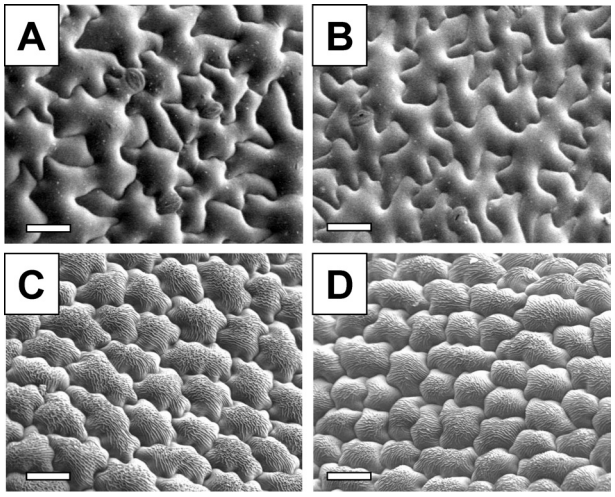

Figure 5. Coordinated increase in cell number and cell size of plants expressing *IPT* from the cyclin promoter. Scanning electron micrographs of the adaxial epidermal surface of the tips of rosette leaves and flower petals. The leaves were the V6 leaves of 20-day old plants. Relative cell size was determined by comparing the cell number per view of the transgenic and the wild-type control, while cell number per leaf was deduced from the leaf size and calculated average cell size (see Table. 4). (A) Leaf expressing P_{CycD3}*:IPT*, the bar is 50 μm. (B) Leaf of a wild-type plant The bar is 50 μm. (C) Flower petal expressing P_{CycD3}*:IPT*, the bar is 25 μm. (D) Flower petal of a wild-type plant, the bar is 25 μm.

Table 4. Increased cell size and cell number of transgenic plants.

Transgene	Number of plants	Cell size		Cell number	
		mm^2/1 k cells	*p*-value	Number/leaf	*p*-value
WT	4	4.19±0.32		3837±281	
P_{CycD3}*:IPT*	4	5.23±0.64	0.026	10188±1256	0.003

The cell size of V6 leaf was determined by counting the number of cells of adaxial leaf SEM image (250×). The mean±standard error is presented. The cell number per leaf was estimated using predetermined leaf area (see Methods) and image cell number count. The significance of the changes over respective controls is presented as *p* values (*t*-test).

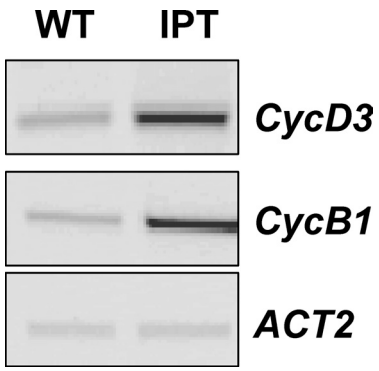

Figure 6. Up-regulation of cyclin expression by *IPT*. Total RNA was prepared from leaves at V6 (plants with 6 rosette leaves) growth stage of wild-type (left lane) and plants expression *IPT* under the control of P_{CycD3} promoter (right lane). The extracted RNA was subjected to semi-quantitative RT-PCR using gene-specific primers, each with same amount of RNA and same number of amplification cycles. The amplification products for each gene were then resolved by gel electrophoresis and photographed. *CycD3*: G1 cyclin; *CycB1*: mitotic cyclin; *ACT2*: an actin encoding gene as a loading control.

Discussion

Proper plant organogenesis requires that cell proliferation, cell growth and cell differentiation are coordinated and integrated into a predetermined organ patterning plan, and any ectopic cellular activity that is not networked with such signaling processes is likely to cause abnormal development. The ectopic expression of *CycD3* and the cytokinin biosynthetic gene (*IPT*) using an array of promoters in previous studies has provided a good illustration in this regard. In both cases, the transgenes were robust in enhancing cell division activity and/or rendering differentiated cells totipotency, but the *de novo* cell proliferation was often uncoupled with proper organ formation, resulting in negative pleiotropic morphogenesis.

To prevent the negative pleiotropic phenotypes caused by mis-production of cytokinin and to better understand the mode of cytokinin action in dividing cells, we targeted *IPT* expression specifically to dividing cells. Analysis of the transgenic plants has allowed us to learn some important aspects on cytokinin function in plants.

First, cytokinin over-production was tolerated only when restricted to dividing cells, and such cell cycle specific production was sufficient to enhance cell division and cell growth. When *IPT* was specifically expressed in actively dividing cells using the cell cycle specific promoters (P_{CycD3} and P_{CycB1}), the transgenic plants were developmentally normal, undergoing similar morphogenesis as wild-type plants both temporally and spatially. Organs of the transgenic plants exhibited dramatically increased cell number and to a lesser degree increased cell size, resulting in enlarged organ size and plant size. The cyclin promoters used in this study were expressed in different organs including roots (Figure 4), and interestingly, the transgenic plants also displayed enhanced root growth (Figure 2A), suggesting that, when properly expressed, cytokinin may even be a positively regulator for root development. We also expressed *IPT* under the control of P_{CDC2a}, which directed a similar but leaky pattern and lower level of expression in dividing cells as compared to P_{CycD3}. In striking contrast, expression of *IPT* by P_{CDC2a} did not enhance cell proliferation and organ development. Furthermore, the transgenic plants exhibited deleterious pleiotropic morphogenesis including root, leaf and flower. Actively dividing cells are known to produce cytokinins that fluctuate as cell cycle progresses (Redig et al. 1996). Our data provide direct evidence that the endogenous cytokinins produced in dividing cells are indeed a rate-limiting factor for cell cycle progression and cell division activity.

Secondly, cytokinin endogenous to dividing cells appeared engaged in an autocrine signaling pathway to enhance cell proliferation and organ size while keeping organ patterning and organ formation under check. Traditionally, cytokinins are believed to be produced in the roots and then translocated to the upper parts of the plant to regulate its growth and development (Letham 1994). More recent evidence, however, showed that much of the cytokinin-dependent morphogenesis was determined by locally produced cytokinins (Faiss et al. 1997). This work showed that normal morphogenesis was achieved only when *IPT* was expressed in dividing cells, and even a low level of leaky expression of *IPT* in scattered non-dividing cells would result in the disruption of proper organ formation, suggesting autocrine signaling for cell division. Consistent with this hypothesis, it has been shown that cytokinins are indeed produced in actively dividing cells, and the same form of endogenous cytokinin as that encoded by *IPT* (zeatin) was shown to be localized within the nucleus of dividing cells (Miyawaki et al. 2004; Moncalean et al. 2001).

Thirdly, the cytokinin over-produced in actively dividing cells was able to coordinate cell division with cell growth, ensuring the accelerated cell cycle being accompanied by accelerated cell volume expansion. Previous manipulation of cell cycle genes has often encountered the dilemma of increasing cell number at the expense of reduced cell size, seemingly supporting the "organismal theory" in that cell division has no role in determining organ size but only serves to fill the space of predetermined size. Thus ectopic expression of *CycD3*, although capable of increasing cell number, displayed much reduced cell size (Dewitte et al. 2003). Our data showed that cell size and cell number can be increased simultaneously to increase organ size, arguing the negative correlation between cell size and cell number in previous studies was not a result of programmed size restraint but more likely a result of the

disruption of the coupling process.

Finally, *CycD3* is a key downstream gene of cytokinin action in promoting cell division. Previously, it has been observed that exogenous addition of cytokinins to cultured cells or liquid-grown seedlings was accompanied by elevated *CycD3* expression (Riou-Khamlichi et al. 1999; Helliwell et al. 2001), suggesting that *CycD3* might be the mediator of cytokinin in controlling cell cycle. We also observed increased expression of *CycD3* in transgenic plants over-expressing *IPT*. *CycD3* was recently shown to be required not only for cell cycle progression but also for preventing cell cycle exit (Dewitte et al. 2003). Conceivably, the increased expression of *CycD3* in dividing cells in this study may have accelerated cell cycle progression by shortening the G1/S phase, leading to the observed faster growth rate, and at the same time may have prevented the dividing cells from an early exit, maintaining a similar developmental time plan as wild-type plants. Both of these actions of *CycD3*, taken together, provided a molecular explanation for the increased final organ size. Significantly, the increased expression of *CycD3* in the transgenic plants over-expressing *IPT* in the dividing cells did not lead to pattern disruption as it would when ectopically expressed (Dewitte et al. 2003), stressing that *CycD3* activity, though cell autonomous, does not trigger pattern formation. We also observed elevated expression of *CycB1* in transgenic plants expressing *IPT* from the *CycD3* promoter. These findings provide a promising genetic approach in manipulating cytokinin production for plant improvement.

Acknowledgements

We would like to thank Todd Ziegler for *Agrobacterium* transformation, John Brooks and Bonnie Denother for line advancement and plant care, and Mike Thompson for thoughtful review of the manuscript.

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