Investigation of cytokinin-deficient phenotypes in *Arabidopsis* by ectopic expression of orchid *DSCKX1*

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Abstract The plant hormone cytokinin plays a major role in regulating plant growth and development. Here we generated cytokinin-reduction *Arabidopsis* plants by overexpressing a heterologous cytokinin oxidase gene *DSCKX1* from *Dendrobium* orchid. These transgenic plants exhibited reduced biomass, rapid root growth, decreased ability to form roots in vitro, and reduced response to cytokinin in growing calli and roots. Furthermore, the expression of *KNAT1*, *STM*, and *CycD3* genes was significantly reduced in the transgenic plants, suggesting that cytokinin may function to control the cell cycles and shoot/root development via regulation of these genes.

Key words: Cytokinin; Cytokinin oxidase; *DSCKX1*; Shoot/root formation; Transgenic *Arabidopsis*

1. Introduction

Cytokinins are phytohormones that influence many essential plant developmental processes including cell division, cell differentiation, apical dominance, flower and fruit development, and leaf senescence [1,2]. It is therefore important to study the biosynthetic and metabolic regulation of cytokinins in plant cells to understand the regulation of hormone levels and the corresponding effect on plant growth and development. The mechanisms of cytokinin homeostasis are being elucidated by current progress in the study of cytokinin biosynthesis and metabolism. Several genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, have been recently identified and characterized in *Arabidopsis* [3,4]. Meanwhile, extensive investigation has provided insights into cytokinins metabolism. Various forms of cytokinins which can be interconverted by specific enzymes [1,5] are either broken down by cytokinin oxidase (CKX) or conjugated into storage forms [6–9]. In particular, CKX, the only known plant enzyme inactivating naturally occurring cytokinins [10,11], is considered to be crucial in regulating endogenous cytokinin levels and distributing native cytokinins during plant development [5,12]. The *CKX* gene was first cloned from maize [13,14], and several of its homologs were subsequently identified in *Arabidopsis* [15]. More recently, it was reported that *Ckx1* was expressed in a developmental manner in the maize kernel, predominantly in the vasculature, suggesting that *Ckx1* in maize plays a role in controlling growth and development via regulation of cytokinin levels transiting in xylem [16].

We have isolated a novel CKX gene, *DSCKX1*, from *Dendrobium* orchid using mRNA differential display [17]. *DSCKX1*, induced by cytokinins, encodes a functional CKX that plays a critical role in the control of cytokinin level in orchids. We also identified important regulatory regions essential for cytokinin-inducible transcription of *DSCKX1* gene [18]. A limited number of studies have investigated the effects of exogenous cytokinins on various developmental aspects of *Arabidopsis*, including shoot and root development [19], cell cycle progression [20,21], flowering [22], and primary and secondary metabolism [23], partly because cytokinin-deficient mutants are not yet available for such studies. Although the studies on *amp1* [24] and *sps* [25], two cytokinin-overproduction mutants have gained insights into the function of cytokinin in plant development, the essential role of cytokinin in plant development needs to be further clarified in 'loss-of-cytokinin' background. Recently, cytokinin-deficient tobacco plants were generated by the ectopic expression of *Arabidopsis* CKX genes. These plants exhibited stunted shoots with smaller apical meristems, prolonged plastochrone, and slower leaf cell production [26]. In contrast to the inhibition of shoot development, their root growth was enhanced dramatically [26].

The generation of cytokinin-deficient *Arabidopsis* plants by overexpression of *DSCKX1* allowed us to examine the developmental consequences of decreased endogenous cytokinin level and the related molecular mechanisms in *Arabidopsis*.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana (Columbia ecotype) plants were grown in conditions described previously [18]. For shoot regeneration from roots, root segments (approximately 7 mm) were excised from 10-day-old seedlings grown on Murashige–Skoog (MS) medium and were subcultured onto the callus-inducing media (CIM) as previously described [27]. These explants were subsequently transferred to shoot-inducing medium (SIM) after 2 days. To reduce effects of plate-to-plate variation, the root explants per line were divided and incubated in four separate plates (five roots each).

The *amp1*/*35S::DSCKX1se* plants were generated by crossing

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Abbreviations: CIM, callus-inducing media; CKX, cytokinin oxidase; iPA, isopentenyladenosine; ZR, zeatin riboside

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35S::DSCKX1se plants with *amp1* mutant and identified by polymerase chain reaction (PCR) with gene specific primers for *DSCKX1* and derived cleaved amplified polymorphic sequences for *amp1* [33].

2.2. Plant transformation

The *Agrobacterium tumefaciens* strain LBA4404 harboring the binary 35S-DSCKX1 plasmid constructed previously [17] was used to transform *A. thaliana* via floral dip transformation [28]. Kanamycin-resistant seedlings of independent primary transformants whose progeny segregated 3:1 for kanamycin resistance were allowed to set seeds, and homozygous lines were selected for further studies.

2.3. Southern and Northern blot analyses

For Southern blot analysis, genomic DNA was digested with *Sac*I, electrophoresed through a 1% agarose gel, then blotted onto nylon membranes (positively charged, Roche Diagnostics). For Northern blot analysis, total RNA was fractionated by 1% glyoxal–agaroses and transferred onto nylon membranes. Hybridizations and detection were performed as described [17].

2.4. CKX activity and cytokinin analysis

The CKX activity was determined according to the method of Libreros-Minotta and Tipton [29]. Protein concentrations were determined using a protein assay kit [30] with bovine serum albumin as the standard.

Cytokinins were extracted and quantified as described [31,17]. Cytokinins were extracted with 100% methanol, purified using C18 Sep-Pak (Waters Associates), further purified using reverse-phase high-performance liquid chromatography (HPLC) on a Luna 5μ C18 column (150×4.6 mm, Phonemenex), and quantified by immunoassay with monoclonal antibody isopentenyladenosine (iPA) and zeatin riboside (ZR) using iPA and ZR detection kits (Sigma). Three separate samples were analyzed, and three replicate ELISA tests were carried out for each HPLC fraction.

2.5. Reverse transcription (RT)-PCR

Total RNA was isolated from 14-day-old seedlings using TRI reagent (Fisher), followed by treatment with RNase-free DNase I (Promega) at 37°C for 1 h to avoid contamination of genomic DNA. The treated RNA (50 ng) was subjected to RT-PCR in a one-step RT-PCR assay (Qiagen). PCR products were electrophoresed, blotted, and hybridized with the specific probes. RT-PCR was repeated at least three times for each harvested sample. The primers used for RT-PCR were as follows: TUB2-1 (5′-ctcaagaggttctcagcagta-3′) and TUB2-2 (5′-tcaccttcttcatccgcagtt-3′) for *β-tubulin* (*TUB2*), used as a positive control; STM-1 (5′-atggagagtggttccaacagcacttc-3′) and STM-2 (5′-gacagaggctgaagaagagaaagagg-3′) for *STM*; KNAT1-1 (5′-ctcttccatgtcacttccttgacgaattc-3′) and KNAT1-2 (5′-taggagggtagagtagtgaggatgagcaa-3′) for *KNAT1*; CycA2-1 (5′-ttcagaatcggcgatcttcgtt-3′) and CycA2-2 (5′-ttagcctcagaagcacgaagat-3′) for *CycA2*; CycC-1 (5′-tggcttccaatttctggacttc-3′) and CycC-2 (5′-gattccagaatcctgcaagaac-3′) for *Cyc*C; CycD1-1 (5′-tatggggagagaatgaggagtt-3′) and CycD1-2 (5′-tatactcgatggccagtactca-3′) for *CycD1*; CycD2-1 (5′-catggctgagaatcttgcttgt-3′) and CycD2-2 (5′-gagttagagcttgcaatctcca-3′) for *CycD2*; CycD3-1 (5′-acgatcttgatgacgatggaga-3′) and CycD3-2 (5′-cctcataaacctcgtatcagca-3′) for *CycD3* (At5g67260).

3. Results

3.1. Generation of cytokinin-deficient 35S::DSCKX1se Arabidopsis transformants

We previously isolated and characterized a novel CKX gene, *DSCKX1*, from *Dendrobium* Sonia [17]. To assess the function of *DSCKX1* in transgenic *Arabidopsis* plants, wild-type *Arabidopsis* plants were transformed with *A. tumefaciens* harboring a construct containing the *DSCKX1* cDNA under the control of the cauliflower mosaic virus 35S promoter (*35S::DSCKX1se*). We isolated a total of 36 independent sense transgenic lines using kanamycin selection, among which 14 plants showed similar non-wild-type phenotypes (described below). Southern blot analyses were carried out on these primary transformants to verify the presence and integrity of the *DSCKX1* gene in transgenic plants. As illustrated in Fig. 1A, there are one to more than seven copies of the transgene integrated in the genome of each individual *Arabidopsis* line. Cytokinin analyses showed no correlation between transgene copy number and cytokinin content (data not shown). To reduce possibilities of position effects of transgene integration or transgene silencing, we chose the transformants (se1, se2, and se8) containing one transgene for further analyses.

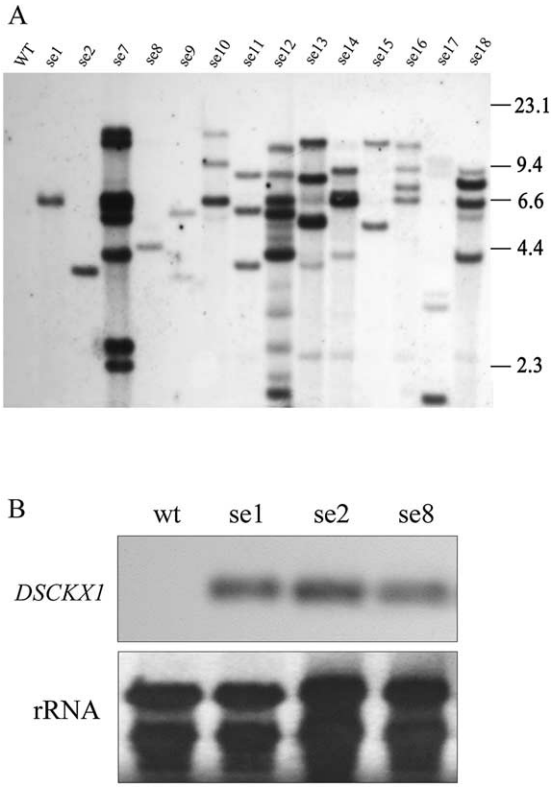

Fig. 1. Gel blot analyses of *Arabidopsis 35S::DSCKX1se* transgenic plants. A: DNA gel blot analysis of *Arabidopsis 35S::DSCKX1se* transgenic plants. A gel blot containing genomic DNA from primary independent transgenic lines (10 μg per lane) digested with *Sac*I was hybridized with the digoxigenin-labeled full-length *DSCKX1* DNA probe. The sizes of the DNA markers are indicated at right in kb. B: RNA gel blot analysis of *Arabidopsis 35S::DSCKX1se* transgenic plants. Total RNA was isolated from 14-day-old seedlings of wild-type (wt) plants and *35S::DSCKX1se* transformants (lines se1, se2, and se8). The gel blot containing 20 μg of total RNA in each lane was hybridized with the *DSCKX1* DNA probe. Equivalent loading of RNA samples was verified by visualizing the methylene blue-stained rRNA bands.

Expression of the orchid *DSCKX1* transgene was investigated in those single-copy transgenic *Arabidopsis* lines (Fig. 1B). Transcript hybridizing to *DSCKX1* was not detected in wild-type seedlings, whereas *DSCKX1* mRNA accumulation was observed in all transgenic lines examined. As expected, CKX activity in *35S::DSCKX1se* transformants was elevated two- to four-fold as compared to that of in wild-type plants (Fig. 2A), while the levels of iPA and ZR were considerably reduced in *35S::DSCKX1se* transformants in all three stages examined (Fig. 2B,C).

3.2. Phenotype of DSCKX1 transgenic plants

In general, *35S::DSCKX1se* transformants were much smaller in stature than wild-type plants (Fig. 3A, Table 1).

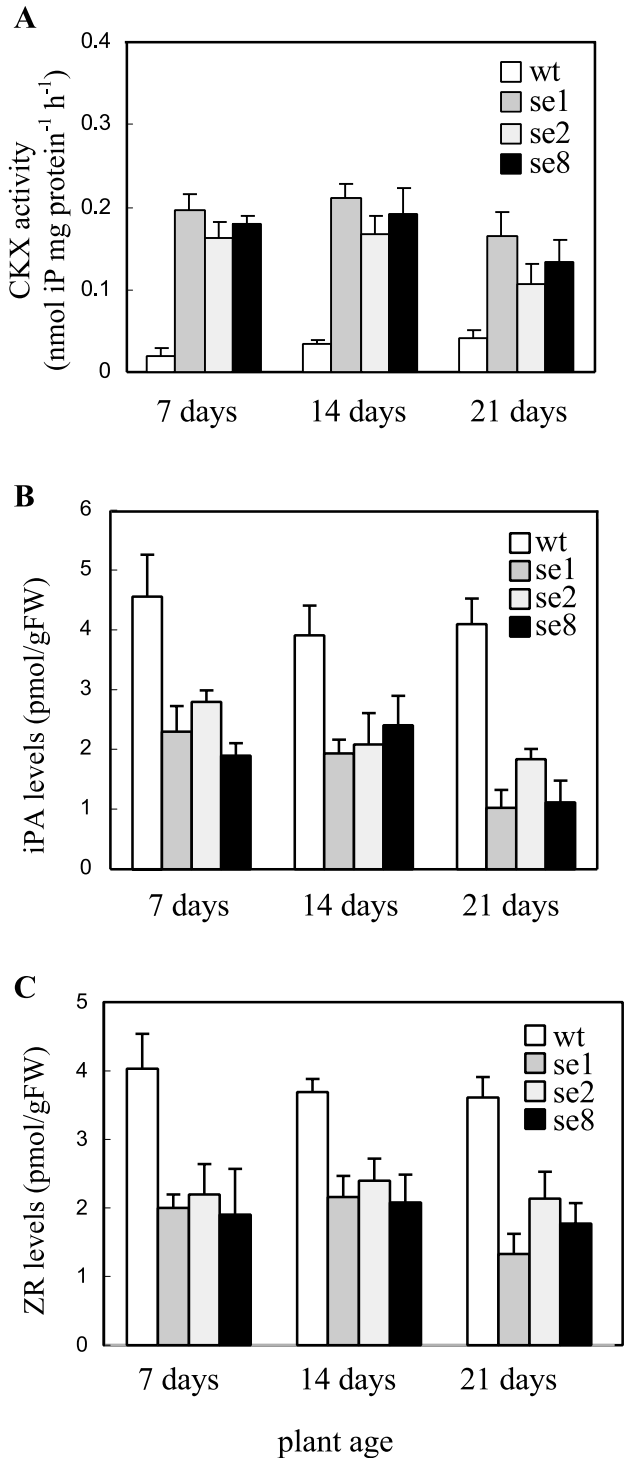

Fig. 2. CKX activity (A) and cytokinin levels (B,C) in wild-type (wt) and *35S::DSCKX1se* transgenic *Arabidopsis* plants (bars se1, se2, and se8). Cytokinin was extracted from leaves of wild-type and transgenic plants at various stages. Cytokinin content is expressed as ZR or iPA equivalents and is based on the reactivity of HPLC-purified fractions with anti-ZR and anti-iPA monoclonal antibodies in an immunoassay.

Twenty-two days after sowing, the biomass of *35S::DSCKX1se* transformants was only about 50% that of wild-type (Table 1). Furthermore, the in vitro study also showed that the shoot regeneration capacity from root explants in *35S::DSCKX1se* transformants was significantly reduced as compared to that in wild-type plants (Table 1, Fig. 3B). In contrast to their inhibited shoot generation and development, the root growth of *35S::DSCKX1se* transformants was greatly enhanced. The primary root length (Table 1) and the number of lateral root branches (data not shown) increased in these transformants as compared to wild-type plants. As for reproductive growth, *35S::DSCKX1se* transformants exhibited slightly earlier flowering time than wild-type but comparable wild-type floral phenotype.

A cross between a *35S::DSCKX1se* transformant (se1) and the *amp1* mutant exhibiting high cytokinin levels was performed to study the possible link between cytokinin and AMP1. The *amp1* mutant shows pleiotropic phenotypes, such as altered shoot apical meristems, increased cell proliferation, polycotyly, constitutive photomorphogenesis, and increased levels of *cyclin D3* [20,32]. AMP1 encodes a putative glutamate carboxypeptidase with significant similarity to *N*-acetyl α-linked acidic dipeptidases [33]. All F1 plants displayed the *35S::DSCKX1se* transformant phenotype because *amp1* is a recessive mutant. Among the 85 F2 progeny, 13 exhibited an intermediate phenotype between *35S::DSCKX1se* transformants and *amp1* (Fig. 3C). We further identified five homozygous plants for both *amp1* and *35S::DSCKX1se*. These plants show similar phenotypes to the *amp1* mutant containing heterozygous *35S::DSCKX1se*. Genetic analyses support the hypothesis that *DSCKX1* overexpression can partially suppress the *amp1* phenotype. Thus, overexpression of *DSCKX1* in *amp1* may downregulate endogenous cytokinin as in wild-type plants, partially rescuing the morphological phenotype of *Arabidopsis amp1* mutant. It is interesting to note that *DSCKX1* cannot totally suppress *amp1* phenotype. This is consistent with the possibility that the pleiotropic phenotypes of the *amp1* mutant only partly result from an increase in cytokinin because application of cytokinins failed to mimic all the *amp1* phenotypes [33].

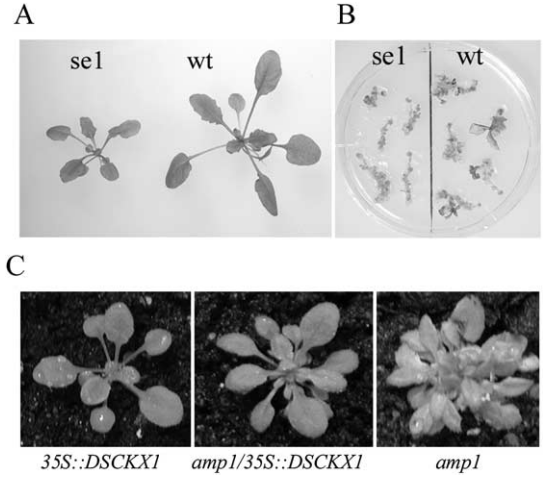

Fig. 3. Phenotype of *Arabidopsis 35S::DSCKX1se* transgenic plants. A: Phenotypes of a 22-day-old wild-type (wt) plant and a *35S::DSCKX1se* transformant (se1). B: Shoot regeneration from roots of wild-type (wt) and *35S::DSCKX1se* transformant (se1). Roots of wild-type (right) and se1 (left) were grown on SIM after being exposed to CIM for 2 days. Shoot initiation was examined after 3 weeks. C: Phenotypes of *35S::DSCKX1se* transformants, *amp1/35S::DSCKX1se* plants, and *amp1* mutant.

3.3. Effects of exogenous cytokinin on the growth of 35S::DSCKX1se transformants

The calli from *35S::DSCKX1se* transformants were further examined for their response to exogenous cytokinins. As shown in Fig. 4A, the enhanced growth of wild-type and se1 and se2 plants was concomitant with increased iPA concentration. Nevertheless, calli of *35S::DSCKX1se* transformants always grew slower than those of wild-type in the presence of iPA at low concentrations, indicating that there was a higher requirement for cytokinin in the growth of *35S::DSCKX1se* transformant calli. However, this effect was diminished at higher doses of iPA. Furthermore, the study on root growth also revealed that the *35S::DSCKX1se* transformants exhibited lower cytokinin sensitivity, as indicated by their response to 10^{-7} M of external iPA, while the root growth of wild-type plants was severely inhibited under the same concentrations (Fig. 4B). Effects of several other exogenous cytokinins, such as zeatin and ZR, on the growth of *35S::DSCKX1se* transformants were similar to that of iPA (data not shown). Taken together, these results indicated that DSCKX1 may oxidize exogenous cytokinin to increase plant tolerance to cytokinin in *35S::DSCKX1se* transgenic plants.

3.4. Expression of cytokinin-regulated genes in 35S::DSCKX1se transformants

Phenotypic similarities between cytokinin-overproducing plants and *KNAT1* overexpressing plants implied a possible link between cytokinins and homeobox genes that play important roles in shoot meristem establishment and maintenance [34]. Thus, we examined the steady state mRNA levels of homeobox genes *KNAT1* and *STM* in *35S::DSCKX1se* transformants. An apparent reduction in both *KNAT1* and *STM* transcripts was observed in all transgenic lines examined (Fig. 5A), which is consistent with the reduced levels of endogenous cytokinin in these plants.

There is evidence that cytokinins have important regulatory functions in the G1 to S transitions in cell cycle progression [10,35,36]. In order to determine if the reduction of cytokinin in transgenic plants affected the activities of cyclins that are involved in the G1 to S phase transition in the cell cycle, expression levels of three different D-type cyclins (*CycD1*, *CycD2*, and *CycD3*) and one C-type cyclin (*CycC*) were investigated by RT-PCR (Fig. 5B). Our data showed that in the rosette leaves there were no significant differences in the expression of *CycC*, *CycD1*, and *CycD2* genes between wild-type and the *35S::DSCKX1se* transformants. However, *CycD3* expression was dramatically reduced in *35S::DSCKX1se* transformants (Fig. 5B). The expression level of a mitotic cyclin (*CycA2*), which acts at G2 to M phase transition, was not affected in the transgenic plants (Fig. 5B).

4. Discussion

Endogenous cytokinin levels in plants are tightly maintained by the balance between biosynthesis and metabolism. Upregulation of a metabolic enzyme, CKX, affects endogenous cytokinin levels and thus cytokinin-related developmental processes. In this study, transgenic *Arabidopsis* plants with ectopic expression of *DSCKX1* mRNA showed a reduction in cytokinin levels. They also exhibited other morphological characteristics including elongated primary roots, reduced frequency of shoot regeneration, and altered sensitivity to exogenous cytokinin. Furthermore, the transgenic plants had shortened petioles and reduced leaf size as compared to wild-type plants. Similar morphological phenotypes occur in transgenic tobacco plants that overexpress CKX genes from *Arabidopsis* [26]. The above phenomena were unsurprisingly in contrast to the typical phenotypes observed in transgenic plants that overproduce cytokinins [34,38], and to the cytokinin-overproduction mutants *amp1* [24] and *sps* [25]. Previously published phenotypes of cytokinin-overproduction mutants and transgenic cytokinin-overproduction plants [38,24,34], and the current study demonstrating the phenotypic consequences of cytokinin-reduction *Arabidopsis* plants provide valuable information for the screening of mutants defective in cytokinin production.

Cytokinins are required, in concert with auxin, for cell division in a wide variety of cultured plant cells. A recent study indicated that cytokinin-dependent cell division activity in *Arabidopsis* was mediated by the transcriptional regulation of *CycD3* gene at the G1 to S transition [21]. The fact that *CycD3* is highly expressed in the cytokinin-overproducing *amp1* mutant and three fast-growing mutants (*shooty callus 6*, *rooty callus 3*, and *callus 2*) suggests a physiological and molecular link between cytokinin and *CycD3* [21,32,37]. Furthermore, tissues overexpressing *CycD3* were cytokinin independent in culture, which implies that *CycD3* may act downstream of cytokinin in promoting cell division and/or differentiation. Our results demonstrated that *CycD3* expression was dramatically reduced in the cytokinin-downregulated plants, while the expression of other D-type cyclins (*CycD1*

Table 1
Comparison of morphology in wild-type and transgenic lines se1, se2, and se8

	Wild-type[a]	Sense transformants[a]		
		se1	se2	se8
Root length (cm)[b]	1.34 ± 0.11	1.98 ± 0.20	1.67 ± 0.23	1.68 ± 0.15
Petiole length (cm)[c]	1.15 ± 0.06	0.72 ± 0.03	0.79 ± 0.08	0.71 ± 0.10
Seedling weight (mg) (6 days old)	4.76 ± 0.32	2.94 ± 0.17	3.31 ± 0.27	2.99 ± 0.19
Seedling weight (mg) (22 days old)	28.0 ± 0.97	14.8 ± 0.19	16.1 ± 0.42	15.0 ± 0.39
Rosette leaf number at flowering[d]	12.4 ± 1.5	10.2 ± 1.4	11.3 ± 1.7	10.8 ± 1.1
No. of shoots/explants[e]	4.6 ± 0.9	1.1 ± 0.7	1.9 ± 0.9	1.5 ± 0.3

[a] $n = 20$.
[b] The lengths of primary roots were measured from 6-day-old seedlings grown on half-strength MS medium under a 16-h light/8-h dark cycle at 22°C.
[c] The petiole length of the largest rosette leaf from 22-day-old plants was measured.
[d] Rosette leaf number was scored as the number of rosette leaves present before the first flower opened.
[e] Root segments excised from 10-day-old seedlings were incubated on CIM for 2 days, then transferred to SIM for 3 weeks.

and *CycD2*), a C-type cyclin (*CycC*), and a mitotic cyclin (*cycA2*) was not affected. These studies suggest that cytokinin may regulate *Arabidopsis* cell cycle progression at least partially by controlling *CycD3* transcription in the tissues we tested.

It is noteworthy that transgenic plants overexpressing the maize *KN1* gene or the *Arabidopsis* homolog *KNAT1* have phenotypes reminiscent of transgenic plants expressing the bacterial cytokinin biosynthetic gene *IPT* [39,40]. Consistent with this observation, elevated *KNAT1* and *STM* transcript levels were detected in both *IPT* transgenic *Arabidopsis* and the cytokinin-overproducing mutant *amp1* [39]. Further study showed that *STM*- and *KNAT1*-overexpressing *shooty callus* mutant lines did not have altered cytokinin content [37]. These results support the hypothesis that cytokinins act upstream of *KNAT1* and *STM*. In contrast, ectopic expression of *KN1* resulted in the cytokinin-autotrophic growth of cultured tobacco tissues with an increase in endogenous cytokinin levels, suggesting that *KN1* may mediate the action of cytokinins in regulating plant development [20]. This is further enhanced by the fact that overexpression of *KNAT1* in lettuce shifted leaf determinate growth to shoot-like indeterminate growth associated with an accumulation of isopentenyl-type cytokinins [41]. Based on the above results, it is difficult to define a cause-and-effect relationship between the *KN1* and *STM* genes and cytokinin. Our study demonstrated that the decrease in cytokinin levels in transgenic plants overexpressing *DSCKX1* caused reduction in *KNAT1* and *STM* expression, suggesting that cytokinin may function through regulation of *KNAT1* and *STM* expression. However, we cannot exclude the possibility that a positive feedback regulation exists between cytokinin levels and homeobox gene expression. Further investigation of endogenous cytokinins in *KNAT1* and *STM* loss-of-function mutants will help unravel the interaction of cytokinin and homeobox genes during plant growth and development.

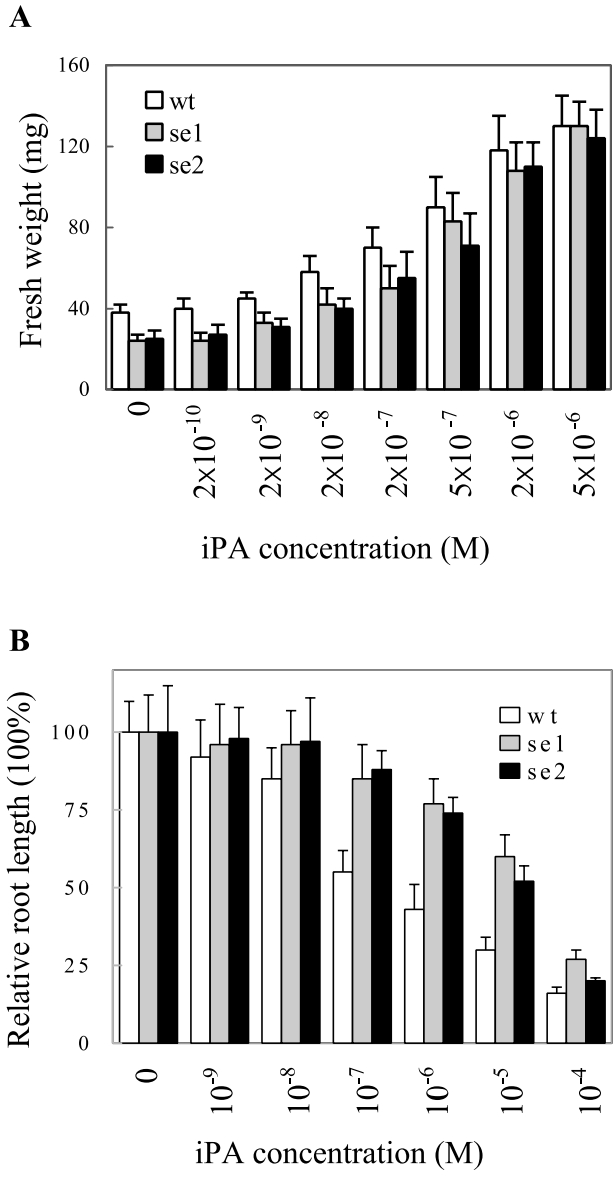

Fig. 4. Effects of exogenous cytokinin on callus and root growth. A: Callus growth of wild-type and *35S::DSCKX1se* transformant (se1, se2) tissues. Stock lines weighing 8 mg were cultured on CIM medium, then subcultured on MS medium supplemented with 0.1 μM of NAA and different concentrations of iPA. The mean callus weight (fresh weight ± S.D., $n = 20$) was determined after 14 days. B: Root growth of wild-type and *35S::DSCKX1se* transformant (se1, se2) tissues. Seeds were sown on MS medium containing 2% sucrose and various iPA concentrations. The length of primary roots is represented as the relative ratio to that of wild-type plants on the iPA-free medium after 8 days of culture. Bars indicate standard deviation ($n = 20$).

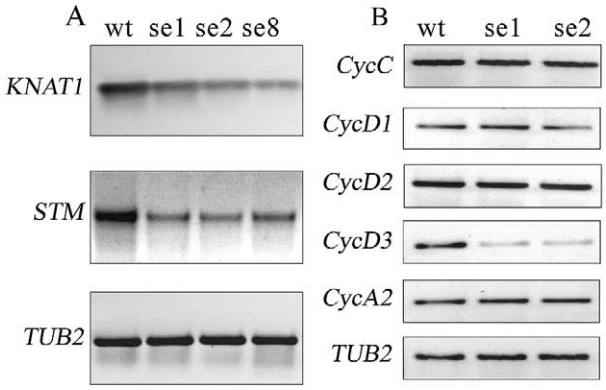

Fig. 5. Expression of *KNAT1*, *STM* (A) and cyclins (B) in *35S::DSCKX1se* transformants determined by RT-PCR. Total RNA used for one-step RT-PCR analysis was isolated from 14-day-old seedlings of wild-type (wt) and *35S::DSCKX1se* transformants (se1, se2, and se8). The *β-tubulin* gene (*TUB2*) was amplified as a quantitative control.

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