The Gravity-Regulated Growth of Axillary Buds is Mediated by a Mechanism Different from Decapitation-Induced Release

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When the upper part of the main shoot of the Japanese morning glory (Pharbitis nil or Ipomoea nil) is bent down, the axillary bud situated on the uppermost node of the bending region is released from apical dominance and elongates. Here, we demonstrate that this release of axillary buds from apical dominance is gravity regulated. We utilized two agravitropic mutants of morning glory defective in gravitropism and shoot bending-induced release from apical dominance required gravitropism cells. Previous studies have shown that basipetal translocation of auxin from the apical bud inhibits axillary bud growth, whereas cytokinin promotes axillary bud growth. We therefore compared the roles of auxin and cytokinin in bending-induced axillary bud growth. In the wild-type and we plants, decapitation increased cytokinin levels and reduced auxin response. In contrast, shoot bending did not cause significant changes in either cytokinin level or auxin response, suggesting that the mechanisms underlying gravity- and decapitation-regulated release from apical dominance are distinct and unique.

Keywords: Apical dominance — Auxin — Gravitropism — Cytokinin — Morning glory — weaving.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; EST, expressed sequence tag; IPT, isopentenyltransferase; ORF, open reading frame; RT–PCR, reverse transcription–PCR; t-ZR, trans-zeatin riboside; we, weaving; WT, wild type.

The nucleotide sequence reported in this paper has been submitted to GenBank data libraries under accession numbers: PnIAA1 (AB371299), PnIPT1 (AB371300) and PnIPT2 (AB371301).

Introduction

Apical dominance is the central regulatory system for maintaining plant shoot architecture, wherein the growing apical shoot suppresses the growth of axillary buds on the axis of leaves below it (reviewed in Cline 1991). Apical dominance is best demonstrated via shoot tip removal, also known as decapitation, which releases axillary buds from apical dominance and initiates their outgrowth. Although the concept of apical dominance has been known for many centuries, the mechanisms underlying this phenomenon are not well understood and have been the center of debate for 100 years.

The plant hormones auxin and cytokinin are thought to have major roles in controlling apical dominance, with auxin production being inhibited and cytokinin stimulation of auxin but promoted by cytokinin (reviewed in Shimizu-Sato and Mori 2001, Ongaro and Leyser 2008). Following decapitation of the shoot apex, auxin levels in the stem decrease, cytokinin levels increase and the axillary buds elongate. Furthermore, direct application of cytokinin to axillary buds of intact plants is sufficient to promote their outgrowth, suggesting a preferential role for cytokinin in apical dominance (Pillay and Railton 1983, Cline et al. 1997).

Apical dominance in plants, although regulated by intrinsic signals such as the developmental stage of the plant, is plastic in its response to various environmental cues such as light and nutrient conditions (Snowden and Napoli 2003), and may also be affected by the influence of gravity (Cline 1983, Prasad and Cline 1987). For example, horizontal placement of the Japanese morning glory results in the outgrowth of several axillary buds in random locations on the main shoot, while inversion of these plants results in elongation of the buds near the shoot base (Prasad and Cline 1985). Furthermore, bending the upper part of the main shoot releases the axillary bud at the bending region from apical dominance and initiates elongation of the bud. This release is prevented by clinorotation of the bent plants (Prasad and Cline 1987). These results suggest that release from apical dominance in morning glory is due, at least in part, to gravistimulation. Similar phenomena have also been observed in agriculture and horticulture. For instance, it is known that shoot bending induces lateral branching and flower bud development in Japanese pear, cherry and plum trees.

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through comparisons between these mutants lack gravisensing cells, allowing extraction of Kitazawa et al. 2005, Kitazawa et al. 2008). The shoots of endodermal cells in morning glory (Hatakeda et al. 2003, defects in the normal differentiation of shoot-gravisensing genes of the movings are impaired in WT plants. For example, we recently demonstrated that shoot bending-induced release from apical dominance is governed by the same mechanisms as decapitation-induced release.

The Japanese morning glory (Pharbitis nil or Ipomoea nil) is characterized as an absolute short-day plant, which is advantageous for the study of apical dominance by enabling researchers to carry out experiments in the vegetative stage without floral transition of meristems. To elucidate the mechanisms of the gravity-regulated apical dominance, we utilized two agravitropic mutants of the Japanese morning glory, weeping (we) and weeping2 (we2). These mutants display agravitropism due to mutations in the SCARECROW (PnSCR) and SHORT-ROOT (PnSHR) genes of the we and we2 plants, respectively, which confer defects in the normal differentiation of shoot-gravisensing endodermal cells in morning glory (Hatakeda et al. 2003, Kitazawa et al. 2005, Kitazawa et al. 2008). The shoots of these mutants lack gravisensing cells, allowing extraction of plant gravisresponse phenotypes and genetic analyses through comparisons between we, we2 and wild-type (WT) plants. For example, we recently demonstrated that gravisensing cells are required for shoot circummutation and for the winding movements of morning glory, since both movements are impaired in we and we2 shoots (Kitazawa et al. 2005, Kitazawa et al. 2008). We believe that these mutants may also be useful for verifying the role of gravity in the release of the axillary bud from apical dominance.

In this study, we first verified gravity-regulated release from apical dominance in the Japanese morning glory by examining whether bending the main shoots of we and we2 plants caused an outgrowth of the axillary buds. We then investigated the involvement of the plant hormones auxin and cytokinin in two modes of axillary bud outgrowth, bending- and decapitation-induced release from apical dominance.

**Results**

**Effect of shoot bending on axillary bud growth**

Upon bending an upright morning glory shoot downward, the axillary bud situated on the uppermost node is released from apical dominance and begins to elongate, a response that is obliterated with clinorotation (Prasad and Cline 1987). These results suggest that the bending-induced outgrowth of the axillary bud depends on gravisresponse. We verified this hypothesis in our system, using the agravitropic mutants of morning glory. The axillary buds located at nodes 1–3 of 4-week-old WT (cv. Violet), we and we2 plants grown under conventional conditions were approximately 2 mm in length. There was no statistical difference between the lengths of these axillary buds. Using WT, we and we2 mutant shoots, we performed ‘shoot bending treatment’, according to the procedure described by Cline (1983). When the main shoots of 4-week-old plants were bent down above node 6, the axillary bud on node 6 (the uppermost node) began to elongate in WT plants, whereas no outgrowth of the axillary bud was observed in either the we or we2 mutants (Fig. 1A, B). To investigate whether this aberrant response of the axillary bud was genetically linked to the agravitropism of these mutants, we performed a linkage analysis of F2 generations. As shown in Fig. 2, all gravitropic F2 generations responded to shoot bending by an outgrowth of the axillary buds on their uppermost nodes. In contrast, elongation was not observed in any of the agravitropic F2 generation plants. These results clearly demonstrated the idea that gravisresponse was involved in apical dominance.

**Effect of decapitation on axillary bud growth**

Classical studies have shown that pinching off the shoot tip (decapitation) induces the outgrowth of the axillary buds in many plant species, a process that is often used for the study of apical dominance (reviewed in Cline 1991). To examine whether endodermis-mediated gravisresponse also affects the regulatory mechanism of decapitation-induced axillary bud outgrowth, we decapitated the shoots above node 1 of 2-week-old WT, we and we2 plants. We also investigated the response of these decapitated plants to the application of exogenous auxin to the stump, and determined whether the presence or absence of an endodermis affected the apical dominance-associated basipetal auxin translocation.

Decapitation of WT plants caused a rapid release of the axillary buds from apical dominance (Fig. 3, upper panel). Likewise, decapitation induced outgrowth of the axillary buds in both we and we2 plants (Fig. 3, middle and lower panel). Exogenous IAA applied to the cut surface of the decapitated stem inhibited axillary bud outgrowth in WT, we and we2 plants (Fig. 3), suggesting that abnormal development of the endodermis does not affect the apical dominance-related auxin translocation. These results further suggest that endodermis-mediated gravisensing does not influence decapitation-induced axillary bud growth.
Molecular identification of auxin and cytokinin reporter genes

The molecular mechanisms underlying shoot bending-induced release from apical dominance remain unclear. Auxin and cytokinin are known to play a role in decapitation-induced release, and it is therefore important to clarify whether the dynamics of these hormones are also involved in bending-induced release. In this study, we examined the involvement of auxin and cytokinin in bending- and decapitation-induced axillary bud growth. An auxin-inducible gene and a biosynthetic gene of cytokinin were adopted as molecular markers.

Aux/IAA is a well-characterized family of auxin-inducible genes (Tiwari et al. 2004). We isolated Aux/IAA homologous genes from the Japanese morning glory. Using a PCR-based strategy (see Materials and Methods), we isolated a cDNA clone homologous to Aux/IAA from the Japanese morning glory cv. Violet, based on the sequence information of the expressed sequence tags (ESTs) of the Japanese morning glory cv. TKS (Morita et al. 2006). The predicted polypeptide of the obtained cDNA sequence contains domains I, II, III and IV, which are highly conserved among Aux/IAA protein families (Tiwari et al. 2004). We then verified whether the corresponding gene was responsive to auxin using a stem section of the morning glory, according to a method previously described (Fujii et al. 2000). Expression of the gene in the excised sections of the WT plant was reduced by auxin starvation, while subsequent treatment of the sections with exogenous IAA elevated its expression in a dose-dependent manner (Supplementary Fig. S1). These data indicate that cDNA isolated from the stem of the Japanese morning glory plant is a gene belonging to the auxin-responsive Aux/IAA family. We named this gene PnIAA1.

It has been reported that one of the cytokinin biosynthetic genes isopentenyltransferase (IPT) contributes to decapitation-induced axillary bud growth in the pea plant (Pisum sativum L.) (Tanaka et al. 2006). Following decapitation, levels of cytokinin and PsIPT1 and PsIPT2 mRNA were markedly increased in the nodal stem. Expression of these genes was repressed by application of IAA.

We searched IPT homologous genes in the morning glory EST database. In Arabidopsis, the IPT family is known for its diverse function (Miyawaki et al. 2006). ATP/ADP IPT and their homologs, AtIPT1 and AtIPT3–AtIPT8

Fig. 1  Shoot-bending treatment on Violet and weeping mutants. (A) Photographs of the bent plants (upper panel) and magnification of one of their axillary buds on the uppermost node in the bending region (lower panel). Arrowheads indicate the axillary bud on the uppermost node. Arrow (g) indicates the orientation of gravity. Bars = 5 cm. (B) Measurement of the axillary bud length on the uppermost node. After bending treatment, the length of the axillary bud on the uppermost node was measured every day. Data represent the means of 12 individuals. Vertical bars indicate SD. Open circles, Violet without bending treatment; filled circles, Violet with bending treatment; open squares, weeping (we) without bending treatment; filled squares, we with bending treatment; open triangles, weeping2 (we2) without bending treatment; filled triangles, we2 with bending treatment.
are Arabidopsis enzymes that catalyze the first step of cytokinin biosynthesis (Kakimoto 2003, Miyawaki et al. 2004). AtIPT2 and AtIPT9 are thought to catalyze the isopentenylation of tRNA, and are probably not involved in cytokinin biosynthesis (Golovko et al. 2002).

We performed a phylogenetic analysis of members of the IPT family in various plants based upon the information in the EST database (Supplementary Fig. S2). Since two EST sequences were likely to be transcribed from genes that belong to the cytokinin biosynthetic IPT family, we used a PCR-based strategy to isolate the corresponding cDNAs from the WT morning glory (see Materials and Methods). The obtained cDNAs of these two IPT-homologous genes, PnIPT1 and PnIPT2, encode putative proteins with sequence identities of 50 and 52%, respectively, to PsIPT1; and 44 and 49%, respectively, to PsIPT2. We therefore designated these IPT-homologous genes PnIPT1 and PnIPT2, respectively.

Fig. 2 F2 linkage analysis between gravitropic response and axillary bud growth. Ten days after shoot-bending treatment, the axillary buds on the uppermost node were measured. Distributions of the length of the axillary buds on the uppermost node of F2 plants are shown: we mutant F2 segregants (upper panel), we2 mutant F2 segregants (lower panel). Open column, F2 generation showing proper gravitropism; filled column, F2 generation showing abnormal gravitropism. Twenty-five individuals of F2 plants showing a gravitropic or agravitropic response were tested.

Fig. 3 Effects of decapitation and auxin application to the stump on axillary bud growth in Violet and agravitropic mutants. Two-week-old plants were decapitated, and lanolin paste containing 0.1% (w/v) IAA was applied to the cut surface of the decapitated stem. Plants without decapitation and decapitated plants to which lanolin paste without IAA was applied (mock treatment) were also grown as controls. Axillary bud growth was monitored every day, and lanolin paste was renewed every other day. Lengths of axillary buds of 24 individuals were examined in each experiment. Data are the means ± SD. Filled circles, plants without decapitation; open circles, decapitated plants with mock treatment; open triangles, decapitated plants with IAA application to the stump.
Effect of decapitation on the expression of PnIAA1 and PnIPT1 genes

To examine whether PnIAA1 and PnIPT genes respond to decapitation, we studied their expression patterns in the first node of the morning glory shoots before and after decapitation. The mRNA level of PnIAA1 began to decrease 1 h after decapitation, and reached a minimum 3 h after decapitation (Fig. 4A). In contrast, the mRNA level of PnIPT1 began to increase after 1 h, reached a maximum level at 3 h, and then began to decrease 6 h after decapitation (Fig. 4B). This expression pattern was similar to that of PsIPT2 reported previously (Tanaka et al. 2006). In the current study, the mRNA level of PnIPT2 was low, and did not show an explicit expression pattern in response to the decapitation (data not shown).

To examine the correlation between the expression of PnIPT1 and the endogenous cytokinin level, we used an enzyme-linked immunosorbent assay (ELISA) method to quantify trans-zeatin riboside (t-ZR) before and after decapitation (Fig. 4C) (Weiler 1980, Weiler 1984). Decapitation induced a marked increase in the level of t-ZR in the node situated below the decapitated stump in the WT plant, implying a correlation between PnIPT1 expression and the endogenous cytokinin level. We therefore concluded that the PnIPT1 gene could be used as a reporter for analysis of cytokinin dynamics in the regulation of apical dominance.

Effect of auxin application on the expression of PnIAA1 and PnIPT1 genes

To elucidate the relationship between basipetal auxin transport and cytokinin biosynthesis, we investigated both PnIAA1 and PnIPT1 expression in the node by applying IAA to the stump after decapitation. Furthermore, to examine whether decapitation-induced release from apical dominance is regulated by the same mechanisms in WT and we plants, we analyzed the expression of the marker genes in we plants. Immediately following decapitation of WT or we plants, lanolin, with or without IAA was applied to their stumps. When measured at 3 h after application, in either the WT or we plants, the levels of the PnIAA1 transcript were decreased in plants treated with lanolin alone, and were increased in plants treated with IAA (Fig. 5A). This suggests that the removal of the shoot tip (auxin source)

Fig. 4 Expression patterns of auxin and cytokinin marker genes and level of endogenous t-ZR in the nodal stem after decapitation. Quantitative real-time PCR analysis of the expression of PnIAA1 (A) and PnIPT1 (B) was carried out. The shoot apex was removed 
1 cm above the first node, then the first nodal stems were collected at the indicated times. The expression levels of these genes were normalized to F1F0 ATP synthase γ-subunit gene expression levels. Relative expression levels are shown, where those at time zero were set as 1. Data are the means ± SD. Experiments were performed in triplicate using independent RNA samples. (C) Endogenous t-ZR contents of the nodal stem after decapitation. The shoot apex was removed 1 cm above the first node, and then the first nodal stems were collected at the indicated times. The t-ZR contents were analyzed by ELISA as described in Materials and Methods. Data are the means ± SE.
causes deprivation of the available endogenous auxin in the node and that the IAA applied to the stump basipetally translocates from the stump to the node. In contrast, levels of the PnIPT1 transcript were increased by lanolin alone, but not by IAA, in WT plants (Fig. 5B), strongly suggesting that PnIPT1 transcription is negatively controlled by the basipetal transport of auxin in the stem. In the we mutant, PnIAA1 and PnIPT1 expression was similar to that observed in WT plants (Fig. 5A, B). This result demonstrates that decapitation induces the release from apical dominance in both WT and we plants, and that this mechanism of release mediates auxin and cytokinin dynamics.

Effect of shoot bending on the expression of PnIAA1 and PnIPT1 genes

We next examined the involvement of auxin and cytokinin in the gravity-regulated growth of the axillary bud. We analyzed PnIAA1 and PnIPT1 expression in node 6 at the bent region (uppermost node) or in the corresponding node (node 6) of control upright plants of WT and we plants. Surprisingly, in WT plants, shoot bending resulted in minimally significant changes in the mRNA levels of PnIAA1 and PnIPT1 at 72 h after bending treatment (Fig. 6A). Likewise, levels of PnIAA1 transcripts in the we mutant did not display significant fluctuations, nor were there significant differences between the WT and we PnIAA1 transcript levels (Fig. 6A). The level of PnIPT1 in we did not differ from those of the WT by 24 h after shoot bending, although its level became lower in the WT thereafter (Fig. 6A). To confirm these results, we used an ELISA method to measure the endogenous cytokinin content in the uppermost node of the bent plants. In contrast to the results of the decapitation experiments, in both WT and we, endogenous t-ZR levels did not significantly increase in the uppermost node within 24 h of bending (Fig. 6B). This result was consistent with the expression pattern of PnIPT1. These observations imply that a dramatic change of either basipetal auxin transport or cytokinin biosynthesis in the uppermost node is not involved in the bending-induced release from apical dominance. Consequently, we conclude from these data that the mechanism of gravity-regulated release from apical dominance is unique from that of decapitation-induced release, and possibly involves a novel pathway that requires neither a reduction of basipetal auxin transport nor cytokinin biosynthesis.

Discussion

Our results in this study with agravitropic shoots of the Japanese morning glory demonstrate that endodermis-mediated gravisensing plays an important role in the bending-induced release of axillary buds from apical dominance. Bending the shoot down stimulated the outgrowth of the axillary bud situated on the uppermost node (Fig. 1, 2). Interestingly, these mutants showed a normal response to decapitation, stimulating outgrowth of axillary buds just below the cut stump (Fig. 3). Auxin applied to the stump suppressed the decapitation-induced release of axillary buds
from apical dominance in both WT and mutant plants (Fig. 3). These results suggest that the gravisresponse mediates the bending-induced release from apical dominance but not the decapitation-induced release. Thus, the mechanisms for the two modes of axillary bud growth may differ from one another.

The mechanism for gravity sensing has been well studied in *Arabidopsis*, i.e. a gravisensing apparatus for shoot gravitropism resides in the endodermal cells (Morita and Tasaka 2004). Shoots of *we* and *we2* morning glory do not show a gravitropic response because of their defects in normal differentiation of endodermal cells (Kitazawa et al. 2005, Kitazawa et al. 2008). Also, the aberrant response of *we* and *we2* shoots in the bending-induced outgrowth of axillary buds is genetically linked to their agravitropic response (Fig. 2). It is therefore assumed that the same gravisensing mechanism is shared by both bending-induced release from apical dominance and shoot gravitropism. At present, however, we cannot rule out the possibility that endodermal cells exert different gravisensing mechanisms for the two gravisponses.

Auxin and cytokinin are thought to be key regulators of apical dominance. Basipetal translocation of auxin from the shoot apex inhibits axillary bud growth, while cytokinin promotes this process. In many plant species, the direct application of cytokinin to axillary buds promotes their outgrowth, and endogenous cytokinin levels rise in and around axillary buds during growth initiation (Li et al. 1995, Turnbull et al. 1997, Emery et al. 1998). There is unequivocal evidence for cytokinin biosynthesis in shoots (Nordström et al. 2004). To date, cytokinin is the only plant hormone known to induce the outgrowth of axillary buds. Tanaka et al. (2006) proposed that one role of auxin is to repress *IPT* gene expression, i.e. local biosynthesis of cytokinin in the nodal stem is negatively regulated by auxin through the control of *IPT* expression in apical dominance. In agreement with this hypothesis, *PnIPT1* gene expression was up-regulated by removal of the auxin source, and was repressed by exogenous IAA application to the cut stump in the morning glory. This suggests that the mechanism underlying the relationship between auxin and

For non-treated plants, the node corresponding to the uppermost node (the sixth node) of bent plants was sampled. (A) Quantitative real-time PCR analysis of the expression of *PnIAA1* (upper panel) and *PnIPT1* (lower panel) was performed. The expression levels of these genes were normalized to *F0F1 ATP synthase* γ-subunit gene expression levels. Relative expression levels are shown, where that of Violet plants at time zero (0 h) was set as value 1. Data represent means ± SD. (B) Endogenous t-ZR contents of the nodal stem on the uppermost node during shoot bending treatment. The nodal stems on the uppermost node (the sixth node) were collected at the indicated times. The t-ZR contents were analyzed by ELISA as described in Materials and Methods. Data are the means ± SE. Open circles, Violet; filled circles, *weeping*.

![Fig. 6](https://academic.oup.com/pcp/article-abstract/49/6/891/1811476)
cytokinin in the regulation of apical dominance is universal among various plant species.

When the upper shoot of the WT plant is bent down, the axillary bud on the uppermost node begins to grow out within 24–36 h (Cline and Riley 1984). The kinetics of the axillary bud growth induced by shoot bending are similar to those induced by decapitation (Figs. 1, 3). It would thus be possible for bending-induced release from apical dominance also to be mediated by auxin and cytokinin. However, surprisingly, shoot bending (gravistimulation) did not affect both auxin and cytokinin dynamics, despite being accompanied by axillary bud outgrowth. Prasad et al. (1993) reported that shoot bending treatment did not significantly cause the reduction of basipetal auxin transport from the shoot apex. The expression of an auxin-inducible gene (\textit{PnIAA1}) hardly changed during bending-induced release from apical dominance (Fig. 6), supporting this observation. In addition, we measured the basipetal auxin transport activity using \textit{\textsuperscript{3}H-labeled IAA} in WT and \textit{we} plants. Our results showed that the basipetal transport activity of exogenously applied \textit{\textsuperscript{3}H]IAA} in \textit{we} did not differ from that of the WT (Supplementary Table S1). This observation also suggests that the gravity-regulated release of axillary buds from apical dominance is not related to basipetal auxin transport. Our present study is the first report investigating cytokinin dynamics during bending-induced release from apical dominance. The expression of 	extit{PnIPT1} and the level of endogenous \textit{i-ZR} did not change significantly during shoot bending, in sharp contrast to those in decapitation-induced plants (Fig. 6A, B). In the time-course study, the expression level of \textit{PnIPT1} declined 48–72 h after bending treatment in WT but not in \textit{we} plants. However, this change in the WT may not be a cause of the bending-induced release from apical dominance because axillary bud outgrowth commences within 24 h after bending and because the outgrowth is expected to accompany an increase in cytokinin level. In addition, we isolated cDNA of a gene (designated as \textit{PnRR1}) homologous to type-A \textit{Arabidopsis Response Regulator} (type-A \textit{ARR}) from morning glory and analyzed its expression by real-time PCR in both decapitated and bent shoots of morning glory (Supplementary Figs. S3, S4). The result was similar to that for \textit{i-ZR}, i.e. decapitation unequivocally increased the expression level of \textit{PnRR1} in both WT and \textit{we} morning glory, but no significant change in \textit{PnRR1} expression was observed following bending treatment in both WT and \textit{we} plants. Type-A \textit{ARRs} are rapidly and specifically induced by cytokinin and characterized as primary cytokinin response regulator genes (D’Agostino et al. 2000).

Taking all of this information into consideration, we conclude that a change in neither basipetal auxin transport nor de novo cytokinin biosynthesis in the uppermost node is necessarily required for the gravity-regulated axillary bud growth, which implies the existence of a novel system in the regulation of axillary bud growth.

A key to elucidating the mechanism of bending-induced release from apical dominance may be hidden in the cascade mediated by the gravitropism endodermal cells. The agravitropic mutants used here lack endodermal cells (Kitazawa et al. 2005, Kitazawa et al. 2008). Since these mutants do not respond to shoot bending and respond normally to decapitation, it is clear that endodermal cells are required specifically for the bending-induced release from apical dominance. The present study utilizes agravitropic mutants of morning glory as a useful tool for the study of the gravity-regulated release from apical dominance. One of the most intriguing questions in the shoot bending-induced release from apical dominance is how plants recognize the highest position of the axillary meristem, and how they direct the buds located in these nodes to initiate growth of a new apical meristem. Gravistimulation is quite a novel mechanism that plants have acquired to address such concepts.

Materials and Methods

Plant materials and growth conditions

\textit{Pharbitis nil} Choisy cv. Violet was used as the WT strain throughout these studies. Seeds of the WT plants were purchased from Marutane Seed Co., Kyoto, Japan. Seeds of the \textit{weeping} and mutants were propagated as described previously (Kitazawa et al. 2005, Kitazawa et al. 2008). For all experiments, we \textit{we} and \textit{we2} mutants that had been crossed with WT plants more than twice to obtain uniform genetic backgrounds. The morning glory plants were grown in a greenhouse as described by Kitazawa et al. (2005).

Shoot bending experiment

For shoot bending experiments, when plants were 4 weeks old, the internode above node 6 was gently bent down so that the upper part of the shoot was inverted. The length of the axillary bud on node 6 (uppermost node) was monitored every day. Twelve individuals were examined on each morning glory strain. Linkage analysis of \textit{F2} plants between gravitropic response and axillary bud growth due to shoot bending was performed as follows: the \textit{we} and \textit{we2} mutants were each crossed with Violet, and the resulting \textit{F1} plants were self-pollinated to generate the \textit{F2} population. Twenty-five individuals of \textit{F2} plants showing gravitropic or agravitropic response were cultivated, respectively, and subjected to shoot bending treatment when plants were 4 weeks old. Ten days after the start of bending treatment, we measured the lengths of the axillary buds on the uppermost node.

Decapitation

For decapitation studies, 2-week-old plants were decapitated, and lanolin paste (lanolin-dehydrate; Wako, Osaka, Japan) containing 0.1% (w/v) IAA (Wako) was applied to the cut surface of the decapitated stem, and the plants were grown for 6d. Plants which had not been decapitated and decapitated plants to which lanolin paste without IAA was applied were also grown. The length of the axillary bud was monitored every day, and lanolin paste was
renewed every other day. Twenty-four individuals were examined in each experiment.

RNA isolation and cloning of full-length PnIAA1 and PnIPT1 cDNAs
Total RNA was extracted from the stems of 2-week-old WT plants grown in a greenhouse, by using an RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. First-strand cDNA was synthesized by using a ReverTra Ace z kit (TOYOBO, Osaka, Japan) following the supplier’s instructions. The EST database of the Japanese morning glory contained a sequence that was likely to be transcribed from a gene homologous to Aux/IAA and contained two sequences that were likely to be transcribed from genes homologous to ATP/ADP IPT. These EST sequences contained full-length cDNA sequences of the corresponding genes. These were amplified using gene-specific primers: 5'-AATGACGGCGGAAGGTGAA-3' (forward) and 5'-GGATCAGAAGCCATTGGGACT-3' (reverse) for PnIAA1, and 5'-TAACCAAGGTTCGCCGACGG-3' (forward) and 5'-AATGCGCTCCTCCAGTGCACC-3' (reverse) for PnIPT1. Amplified cDNA fragments were then cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). DNA sequences were determined by using an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to standard procedures.

Quantitative reverse transcription–PCR (RT–PCR) analysis
First-strand cDNA was synthesized as described above. Quantitative real-time PCR was performed on a Mx3000P detection system using an iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions. The expression of target genes was normalized to mitochondrial F1F0 ATP synthase γ-subunit expression, which is commonly used as an internal standard in morning glory (GenBank accession No. AB194067). PCR was carried out using the following program: once for 120 s at 95°C, 45 cycles of 10 s at 95°C, 30 s at 55°C (PnIAA1 and ATP synthase) or 30 s at 57°C (PnIPT1), 45 s at 72°C, followed by melting curve analysis. Calibration was performed using a cDNA fragment cloned in a plasmid as a template. The following primers used for amplification were set at the specific region for each gene: PnIAA1 F1 (5'-AATGACGGCGGAAGGTGAA-3') and PnIAA1 R1 (5'-GGATCAGAAGCCATTGGGACT-3') for PnIAA1, PnIPT1 F2 (5'-TAACCAAGGTTCGCCGACGG-3') and PnIPT1 R2 (5'-AATGCGCTCCTCCAGTGCACC-3') for PnIPT1, ATPS F (5'-CAGTGGATCTGTGATGACATCCTTAAAAATG-3') and ATPS R (5'-CTTCCTCGAGTTTTATTTCATCACCATCAG-3') for ATP synthase γ-subunit.

Measurement of trans-zeatin riboside (t-ZR) content
The t-ZR standard was purchased from Sigma Chemical Co. (St Louis, MO, USA), and prepared via a dilution series with absolute methanol to make a standard curve. Collected samples of morning glory were frozen once with liquid nitrogen, and then homogenized in absolute methanol. After centrifugation of the homogenate, the supernatant was collected as a crude extract. We used a commercial competitive ELISA kit (trans-zeatin riboside immunoassay detection kit; Sigma). The crude extracts were pre-incubated in wells for 3 h at 4°C and then placed in an incubator at 37°C for 1 h. The resultant products of the enzymatic reactions were quantified by a spectrophotometer at 405 nm. The detailed procedure was as per the manufacturer’s instructions.

Supplementary material
Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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