Title: Auxin controls local cytokinin biosynthesis in the nodal stem in apical dominance.

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Summary

In intact plants, the shoot apex grows predominantly and inhibits axillary bud outgrowth. After shoot apex decapitation, axillary bud outgrowth begins. This phenomenon is called apical dominance. Although the involvement of auxin, which represses axillary bud outgrowth, and cytokinin (CK), which promotes axillary bud outgrowth, has been proposed, little is known about the underlying molecular mechanisms. Here, we demonstrate that local CK biosynthesis in the nodal stem is negatively regulated by auxin by controlling expression of the gene pea *adenosine phosphate-isopentenyltransferase* (*PsIPT*), which encodes a key enzyme in CK biosynthesis. Before decapitation, *PsIPT1* and *PsIPT2* transcripts were undetectable; after decapitation, they were markedly induced in the nodal stem along with CK accumulation. *PsIPTs* expression was repressed by the application of indole-3-acetic acid (IAA). In excised nodal stem, *PsIPTs* expression and CK levels also increased under IAA-free conditions. Furthermore, β-glucuronidase expression under the control of the *PsIPT2* promoter region in transgenic *Arabidopsis* was

repressed by IAA. Our results indicate that, in apical dominance, one role of auxin is to repress local CK biosynthesis in the nodal stem and that, after decapitation, CKs, which are thought to be derived from the roots, are locally biosynthesized in the nodal stem rather than in the roots.

Introduction

In many plant species, the shoot apexes repress axillary bud growth and grow predominantly. This phenomenon is called apical dominance, and it is regulated by plant hormones, auxin and cytokinin (CK). Auxin derived from the shoot apex inhibits axillary bud growth, while CK, thought to be derived from the roots, promotes axillary bud growth (Cline, 1991; Leyser, 2003; Shimizu-Sato and Mori, 2001). The role of auxin *in vivo* is supported by the following observations. Decapitation of *Vicia* plants induces axillary bud outgrowth, but application of auxin to the stump prevents axillary bud outgrowth (Thimann and Skoog, 1933; Thimann and Skoog, 1934). These observations have been confirmed in many plant species (Cline, 1996). Furthermore, application of the auxin-transport inhibitor 2, 3, 5-triiodobenzoic acid in lanolin to the stems of intact plants reduces or abolishes apical dominance (Panigrahi and Audus, 1966; Snyder, 1949). These data strongly support the hypothesis that apically-derived auxin is transported basipetally and inhibits axillary bud outgrowth. In addition, direct application of auxin to the axillary buds does not

prevent bud outgrowth (Leyser and Day, 2003). Radio-labeled auxin applied to the stump is not translocated into the axillary buds (Everat-Bourbouloux and Bonnemain, 1980; Hall and Hillman, 1975; Lim and Tamas, 1989; Prasad *et al.*, 1993). Indoleacetic acid (IAA) levels in dormant axillary buds are low and increase in the axillary buds after decapitation of the apex shoot (Gocal *et al.*, 1991; Pearce *et al.*, 1995).

On the other hand, direct application of CK to axillary buds promotes axillary bud outgrowth, even though the shoot apex is intact (Panigrahi and Audus, 1966; Sachs and Thimann, 1964; Sachs and Thimann, 1967). As with auxin, these observations have been confirmed in many plant species. Axillary bud outgrowth is well correlated with bud CK levels. Auxin is thought to control the concentration of CK derived from the roots (Letham, 1994). CK concentrations in bean xylem exudate increase within 16 h after decapitation and gradually return to basal levels (Bangerth, 1994), and those in chickpea axillary buds after decapitation increase 7-fold by 6 h and 25-fold by 24 h (Turnbull *et al.*, 1997), suggesting that CKs may be an activator

involved in axillary bud outgrowth.

The involvement of auxin and CK is further supported by phenotypic observations of many mutants with altered shoot branching patterns (Ward and Leyser, 2004). For example, the *Arabidopsis* mutant *axr1*, which has a reduced response to auxin, has reduced apical dominance (Lincoln *et al*., 1990). The petunia mutant *sho* (Zubko *et al*., 2002), which was identified by activation tagging, and the *Arabidopsis* mutant *hoc* (Catterou *et al*., 2002) have increased CK levels and reduced apical dominance. Transgenic plants with elevated CK levels due to overexpression of *ipt* from *Agrobacterium tumefaciens* (Medford *et al*., 1989), and those with reduced IAA levels due to overexpression of IAA-lysine synthetase from *Pseudomonas savastanoi*, have reduced apical dominance (Romano *et al*., 1991). In addition, transgenic plants with elevated IAA levels due to overexpression of indoleacetamide hydrolase and tryptophan monooxygenase from *Agrobacterium tumefaciens*, have increased apical dominance (Romano *et al*., 1993; Sitbon *et al*., 1992).

These results provide clear evidence that auxin and CK are involved in apical

dominance. Little is known, however, about the underlying molecular mechanisms. It appears paradoxical that auxin, which is considered to be a growth promoter, actually inhibits axillary bud outgrowth. We report here that a role of auxin in apical dominance is to repress gene expression of *adenosine phosphate-isopentenyltransferase* (*IPT*, EC 2.5.1.27), which encodes a key enzyme in CK biosynthesis (Kakimoto, 2001; Takei *et al.*, 2001a). Furthermore, after decapitation, CKs are locally biosynthesized in the stem rather than in the roots.

Results

Identification of PsIPT genes expressed in the stem after decapitation

To investigate the role of basipetal auxin transport at the node in apical dominance, we examined genes expressed on the nodal stem before and after decapitation. Using a subtraction method, we isolated various genes including *IPT* and GA2 oxidase, expressed specifically at the nodal stem 3 h after decapitation. *PsGA2ox1* gene expression is induced in the stem after decapitation in pea (Ross *et al.*, 2000). *IPT* encodes a key enzyme in CK biosynthesis. Because direct application of CKs to the axillary buds promotes axillary bud outgrowth, even in intact plants (Panigrahi and Audus, 1966; Sachs and Thimann, 1964; Sachs and Thimann, 1967), we investigated the relationship between auxin and *IPT* expression.

We attempted to exhaustively identify all *IPT* genes expressed in the stem after decapitation. Based on the amino acid sequences of *Arabidopsis* IPT, we designed degenerated primers and performed RT-PCR. Using the amplified DNA fragments as a probe, we screened a cDNA library prepared from the stem 3 h after

decapitation, and obtained two full-length *IPT* cDNAs named *PsIPT1* and *PsIPT2*. The *IPT* clone identified in the subtraction experiment was *PsIPT2*. *PsIPT1* cDNA had 1463 bp and consisted of 328 amino acid residues. *PsIPT2* cDNA had 1462 bp and consisted of 341 amino acid residues. The deduced amino acid sequences of PsIPT1 share 58.7% identity with those of PsIPT2.

Effect of auxin on PsIPT gene expression

To clarify that basipetal auxin transport affect *PsIPT* expression, we examined *PsIPT* expression patterns in the second nodal stem before and after decapitation (Figure 1). The mRNA levels of both genes were undetectable in the nodal stem before decapitation by Northern blot analysis. *PsIPT2* mRNA was increased at 1 h, reached a maximum level at 3 h, and decreased from 9 h after decapitation 1 cm above the second node. In contrast, *PsIPT1* mRNA markedly increased at 3 h, reached a maximum level by 5 h, and decreased from 9 h after decapitation. Both *PsIPT1* and *PsIPT2* mRNA levels were undetectable 24 h after decapitation. Together, these

results and the report that basipetal auxin transport in pea stem is 1 cm/h (Johnson and Morris, 1989) suggest that auxin derived from a shoot apex represses *PsIPT2* expression at the nodal stem. We then investigated whether *PsIPT* expression was repressed by exogenous IAA in the nodal stem. Stem segments excised from seedlings 3 h after decapitation, in which apically-driven auxin was depleted and *PsIPT1* and *PsIPT2* transcripts were expressed, were incubated in MES buffer with or without IAA (Figure 2a). *PsIPT1* and *PsIPT2* transcript levels decreased soon after incubation with IAA, whereas they persisted in the IAA-free buffer. These results suggest that accumulation of *PsIPT* transcripts in the nodal stem is negatively regulated by IAA. We then examined whether *PsIPT* expression in the stem is induced by the depletion of auxin. Stem segments excised from intact seedlings, in which *PsIPT* transcripts were not expressed, were incubated in MES buffer with or without IAA (Figure 2b). *PsIPT1* and *PsIPT2* transcripts were increased in the IAA-free buffer, but not in the buffer with IAA. When incubated in the IAA-free buffer, the induction of *PsIPT* seemed to be due to the diffusion of auxin from the stem into

the buffer. Moreover, to elucidate the relationship between *PsIPT* expression and basipetal auxin transport in the stem, we investigated *PsIPT* expression by applying IAA to the stump after decapitation (Figure 3). Since *PsPIN1* gene, which was isolated as a gene expressed specifically in the nodal stem of intact seedlings in the subtraction experiment, was induced by exogenous auxin (data not shown), transcript levels of *PsPIN1* were also examined as a positive control for the effects of auxin. After decapitation, lanolin with or without IAA was immediately applied to the stump. Three hours after application, *PsIPT1* and *PsIPT2* transcripts were increased by lanolin alone, but not by IAA. These results indicated that *PsIPT* is controlled by auxin transported basipetally in the stem.

Analyses of PsIPT gene expression in various organs and by other treatments

We examined the expression of *PsIPT1* and *PsIPT2* in other organs: terminal buds, and leaflets in intact seedlings; axillary buds, and roots before/after decapitation using real-time PCR (Figure 4). The expression levels of *PsIPT1* and

PsIPT2 in terminal buds, leaflets, axillary buds, and roots were very low. After decapitation, the *PsIPT1* and *PsIPT2* expression were not induced in the axillary buds, slightly induced in the roots but at low level. Marked induction of *PsIPT* genes was observed only in the stems. These results confirmed that the stem is the main contributor of cytokinin biosynthesis to the axillary buds after decapitation.

We also examined the effect of 2, 3, 5-triiodobenzoic acid (TIBA) in lanoline, which was applied to the internode as complete ring around the internode, on the expression of *PsIPT1* and *PsIPT2*. TIBA treatment induced the expression of *PsIPT1* and *PsIPT2*, indicating that auxin transported from the shoot apex represses *PsIPT* expression.

We then examined the *PsIPT* expression in the root segments by application and depletion of IAA. Root segments excised from intact seedlings, in which *PsIPT* transcripts were expressed at very low level, were incubated in MES buffer with or without IAA. *PsIPT1* and *PsIPT2* transcripts were increased in the IAA-free buffer, but not in the buffer with IAA. The induction of *PsIPT* seemed to be due to the

depletion of auxin from the roots into the buffer. These expression patterns in the root segments were similar to those in the stem segments (Figure 2b), suggesting that *PsIPT1* and *PsIPT2* transcriptions can occur in root when auxin level decreases. However, shoot decapitation can not cause such a physiological condition.

Response of PsIPT2 promoter to auxin in Arabidopsis

To confirm whether the promoter region of *PsIPT2* is sufficient for *PsIPT2* repression in response to auxin, we generated transgenic *Arabidopsis* containing the β-glucuronidase (*GUS*) reporter gene under the control of the *PsIPT2* promoter region. Various organs except root tip in 10-d-old transgenic *Arabidopsis* seedlings were GUS-stained probably due to a heterologous system or lack of some promoter regions to respond to spatial and temporal expression. When these plants transferred to new media contained synthetic auxin, the roots were not GUS-stained (data not shown). To investigate the effect of IAA on the promoter activity, we examined *GUS* transcript levels (Figure 5) because GUS protein seems to be stable

in plant cells. *GUS* gene was expressed in whole seedlings and then was repressed by IAA treatment. The response of *GUS* expression to auxin was the same as that of *PsIPT2* expression in the pea stem (Fig. 2a). These results indicated that plants, at least *Arabidopsis,* have molecular mechanisms of repression similar to those of auxin on *PsIPT2* expression in pea.

CK level in axillary buds and stem before and after decapitation

To investigate whether the induction of *PsIPT* increased CK levels, we measured the CK levels in nodal stems in which axillary buds were removed, and axillary buds before and after decapitation (Table 1). The levels of the various CKs examined were low in both tissues before decapitation, but markedly increased in both tissues after decapitation. In the nodal stem, the increase in CK levels corresponded to the *PsIPT* expression patterns (Figure 1). In the axillary buds, although *PsIPT1* and *PsIPT2* transcripts were very low level at least until 6 h after decapitation (Figure 4), CK levels increased 3 h after decapitation and markedly increased 6 h after decapitation.

The CK increment in the axillary buds lagged as compared with that in the nodal stem. These results suggest that the increased CKs were biosynthesized in the stem, not in the axillary buds, and then transported into the dormant axillary buds.

We also measured CK levels in stem segments excised from intact seedlings (Table 2). To eliminate the possibility that CK diffused from the segments during incubation, the basal ends of the excised stem segments were inserted into agar plates. Auxin treatment was performed with application of lanolin with or without IAA to the apical stump. CK levels markedly increased 3 h after application of lanolin alone, and the amount of CK synthesized in the excised stem segments was the same as that in the intact stem, suggesting that CKs are biosynthesized mainly in the stem, at least for several hours after decapitation, although they could be biosynthesized in the root. On the other hand, CKs were not detected when IAA was applied to the stump, suggesting that IAA impeded the increase of CKs in the nodal stem. These results are consistent with the repression of *PsIPT* by IAA.

Discussion

Apical dominance is one of the classical developmental events believed to be controlled by cross-talk between auxin and CK (Cline, 1991; Leyser, 2003; Shimizu-Sato and Mori, 2001). Although auxin is thought to inhibit axillary bud outgrowth, little is known about the underlying molecular mechanisms. Our data clearly show that auxin represses *IPT* gene expression (Figure 1-5). We demonstrated that one role of auxin is to repress the *IPT* gene expression; that is, local CK biosynthesis in the nodal stem is negatively regulated by auxin through the control of *IPT* expression in apical dominance. Although repression of CK biosynthesis by auxin was previously reported (Eklöf *et al*., 1997; Nordström *et al*., 2004), the present study clearly demonstrated that auxin repressed *PsIPT1 and PsIPT2* expression, the first step in the CK biosynthesis pathway. In contrast, auxin positively regulates *AtIPT5* and *AtIPT7* expression in *Arabidopsis* root (Miyawaki *et al*., 2004). In pea root, we demonstrated that auxin negatively regulated *PsIPT2* and *PsIPT2* expression as the same as in pea stem (Figure 4) and at least *PsIPT2*

promoter was also repressed by auxin even in *Arabidopsis* (Figure 5). We did not confirm whether other members of *PsIPT* genes are positively regulated by auxin or not. One possibility is that the different response to auxin reflects differential regulation of specific *IPT* genes. Furthermore, auxin also represses expression of *Arabidopsis CYP735As*, which catalyze hydroxylation in the last step of *trans*-zeatin biosynthesis in Arabidopsis roots (Takei *et al.*, 2004b). These results suggest that auxin is involved in CK biosynthesis negatively and positively, and that the regulation of CK biosynthesis by auxin is complicated and different in each individual case.

While *PsIPT* expression was induced after decapitation, decapitation-induced *PsIPT* expression was transient (Figure 1). This result is consistent with the report by Li *et al.* that the CK concentration in the stem rose quickly after decapitation, peaked after 12 h, and then decreased (Li *et al.*, 1995). De novo-synthesized IAA derived from a new shoot apex, which had previously been a dormant axillary bud, appeared to flow to the stem 10 h after decapitation (data not shown) and again repress *PsIPT* expression.

CK biosynthesis occurred only in the excised stem segments in the absence of auxin (Table 2). Roots are thought to be the major sites of CK biosynthesis because they contain high levels of CKs (Hopkins and Hüner, 2004). Some reports suggest that CKs biosynthesized in the roots are transported to the axillary buds (Bangerth, 1994; Turnbull *et al*., 1997). On the other hand, other tissues, including leaves, stem, shoot apical meristem, and immature seeds can also produce CKs (Chen *et al*., 1985; Letham, 1994). Recent analyses of *IPT* expression patterns in *Arabidopsis* indicated that tissues expressing *AtIPT* are widely distributed throughout the plant (Miyawaki *et al*., 2004; Takei *et al*., 2004a). Nordström *et al.* also reported that CKs are synthesized in the aerial part of the plant (Nordström *et al*., 2004) and contradicted the hypothesis of CKs as long-distance mediators in root-shoot communication. Furthermore, Faiss *et al.* performed reciprocal grafts between wild-type tobacco and transgenic tobacco, which conditionally controlled bacterial *ipt* gene expression and CK enhancement under a tetracycline-dependent gene expression system (Faiss *et al*., 1997). The results of their graft experiments led

them to question the classical view of the role of CK as a root-borne signal in the control of shoot apical dominance, and to suggest that CK might be produced locally when apical control is released. In fact, axillary bud outgrowth occurs in the excised nodal stem segments in the absence of auxin (Chatfield *et al*., 2000; Cline *et al*., 1997; Tamas *et al*., 1989). In addition, although we demonstrated that *PsIPT1* and *PsIPT2* were induced in the root segments by the depletion of auxin, *PsIPT1* and *PsIPT2* were not markedly induced in the roots after decapitation and their expression levels were very low. Based on these reports and our results, we concluded that CKs that promote axillary bud outgrowth after shoot apex decapitation are biosynthesized locally and mainly in the stem, and that there is little root contribution of CK biosynthesis after decapitation. This conclusion is in agreement with many previous findings and adds to the understanding of the mechanisms of apical dominance.

Determination of the site of *PsIPT2* expression in the stem is important to understand the cross-talk between auxin and CK. Booker *et al*. reported that

apically-derived auxin acts in the xylem and/or interfascicular tissue to inhibit axillary bud outgrowth (Booker *et al*., 2003). AtPIN1, which is thought to be part of an auxin efflux carrier, localizes in vascular tissue, especially parenchymatous xylem cells (Gälweiler *et al*., 1998). These results suggest that xylem-associated cells have pivotal roles in the regulation of axillary bud outgrowth, including in *IPT* expression.

Recently, another role of auxin in apical dominance was suggested by analyses of *Arabidopsis max3*, *max4* (Booker *et al*., 2004; Sorefan *et al*., 2003), and pea *rms1* (Foo *et al*., 2005) branching mutants. *MAX4* and *MAX3*, which encode members of the carotenoid cleavage dioxygenase family, might be involved in the synthesis of a mobile branch-inhibitor (Booker *et al*., 2004; Schwartz *et al*., 2004; Sorefan *et al*., 2003). *RMS1* is orthologous to *MAX4* (Sorefan *et al*., 2003). Moreover, *RMS1* expression requires auxin (Foo *et al*., 2005; Sorefan *et al*., 2003), suggesting that auxin also controls axillary bud outgrowth through the synthesis of a branch-inhibitor (Leyser, 2005). There is also a petunia orthologous gene, *Dad* (Snowden *et al*., 2005).

Finally, we propose the following mechanism. In intact plants, auxin is basipetally transported and represses *IPT* gene expression in the stem. Consequently, axillary buds lack the ability to grow out. On the other hand, once the shoot apex is decapitated, the auxin level in the stem decreases, repression of *IPT* gene expression is released, CK levels increase, and axillary buds grow out. After axillary buds grow out, de novo-synthesized IAA derived from a new shoot apex flows to the stem and again represses *IPT* gene expression. Our observations provide a new interpretation regarding apical dominance, which is a well-known phenomenon in the field of plant physiology, and a model for molecular mechanisms of plant hormone cross-talk.

Experimental procedures

Plant growth conditions

Seeds of *Pisum sativum* L. cv. Alaska were soaked in running tap water for 24 h and sown in trays of rockwool. Plants were grown at 25˚C in the dark for 4 d, and then on a 16 h light/8 h dark photoperiod for 3 d (Shimizu and Mori, 1998). Nodal stem segments (1 cm long), from which the axillary buds were removed, were excised (0.5 cm on each side of the node) from the second node in 7-d-old seedlings. Axillary buds at the second node were used. Seedlings were decapitated 1 cm above the second node to stimulate axillary bud outgrowth. *Arabidopsis thaliana* was grown on 1 x MS medium and 0.8% (w/v) agar at 22˚C under continuous light conditions.

Identification of differentially expressed genes in stem before and after decapitation

Two subtracted cDNA libraries were constructed using the PCR-Select^{TM} cDNA subtraction kit (Clontech, Mountain View, CA) according to the manufacturer's

protocol. To construct a 3 h-subtracted library, driver and tester cDNAs were prepared from nodal stem segments before and 3 h after decapitation, respectively, in pea seedlings. To construct a 0 h-subtracted library, tester and driver cDNAs were prepared from nodal stem segments before and 3 h after decapitation, respectively. More than 1000 cDNA clones from each library were randomly picked and sequenced.

Cloning of PsIPTs

To identify any *IPT* genes expressed in the stem after decapitation, degenerate primers were designed based on the conserved regions of amino acid sequences of *Arabidopsis* IPT and RT-PCR was performed with the primers 5'-GA(G/A)AT(A/T/C)(G/A)T(A/T/C)AA(T/C)TCNGA(T/C)AA(G/A)AT-3' and 5'-AC(G/A)TCNACCCANAG(G/A)AA(G/A)CA(G/A)CA-3', where N indicates all four deoxyribonucleotides. PCR amplification was performed with pea cDNA from total RNAs from the second node after decapitation. The amplified fragments were cloned

into pZErO-2.1 (Invitrogen Corp., Carlsbad, CA) and sequenced. A pea cDNA library was constructed using poly $(A)^+$ RNA prepared from the nodal stems 3 h after decapitation with pZErO-2.1, and screened with the partial *PsIPT* cDNA clone that was identified from the subtraction screening and the selected RT-PCR products were labeled by random primer methods with [^{32}P]-dCTP. Hybridization was performed in Perfect Hyb™ Plus Hybridization Buffer (Sigma Chemical Co., St. Louis, MO) at 65˚C. The membrane was washed in 2 x SSPE and 0.1% SDS at 65˚C twice for 30 min each. The image was visualized with a BAS2000 Imaging Analyzer (Fuji Film Co., Tokyo, Japan).

Northern blot analyses

Total RNA was isolated from the tissues using the guanidine thiocyanate/CsCl method. Formaldehyde agarose gel electrophoresis of total RNA (10 μg/lane) was performed using standard procedures. The RNAs were blotted onto a Hybond N+ membrane (Amersham Biosciences, Piscataway, NJ.), and hybridized with *PsIPT1*,

PsIPT2, *PsPIN1*, and *GUS* clone DNAs labeled by random primer methods with [^{32}P]-dCTP. Hybridization was performed as described above.

Quantitative real-time PCR

Total RNA was prepared by RNeasy® Plant Mini Kit (Qiagen). cDNA was synthesized using SuperScript III RT (Invitrogen) with oligo(dT)$_{20}$ primers. Accumulation levels of *PsIPT1* and *PsIPT2* transcripts were analyzed by LightCycler 1.5 (Roche Diagnostics) with SYBR® Premix Ex Taq™ according to the manufacturer's protocol. The primers for PCR were checked for the specific product formation by agarose gel analysis. The sequences of the primers used for PCR were: 5'-ACCGTCTTGATGCTACGGAGGTTGTGC-3' and 5'-TCTAATGGGTTACCCCTGCCACAGACG-3' for PsIPT1; 5'-TGGCAGCAACATCATCCTCTGCCTGC-3' and 5'-ACCTGTGGCCCCCATTATCACTAC-3' for PsIPT2. In each case, amplified PCR products using above the primer set were measured concentration and used as a

template to generate a calibration curve.

IAA treatment

Stem and root segments of pea and whole seedlings of *Arabidopsis thaliana* were incubated in MES buffer (10 mM MES [pH 6.8], 100 μg/ml carbenicillin, and 0.01% Tween 20) with 10^{-5} M IAA for the indicated times. IAA-lanolin paste was prepared with IAA in ethanol to a final concentration of 1% (w/v) IAA.

TIBA treatment

TIBA-lanolin paste was prepared with TIBA in ethanol to a final concentration of 2% (w/v) TIBA. TIBA-lanoline was applied to the third internode 1 cm above the second node as complete ring around the internode. Nodal stem segments (1 cm long), from which the axillary buds were removed, were excised (0.5 cm on each side of the node) from the second node 6 h after TIBA application.

Transformation of Arabidopsis with the pPsIPT2::GUS reporter gene

The genomic DNA fragment of *PsIPT2* was isolated from a pea genomic library using *PsIPT2* cDNA as a probe. A 3179-bp fragment containing the promoter region (2025 bp) and the 5'-flanking region (1154 bp, including the intron) from the 9 amino acid residues of the open reading frame was amplified from genomic DNA by the first PCR with the primers 5'-AAAAAGCAGGCTGTGGAGGGTAGCAATGAGTATGGTG-3' and 5'-AGAAAGCTGGGTATGATGTTGCTGCCACTGGGATAATCAT-3' (underlines indicate partial attB sequences for BP reaction in the Gateway system) and the second PCR with the attB adapter primers. The resulting fragment was cloned into pDONR207 (Invitrogen) using the Gateway BP reaction, and sequenced. The *PsIPT2* gene fragment was cloned into the *Agrobacterium* binary vector pGWB3 (provided by T. Nakagawa, Shimane University), which had the *GUS* reporter gene and the noparine synthase terminator, using the Gateway LR reaction (Invitrogen) according to the manufacturer's protocol. The resulting plasmid was transformed

into *Agrobacterium tumefaciens*, strain GV3101. *Arabidopsis* ecotype Columbia (Col-0) plants were transformed using the floral dip method (Clough and Bent, 1998).

Measurement of CK contents

Axillary buds (ca. 0.5 g) and nodal stem segments (ca. 1 g) were collected from more than 4000 plants and 20 plants, respectively, and frozen at -20˚C until use. Extraction and fractionation of CKs from tissues were performed as described previously (Dobrev, P. I. and KamÍnek, M., 2002). The CK fractions were purified further using immune-affinity columns (Takei *et al*., 2001b). After desalting, the resulting samples were analyzed by liquid chromatography-mass spectrometry as described previously (Yonekura-Sakakibara *et al*., 2004).

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References

Bangerth, F. (1994) Response of cytokinin concentration in the xylem exudate of bean (*Phaseolus vulgaris* L.) plants to decapitation and auxin treatment, and relationship to apical dominance. *Planta* **194,** 439-442.

Booker, J., Auldridge, M., Wills, S., McCarty, D., Klee, H. and Leyser, O. (2004) MAX3/CCD7 is a carotenoid cleavage dioxygenase required for the synthesis of a novel plant signaling molecule. *Curr. Biol*. **14**, 1232-1238.

Booker, J., Chatfield, S. and Leyser, O. (2003) Auxin acts in xylem-associated or medullary cells to mediate apical dominance. *Plant Cell* **15**, 495-507.

Catterou, M., Dubois, F., Smets, R., Vaniet, S., Kichey, T., Onckelen, H.V., Sangwan-Norreel, B.S. and Sangwan, R.S. (2002) *hoc*: an *Arabidopsis* mutant overproducing cytokinins and expressing high *in vitro* organogenic capacity. *Plant J*. **30**, 273-287.

Chatfield, S.P., Stirnberg, P., Forde, B.G. and Leyser, O. (2000) The hormonal regulation of axillary bud growth in *Arabidopsis*. *Plant J.* **24**, 159-169.

Chen, C., Ertl, J.L., Leisner, S.M. and Chang, C. (1985) Localization of cytokinin biosynthetic sites in pea-plants and carrot roots**.** *Plant Physiol*. **78**, 510-513.

Cline, M.G. (1991) Apical dominance. *Bot. Rev*. **57**, 318-358.

Cline, M.G. (1996) Exogenous auxin effects on lateral bud outgrowth in decapitated shoots. *Ann. Bot.* **78**, 255-266.

Cline, M., Wessel, T. and Iwamura, H. (1997) Cytokinin/auxin control of apical dominance in *Ipomoea nil*. *Plant Cell Physiol*. **38**, 659-667.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735-743.

Dobrev, P. I. and KamÍnek, M. (2002) Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction. *J. Chromatogr. A* **950**, 21-29.

Eklöf, S., Åstot, C., Blackwell, J., Moritz, T., Olsson, O. and Sandberg, G. (1997) Auxin-cytokinin interactions in wild-type and transgenic tobacco. *Plant Cell Physiol*.

38, 225-235.

Everat-Bourbouloux, A. and Bonnemain, J.L. (1980) Distribution of labeled auxin and derivatives in stem tissues of intact and decapitated broad-bean plants in relation to apical dominance. *Physiol. Plant* **50,** 145-152.

Faiss, M., Zalubiova, J., Strnad, M. and Schmulling, T.（1997） Conditional transgenic expression of the *ipt* gene indicates a function for cytokinins in paracrine signaling in whole tobacco plants. *Plant J.* **12,** 401-415.

Foo, E., Bullier, E., Goussot, M., Foucher, F., Rameau, C. and Beveridge, C.A. (2005) The branching gene *RAMOSUS1* mediates interactions among two novel signals and auxin in pea. *Plant Cell* **17**, 464-474.

Gälweiler, L., Guan, C., Müller, A., Wisman, E., Mendgen, K., Yephremov, A. and Palme, K. (1998) Regulation of polar auxin transport by AtPIN1 in *Arabidopsis* vascular tissue. *Science* **282**, 2226-2230.

Gocal, G.F., Pharis, R.P., Yeung, E.C. and Pearce, D. (1991) Changes after decapitation in concentrations of indole-3-acetic-acid and abscisic-acid in the larger

axillary bud of *Phaseolus-vulgaris* L. cv Tender Green. *Plant Physiol.* **95,** 344-350.

Hall, S.M. and Hillman, J.R. (1975) Correlative inhibition of lateral bud growth in *Phaseolus vulgaris* L. timing of bud growth following decapitation. *Planta* **123,** 137-143.

Hopkins, W.G. and Hüner, N.P.A. (2004) Cytokinins are synthesized primarily in the root. in *Introduction to Plant Physiology*. New York: Wiley, pp.325-326.

Johnson, C. F. and Morris, D. (1989) Applicability of the chemiosmotic polar diffusion-theory to the transport of indol-3yl-acetic acid in the intact pea (*Pisum sativum* L.). *Planta* **178,** 242-248.

Kakimoto, T. (2001) Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate: ATP/ADP isopentenyltransferases. *Plant Cell Physiol.* **42**, 677-685.

Letham, D.S. (1994) Cytokinin as phytohormones-sites of biosynthesis, translocation, and function of translocated cytokinin. in *Cytokinin: Chemistry, Activity, and Function.* (Mok, D.W.S. and Mok, M.C., eds). Florida: CRC Press, pp. 57-80.

Leyser, O. (2003) Regulation of shoot branching by auxin. *TRENDS in Plant Science*. **8**, 541-545.

Leyser, O. (2005) The fall and rise of apical dominance. Curr. Opin. Genet. Dev. **15**, 468-471.

Leyser, O. and Day, S. (2003) Shoot branching. in *Mechanisms in Plant Development*. Oxford: Blackwell Science, pp. 213-219.

Li, C. J., Guevera, E., Herrera, J. and Bangerth, F. (1995) Effect of apex excision and replacement by 1-naphthylacetic acid on cytokinin concentration and apical dominance in pea plants. *Physiol. Plant.* **94**, 465-469.

Lim, R. and Tamas, A. (1989) The transport of radiolabeled indoleacetic-acid and its conjugates in nodal stem segments of *Phaseolus-vulgaris* L. *Plant Growth Reg.* **8,** 151-164.

Lincoln, C., Britton, J.H. and Estelle, M. (1990) Growth and development of the *axr1* mutants of Arabidopsis. *Plant Cell* **2**, 1071-1080.

Medford, J.I., Horgan, R., El-Sawi, Z. and Klee, H.J. (1989) Alterations of

endogenous cytokinins in transgenic plants using a chimeric isopentenyl transferase gene. *Plant Cell* **1,** 403-413.

Miyawaki, K., Matsumoto-Kitano, M. and Kakimoto, T. (2004) Expression of cytokinin biosynthetic isopentenyltransferase genes in *Arabidopsis*: tissue specificity and regulation by auxin, cytokinin, and nitrate. *Plant J.* **37**, 128-138.

Nordström, A., Tarkowski, P., Tarkowska, D., Norbaek, R., Åstot, C., Dolezal, K. and Sandberg, G. (2004) Auxin regulation of cytokinin biosynthesis in *Arabidopsis thaliana*: A factor of potential importance for auxin-cytokinin-regulated development. *Proc. Natl. Acad. Sci. USA* **101**, 8039-8044.

Panigrahi, D.A. and Audus, L. (1966) Apical dominance in *Vicia faba*. *Ann. Bot.* **30,** 457-473.

Pearce, D.W., Taylor, J.S., Robertson, J.M., Harker, K.N. and Daly, E.J. (1995) Changes in abscisic-acid and indole-3-acetic-acid in axillary buds of *Elytrigia repens* released from apical dominance *Physiol. Plant.* **94,** 110-116.

Prasad, T.K., Li, X., Abdel-Rahman, A.M., Hosokawa, Z., Cloud, N.P., LaMotte,

C.E. and Cline, M.G. (1993) Does auxin play a role in the release of apical dominance by shoot inversion in *ipomoea nil? Ann. Bot.* **71**, 223-229.

Romano, C.P., Cooper, M.L. and Klee, H.J. (1993) Uncoupling auxin and ethylene effects in transgenic tobacco and Arabidopsis plants. *Plant Cell* **5**, 181-189.

Romano, C.P., Hein, M.B. and Klee, H.J. (1991) Inactivation of auxin in tobacco transformed with the indoleacetic-acid lysine synthetase gene of *Pseudomonas savastanoi*. *Genes Dev.* **5**, 438-446.

Ross, J.J., O' Neill, D.P., Smith, J.J., Kerckhoffs, L.H. J. and Elliott, R.C. (2000) Evidence that auxin promotes gibberellin A1 biosynthesis in pea. *Plant J.* **21**, 547-552.

Sachs, T. and Thimann, K.V. (1964) Release of lateral buds from apical dominance. *Nature* **201**, 939-940.

Sachs, T. and Thimann, K.V. (1967) The role of auxin and cytokinins in the release of buds from dominance. *Amer. J. Bot.* **54,** 134-144.

Schwartz, S.H., Qin, X. and Loewen, M.C. (2004) The biochemical

characterization of two careteniod cleavage enzymes from Arabidopsis indicates that a carotenoid-derived compound inhibits lateral branching. *J. Biol. Chem.* **279**, 46940-46945.

Shimizu, S. and Mori, H. (1998) Analysis of cycles of dormancy and growth in pea axillary buds on mRNA accumulation patterns of cell cycle-related genes. *Plant Cell Physiol.* **39**, 255-262.

Shimizu-Sato, S. and Mori, H. (2001) Control of outgrowth and dormancy in axillary buds. *Plant Physiol.* **127**, 1405-1413.

Sitbon, F.B., Hennion, S, Sundberg, B., Little, C.H.A., Olsson, O. and Sandberg, G. (1992) Transgenic tobacco plants coexpressing the *Agrobacterium tumefaciens iaaM* and *iaaH* genes display altered growth and indoleacetic acid metabolism. *Plant Physiol.* **99**, 1062-1069.

Snowden, K.C., Simkin, A.J., Janssen, B.J., Templeton, K.R., Loucas, H.M., Simons, J., Karunairetnam, S., Gleave, A.P., Clark, D.G. and Klee, H.J. (2005) The *Decreased apical dominance1/Petunia hybrida CAROTENOID CLEAVAGE*

DIOXYGENASE8 gene affects branch production and plays a role in leaf senescence, root growth, and flower development. *Plant Cell* **17**, 746-759.

Snyder, W.E. (1949) Some responses of plants to 2, 3, 5-triiodobenzoic acid. *Plant Physiol.* **23**, 195-206.

Sorefan, K., Booker, J., Haurogne, K., Goussot, M., Bainbridge, K., Foo, E., Chatfield, S., Ward, S., Beveridge, C., Rameau, C. and Leyser, O. (2003) *MAX4* and *RMS1* are orthologous dioxygenase-like genes that regulate shoot branching in *Arabidopsis* and pea. *Genes Dev*. **17**, 1469-1474.

Takei, K., Sakakibara, H. and Sugiyama, T. (2001a) Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in *Arabidopsis thaliana*. *J. Biol. Chem.* **276**, 26405-26410.

Takei, K., Takahashi, T., Sugiyama, T., Yamaya, T. and Sakakibara, H. (2001b) Nitrogen-dependent accumulation of cytokinins in root and the translocation to leaf: implication of cytokinin species that induces gene expression of maize response regulator. *Plant Cell Physiol.* **42**, 85-93.

Takei, K., Ueda, N., Aoki, K., Kuromori, T., Hirayama, T., Shinozaki, K., Yamaya, T. and Sakakibara, H. (2004a) *AtIPT3* is a key determinant of nitrate-dependent cytokinin biosynthesis in *Arabidopsis*. *Plant Cell Physiol.* **45**, 1053-1062.

Takei, K., Yamaya, T. and Sakakibara, H. (2004b) *Arabidopsis CYP735A1* and *CYP735A2* encode cytokinin hydroxylases that catalyze the biosynthesis of *trans*-zeatin. *J. Biol. Chem.* **279**, 41866-41872.

Tamas, I.A., Schlossberg-Jacobs, J.L., Lim, R., Friedman, L. and Barone, C.C. (1989) Effect of plant-growth substances on the growth of axillary buds in cultured stem segments of *Phaseolus vulgaris* L. *Plant Growth Regul.* **8**, 165-183.

Thimann, K.V. and Skoog, F. (1933) Studies on the growth hormone of plants. III. The inhibiting action of the growth substance on bud development. *Proc. Natl. Acad. Sci. USA* **19**, 714-716.

Thimann, K.V. and Skoog, F. (1934) On the inhibition of bud development and other functions of growth substance in *Vicia faba*. *Proc. R. Soc. London Ser. B.* **114**, 317-339.

Turnbull, C.G.N., Myriam, A.A., Raymond, I.C.D. and Morris, D.S.E. (1997) Rapid increases in cytokinin concentration in lateral buds of chickpea (*Cicer arietinum* L.) during release of apical dominance. *Planta* **202,** 271-276.

Ward, S.P. and Leyser, O. (2004) Shoot branching. *Curr. Opin. Plant Biol.* **7**, 73-78.

Yonekura-Sakakibara, K., Kojima, M., Yamaya, T. and Sakakibara, H. (2004) Molecular characterization of cytokinin-responsive histidine kinase in maize: differential ligand preferences and response to cis-zeatin. *Plant Physiol*. **134**, 1654-1661.

Zubko, E., Adams, C. J., Macháèková, I., Malbeck, J., Scollan, C. and Meyer, P. (2002) Activation tagging identifies a gene from *Petunia hybrida* responsible for the production of active cytokinins in plants. *Plant J*. **29**, 797-808.

Table 1. CK contents of the second nodal stems and the axillary buds of the second node after decapitation.

CKs (pmol g^{-1}FW)	stem			axillary bud		
	0 h	3 h	6 h	0 h	3 h	6 h
iPRMP	28.47±3.93	93.57±9.82	292.1±12.5	3.48±2.17	10.56±0.27	136.3±31.6
iPR	0.83±0.12	4.69±0.37	10.20±1.48	0.80±0.35	17.27±4.13	12.97±2.02
iP	0.19±0.03	0.44±0.02	0.66±0.10	0.59±0.05	2.06±0.53	1.20±0.35
tZRMP	2.69±0.38	5.79±0.63	35.52±4.27	0.63±0.24	2.34±0.61	129.4±20.8
tZR	0.23±0.02	1.22±0.15	5.59±0.91	0.26±0.04	3.93±2.08	14.16±4.42
tZ	0.18±0.01	0.27±0.03	0.79±0.09	0.31±0.05	0.95±0.53	4.47±2.05
cZRMP	5.78±0.68	4.91±0.45	7.09±0.10	5.21±1.78	1.95±0.05	10.44±0.14
cZR	0.47±0.07	0.57±0.07	0.55±0.05	1.85±0.28	5.89±1.16	2.84±0.82
cZ	0.14±0.02	0.12±0.01	0.10±0.01	0.28±0.02	0.28±0.06	0.16±0.01

Nodal stem segments (ca. 1 g) and axillary buds (ca. 0.5 g) were separately collected at the indicated times (in hours) after decapitation 1 cm above the second node. CK contents were analyzed as described in Experimental procedure. Mean value was calculated from three replicate samples with standard deviation. iP, isopentenyladenine; iPR, iP riboside; iPRMP, iPR 5'-monophosphate; tZ, *trans*-zeatin; tZR, tZ riboside; tZRMP, tZR 5'-monophosphate; cZ, *cis*-zeatin; cZR, cZ riboside; cZRMP, cZR 5'-monophosphate.

Table 2. CK contents of the excised stem segments incubated with or without IAA.

CKs (pmol g^{-1}FW)	- IAA			+ IAA	
	0 h	3 h	6 h	3 h	6 h
iPRMP	9.52±0.62	264.0±100.1	321.0±64.66	6.28±0.82	8.61±0.86
iPR	0.28±0.00	8.90±3.54	9.86±1.40	0.34±0.05	0.45±0.04
iP	0.14±0.01	1.09±0.42	1.16±0.05	0.10±0.01	0.19±0.02
tZRMP	1.46±0.11	12.96±5.02	32.14±6.85	0.19±0.02	0.11±0.01
tZR	0.08±0.01	2.42±0.91	5.33±0.48	0.02±0.00	0.02±0.00
tZ	0.54±0.09	0.52±0.18	0.86±0.07	0.54±0.10	0.41±0.02
cZRMP	6.72±0.37	5.16±1.84	6.83±1.18	1.34±0.14	0.90±0.07
cZR	0.48±0.02	0.55±0.21	0.69±0.06	0.33±0.05	0.33±0.06
cZ	0.15±0.02	0.12±0.05	0.12±0.01	0.03±0.00	0.05±0.00

The stem segments were excised from intact seedlings and their basal ends were inserted into agar plates. Lanolin with or without IAA was applied to the apical stump. The excised stem segments were incubated for the indicated times. Then CK contents were analyzed as described in Experimental procedure. Mean value was calculated from three replicate samples with standard deviation. iP, isopentenyladenine; iPR, iP riboside; iPRMP, iPR 5'-monophosphate; tZ, *trans*-zeatin; tZR, tZ riboside; tZRMP, tZR 5'-monophosphate; cZ, *cis*-zeatin; cZR, cZ riboside; cZRMP, cZR 5'-monophosphate.

Figure legends

Figure 1. Accumulation patterns of *PsIPT* transcripts in the nodal stem after decapitation. The second nodal stems, from which the axillary buds were removed, after decapitation 1 cm above the second node, were collected at the indicated times. RNA was isolated from these stems and subjected to Northern blot analyses. Numbers below each lane indicate the time in hours after decapitation. The bottom panel (rRNA) shows the ethidium bromide-staining RNA gel as the loading control. Results are representative of four separate experiments.

Figure 2. Effects of exogenous IAA on *PsIPT* expression in the nodal excised stem segments. The nodal stem segments, from which the axillary buds were removed, were excised from seedlings 3 h after decapitation 1 cm above the second node (a) and excised from intact seedlings (b). The excised stem segments were incubated with 10^{-5} M IAA or IAA-free buffer. Numbers below each lane indicate the incubation time. The bottom panel (rRNA) shows the ethidium bromide-staining RNA gel as the

loading control. Results are representative of four separate experiments.

Figure 3. Effects of IAA applied to the decapitated stump on *PsIPT* expression. The shoot apex was removed 1 cm above the second node, and lanolin paste with 1% IAA (+) and without (-) was immediately applied to the cut stump. The *PsIPT1*, *PsIPT2*, and *PsPIN1* transcript levels were determined in a 1-cm long piece of stump 3 h after application. The right-hand panel (rRNA) shows the ethidium bromide-staining RNA gel as the loading control. Results are representative of three separate experiments.

Figure 4. Expression patterns of *PsIPT* transcripts in various organs and by TIBA and IAA treatments. Accumulation patterns of *PsIPT1* (a) and *PsIPT2* (b) transcripts in various organs and by TIBA and IAA treatments. Total RNAs were prepared from various organs: terminal buds (tb), and leaflets (lf) in intact seedlings; axillary buds, and roots 0 h and 6 h after decapitation, respectively. Total RNAs were also

prepared from the stems (TI) that were treated with TIBA for 6 h and the root segments that were incubated with 10^{-5} M IAA (+) or IAA-free (-) buffer for 6 h. The total RNAs were subjected to quantitative real-time PCR. Other detail conditions are described in Experimental procedures. Accumulation levels of the transcripts are given as the copy number of mRNA per 1 ng total RNA. Real-time PCR was performed in triplicate, and the mean values with standard deviation are shown.

Figure 5. Effects of IAA on *GUS* expression under the control of the *PsIPT2* promoter region in transgenic *Arabidopsis* seedlings. Ten-day-old transgenic *Arabidopsis* whole seedlings were incubated with 10^{-5} M IAA or IAA-free buffer. After incubation, RNA was isolated from the seedlings and analyzed by Northern blotting with *GUS* DNA as a probe. Numbers below each lane indicate the incubation time. Results are representative of four separate experiments.