The *BUD2* mutation affects plant architecture through altering cytokinin and auxin responses in *Arabidopsis*

Xia Cui^{1,*}, Chunmin Ge^{1,3,*}, Renxiao Wang^{1,4}, Huanzhong Wang^{1,5}, Weiqi Chen², Zhiming Fu¹, Xiangning Jiang², Jiayang Li¹, Yonghong Wang¹

¹State Key Laboratory of Plant Genomics and National Center for Plant Gene Research, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China; ²The Tree and Ornamental Plant Breeding and Biotechnology Laboratory of State Forestry Administration, College of Life Sciences and Biotechnology, Beijing Forestry University, Beijing 100083, China

The ratio of auxin and cytokinin plays a crucial role in regulating aerial architecture by promoting or repressing axillary bud outgrowth. We have previously identified an *Arabidopsis* mutant *bud2* that displays altered root and shoot architecture, which results from the loss-of-function of *S*-adenosylmethionine decarboxylase 4 (SAMDC4). In this study, we demonstrate that *BUD2* could be induced by auxin, and the induction is dependent on auxin signaling. The mutation of *BUD2* results in hyposensitivity to auxin and hypersensitivity to cytokinin, which is confirmed by callus induction assays. Our study suggests that polyamines may play their roles in regulating the plant architecture through affecting the homeostasis of cytokinins and sensitivities to auxin and cytokinin.

Keywords: *BUD2*, shoot branching, auxin, cytokinin

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Introduction

Polyamines, including diamine putrescine, triamine spermidine and tetraamine spermine, are low-molecular-mass organic cations. They exist widely in all living organisms and are essential for their survival, because blocking of the biosynthesis of polyamines leads to lethal phenotypes in animals [1] and higher plants [2–4]. In higher plants, polyamines have been proposed to function in response to environmental stresses and in regulating growth and development [4–11]. Plant molecular and physiological studies over past decades have shown that higher plants defective in producing polyamines often have altered plant architecture, with reduced plant

height or more branches in shoots and roots [4, 10–13]. However, the underlying mechanisms still remain largely elusive.

Branches of higher plants originate from the axillary meristems (AMs) of shoots, and formation of a branch generally consists of the initiation of a new AM and its subsequent outgrowth. However, an AM may arrest its growth under some conditions, forming a dormant bud. The dormant buds will release their outgrowth once they sense a permissible environmental or developmental signal. In many plant species, axillary buds become dormant due to the inhibiting effects of the primary shoot apex on the outgrowth of AMs, a phenomenon known as ‘apical dominance’. Auxin was first regarded as a direct regulator in this process [14], a notion strengthened thereafter by physiological studies on decapitated shoot apices [15–17], and by analyzing auxin biosynthesis, transport and signaling [18–29] in plants. However, when radiolabelled auxin was applied to a decapitated stump, the outgrowth of axillary buds was inhibited even though radiolabelled auxin was not found to accumulate in axillary buds, suggesting an indirect suppression effect of auxin on the AM outgrowth [28, 30] and the presence of second messengers.

*These two authors contributed equally to this work.

Correspondence: Yonghong Wang

Tel: +86-10-64806008; Fax: +86-10-64883428

E-mail: yhwang@genetics.ac.cn

³Current address: The Stowers Institute for Medical Research, Kansas, USA. ⁴Current address: Institute of Biology II, Albert Ludwigs University, Freiburg, Germany. ⁵Current address: The Samuel Roberts Noble Foundation, Ardmore, USA.

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Cytokinin has been proposed as a second messenger that mediates the action of auxin in controlling the apical dominance, because it promotes the outgrowth of lateral buds when directly applied to buds [31]. Although an antagonistic role of auxin and cytokinin in the regulation of apical dominance has been postulated for decades, little is known about the underlying molecular mechanisms. Recent studies have shown that auxin can regulate the expressions of *IPT* genes and that cytokinin biosynthesis is directly affected by auxin in local shoots [32, 33], suggesting that auxin may modulate cytokinin concentration and thus represses AM outgrowth [31, 34–36].

Recent studies on a series of branching mutants, such as more axillary growth (*max*) of *Arabidopsis* [37–40], ramosus (*rms*) mutants of pea [41–43], *decreased apical dominance* (*dad*) mutants of petunia [44, 45] and *dwarf* (*d*) mutants of rice [46–51], have revealed strigolactone as a second messenger of auxin action on the control of AM outgrowth [52, 53]. Strigolactones, a group of terpenoid lactones that have been found in root exudates of diverse plant species, are synthesized from carotenoids in roots and transported acropetally or synthesized locally to repress the outgrowth of shoot branches [38, 54–56]. However, the biosynthesis and signaling pathways still remain to be elucidated.

Moreover, polyamines have been demonstrated to play a regulatory role in controlling the outgrowth of axillary buds in the *Arabidopsis* mutant, *bushy and dwarf 2* (*bud2*) [4], which shows a dwarf and bushy phenotype. The *BUD2* gene encodes *S*-adenosylmethionine decarboxylase 4 (SAMDC4), an enzyme required for the biosynthesis of polyamines in *Arabidopsis thaliana*. In this study, we show that polyamines may play their roles in regulating the plant architecture through affecting the homeostasis of cytokinins and sensitivities to auxin and cytokinin.

Results

The bud2 plant shows altered hypocotyl elongation and lateral bud outgrowth

Our previous study showed that the bushy and dwarf phenotype of the *Arabidopsis bud2* mutant plant results from the defect in the biosynthesis of polyamines [4]. To investigate whether the loss of apical dominance of *bud2* plants involves auxin action, we examined the hypocotyl elongation at higher temperature and the lateral shoot development after decapitation of wild-type and *bud2* mutant plants [43, 57, 58]. Identification of temperature-induced hypocotyl elongation is a simple and rapid assay in the study of an auxin-mediated growth response [59]. As shown in Figure 1A, the hypocotyl length of the

Figure 1 Hypocotyl elongation at high temperature and branching of decapitated plants. **(A)** Induction of hypocotyl elongation by high temperature. Wild-type and *bud2* mutant seedlings were grown on 1/2 MS plates at 20 °C or 29 °C for 9 days and photographed at the same magnification. **(B)** The growth rate of the lowest cauline bud in intact and decapitated plants. The lowest cauline bud length of the intact and decapitated plants (decapitated above the lowest cauline bud 1.0 cm) were measured every 24 h until day 9. Error bars show SE ($n = 5$).

9-day-old *bud2* seedlings grown at 29 °C was seriously inhibited compared with that of the wild-type seedlings, suggesting that *bud2* may have an altered auxin-dependent developmental process. This was further strengthened by the decapitation experiment. As shown in Figure 1B, the outgrowth rate of lateral buds of decapitated wild-type plants was significantly accelerated compared with that of the decapitated *bud2* plants, indicating that the *bud2* plant has an abnormal response to shoot decapitation. These results strongly suggest that the *bud2* mutant plant has an altered auxin-related development.

The bud2 plant is hyposensitive to exogenous auxin

To determine whether the biosynthesis, transport or signaling of auxin is altered in the *bud2* mutant plant, we compared the IAA concentration, polar auxin transport (PAT) and response to auxin between the wild-type and mutant plants. No significant differences in the IAA contents of seedlings and the PAT capacity of

roots and inflorescence stems were found between *bud2* and wild-type plants (see Supplementary information, Figures S1 and S2). However, application of a low concentration of exogenous auxin (1.0 nM 1-naphthalene acetic acid, NAA) to *bud2* seedlings showed no effect on lateral root formation, in contrast to wild-type plants (Figure 2A), indicating that the *bud2* plant is relatively insensitive to exogenous auxin in promoting lateral root formation. Furthermore, we assayed the auxin effect on the axillary bud outgrowth using a split plate procedure reported previously [22]. As shown in Figure 2B, the exogenous NAA almost completely inhibited the axillary bud outgrowth of the wild-type plants, but it showed no significant impeding effect on *bud2* and *axr1-3*, a mutant defective in the auxin-signaling pathway [60]. These results demonstrate that the lateral root formation and axillary bud elongation of the *bud2* plants are hyposensitive

to exogenous auxin, suggesting that polyamines may be involved in regulating root and shoot branching through affecting auxin response in *Arabidopsis*. Consistently, we found that the expression levels of primary auxin-responsive genes, *IAA1* and *IAA4* [61], in the *bud2-2* plant were significantly attenuated after 60 min of IAA treatment (Figure 2C). Taken together, these results indicate that auxin signaling is defective in *bud2* mutant plants.

Induction of *BUD2* by auxin

To determine whether *BUD2* expression is regulated by auxin or cytokinin, we determined the *BUD2* expression levels in wild-type seedlings on auxin and cytokinin treatments. RNA gel blot analysis revealed that the *BUD2* transcripts were obviously accumulated at 30 min after IAA treatment and reached a maximal level at 120 min, while no significant alteration was observed after

Figure 2 Response of the *bud2* plant to exogenous auxin. **(A)** The lateral root formation of *bud2-2* mutant seedlings is less sensitive to exogenous α -NAA. The light-grown 3-day-old seedlings were transferred from the 1/2 MS plate onto the plate containing different concentrations of α -NAA. After 4 days of treatment, the average number of visible lateral roots was measured for each genotype. Experiments were repeated twice. Error bars show SE ($n = 20$). **(B)** Effect of α -NAA on lateral inflorescence outgrowth from excised nodes of the wild-type, *bud2-2* and *axr1-3* plants. The lowermost cauline node was excised from the elongating primary inflorescence of axenically grown plants and inserted between two separate agar slabs. Slabs contained either no α -NAA or 1.0 μ M α -NAA in contact with the apical or basal part of the stem. Lateral shoot lengths (mean $\pm$ SE) were determined every 24 h until day 9 after excision. **(C)** Expression levels of *IAA1* and *IAA4* in wild-type and *bud2-2* plants on IAA (10 μ M) treatment. Data represents the expression alteration of hormone-treated samples from three independent experiments. Bars represent $\pm$ SD.

treatment with 6-BA (Figure 3A). We also examined the expression levels of other SAMDC genes of *Arabidopsis* [4] upon auxin treatment. The result showed that

SAMDC2 and *SAMDC3* could be induced by auxin and had a similar tendency as the *BUD2* response. However, *SAMDC1* transcript was strongly repressed with exogenous auxin treatment (Figure 3B). This result suggests that *SAMDC* genes may interact with auxin signaling.

To determine whether *BUD2* is induced specifically by auxin, we analyzed the expression level of *BUD2* in the auxin receptor *tir1afb2afb3* triple mutant [62] by a quantitative PCR analysis. As shown in Figure 3C, the expression level of *BUD2* increased by 2.5-fold on IAA treatment in the wild-type plants, but by only ~1.2-fold in the *tir1afb2afb3* triple mutant plants, suggesting that *BUD2* can be specifically induced by auxin.

Altered biosynthesis of cytokinins in *bud2* plants

The outgrowth phenotype of the *bud2* axillary buds may also result from altered cytokinin biosynthesis and/or response, because cytokinin has long been proposed as a secondary messenger of IAA in controlling shoot branching [63-65]. We therefore compared the endogenous levels of various cytokinins between *bud2-2* and wild-type seedlings. As shown in Table 1, the homeostasis of various endogenous cytokinins in the *bud2* plant

Figure 3 Induction of *BUD2* by auxin. **(A)** Expression of *BUD2* induced by auxin or cytokinin, showing the RNA gel blot analysis of the *BUD2* expression levels in 10-day-old seedlings treated with IAA (10 μ M) or 6-BA (10 μ M). **(B)** Expression of *SAMDCs* revealed by Q-PCR in 10-day-old seedlings treated with IAA (10 μ M). Bar represents $\pm$ SD. **(C)** Expression of *BUD2* in wild-type and *tir1afb2afb3* plants treated with IAA (10 μ M) for 2 h. Bars represent $\pm$ SD.

Table 1 Cytokinin contents in *bud2* and wild-type plants

	WT	<i>bud2-2</i>	WT (%)
	Content (pg g ⁻¹ FW)	Content (pg g ⁻¹ FW)	
Z7G	1043.87 $\pm$ 63.18	1477.66 $\pm$ 21.21 ^a	141.6
Z9G	547.52 $\pm$ 35.56	826.87 $\pm$ 51.21 ^a	151.0
ZOG	859.74 $\pm$ 59.47	1303.82 $\pm$ 38.40 ^a	151.7
DZOG	10.06 $\pm$ 2.88	7.23 $\pm$ 1.50	71.9
ZROG	766.16 $\pm$ 39.78	942.90 $\pm$ 38.75 ^a	123.1
iP7G	2059.41 $\pm$ 47.34	1903.96 $\pm$ 71.89 ^a	92.5
iP9G	70.96 $\pm$ 2.97	73.13 $\pm$ 9.56	103.1
tZ	28.74 $\pm$ 3.27	23.05 $\pm$ 3.02	80.2
tZR	209.06 $\pm$ 9.97	244.50 $\pm$ 7.53	117.0
tZRMP	424.79 $\pm$ 15.71	464.63 $\pm$ 23.78	109.4
DZR	9.03 $\pm$ 0.39	12.13 $\pm$ 1.63	134.2
DZRMP	97.58 $\pm$ 4.99	99.10 $\pm$ 15.48	101.6
iP	14.92 $\pm$ 1.42	25.62 $\pm$ 3.59 ^b	171.7
iPR	38.49 $\pm$ 2.70	49.31 $\pm$ 3.58 ^a	128.1
iPRMP	925.99 $\pm$ 54.64	788.84 $\pm$ 25.08 ^b	85.2

^aData represents means $\pm$ SE from three independent samples.

^bIndicates the significant difference compared to wild type plants by Student's *t*-test. ^a $P \leq 0.01$, ^b $P \leq 0.05$. The statistical tests were performed using the SPSS v10.0.

Figure 4 Response of *bud2* to exogenous cytokinin. **(A)** Expression levels of *ARR5*, *ARR6* and *ARR15* of wild-type and *bud2* seedlings at 25 DAG. Data represents the relative expression levels of these genes of three independent experiments. Bars represent $\pm$ SD. **(B)** Comparison of the root elongation between wild-type and *bud2-2* plants on 6-BA treatment. Data represents means $\pm$ SE ($n = 30$). **(C)** Expression of *ARR5* and *ARR6* in the aerial parts of wild-type and *bud2-2* plants treated with 6-BA (10 μ M). Data represents the alteration of the expression of hormone-treated samples from three independent experiments. Bars represent $\pm$ SD.

was altered: trans-zeatin *O*-glucoside (ZOG), trans-zeatin 7-glucoside (Z7G), trans-zeatin 9-glucoside (Z9G), trans-zeatin riboside *O*-glucoside (ZROG), *N*6-(D2-isopentenyl) adenosine (iPR) and *N*6-(D2-isopentenyl) adenine (iP) were significantly increased, but *N*6-(D2-isopentenyl) adenine 7-glucoside (iP7G) and *N*6-(D2-isopentenyl) adenosine monophosphate (iPRMP) were apparently decreased. These results demonstrate that the altered polyamine biosynthesis that resulted from the null mutation of *BUD2* is able to cause an abnormal homeostasis of endogenous cytokinins, which may in turn affect the expression of cytokinin-responsive genes. We therefore examined the expression of cytokinin primary responsive genes, *ARR5*, *ARR6* and *ARR15*, in the aerial part of 25-day-old plants [66–68]. The results showed that the expression levels of these cytokinin-responsive genes were significantly elevated in the *bud2* mutant plant compared with the wild-type plant (Figure 4A).

The *bud2* plant is hypersensitive to exogenous cytokinin

To further investigate whether *BUD2* is involved in cytokinin signaling, we examined the sensitivity of *bud2* and wild-type plants on cytokinin treatments. We measured the root elongation of *bud2* and wild-type seedlings at the presence of various levels of exogenous cytokinin 6-BA. Compared to that of the wild-type plants, the inhibition of root elongation by 6-BA in *bud2-2* seedlings took place at a much lower concentration, indicating that *bud2* plants are hypersensitive to cytokinin (Figure 4B).

We further monitored the expression of two cytokinin primary responsive genes, *ARR5* and *ARR6*, in wild-type and *bud2-2* seedlings treated with 6-BA [69]. As shown in Figure 4C, the expression of *ARR5* and *ARR6* was rapidly induced on the treatment of 6-BA and declined in both the wild-type and *bud2-2* after 30-min treatment of 6-BA, but their induction levels in *bud2-2* were much higher than those in the wild-type plants (Figure 4C).

These results indicate that the response of *bud2* to cytokinin is also changed.

Genetic analysis of *bud2* double mutant

To further investigate the function of *BUD2* in regulating axillary bud outgrowth, we generated a *bud2-2 axr1-3* double mutant (Figure 5). The *bud2-2 axr1-3* double mutant showed an extremely dwarf and highly branched shoot phenotype; the length of lateral buds was much longer than the primary inflorescence and sometimes two lateral branches could be developed from a leaf axil (Figure 5D and 5E). Moreover, the high-order branches could also grow out from the axils of the first- and second-order branches in the double mutant (Figure 5E).

Furthermore, the expression levels of *ARR5* and *ARR6* in the axillary buds (< 1.5 mm in length) were enhanced

in the double mutant compared to either parental mutants (Figure 5F). These results suggest that *BUD2* is likely involved in an IAA/cytokinin-regulated shoot branching pathway that is independent of *AXR1*.

Polyamines affect callus growth in response to the cytokinin/auxin ratio

Cytokinins are able to promote cell division and produce callus in concert with auxin in cultured plant tissues [70]. We further compared the callus production of wild-type and *bud2* hypocotyl explants with various combinations of cytokinin zeatin and auxin 2,4-D. As shown in Figure 6, a significant difference in callus growth was observed at different combinations of auxin and cytokinin concentrations. The calli generated from the *bud2* mutant hypocotyl explants grew much faster than those from the wild type at all the tested combinations of

Figure 5 Phenotypes of the *bud2-2axr1-3* double mutant. The phenotypes of wild type (A), *axr1-3* (B), *bud2-2* (C) and *axr1-3bud2-2* (D) plants were photographed at day 10 after bolting. (E) The phenotype of *bud2-2axr1-3* at day 18 after bolting. White arrows indicate two lateral buds in a leaf axil and the red arrow indicates the main shoot apex. Bars = 2 cm. (F) The expression of two cytokinin primary responsive genes, *ARR5* and *ARR6*, in the lateral buds of wild-type, *bud2-2*, *axr1-3* and *axr1-3bud2-2* plants (bud length < 1.5 mm). Data represents the relative expression levels of these genes in three independent experiments. Bars represent $\pm$ SD.

Figure 6 Callus induction of wild-type and *bud2-1* explants. The hypocotyl explants from 5-day-old dark-grown seedlings of wild type (upper panel) and *bud2-1* (lower panel) were cultured on GM plates containing the indicated concentrations of *trans*-zeatin and 2,4-D for 30 days in the dark. One representative callus from each treatment was collected and photographed.

trans-zeatin and 2,4-D, especially at the combination of lower concentration of cytokinin (0.005 μM) and higher concentration of 2,4-D (0.5 μM). This result is consistent with the seedling responses to exogenous cytokinin and auxin (Figures 2 and 4), indicating that the alteration of polyamines in *bud2* results in an altered response to auxin and cytokinin.

Discussion

We have previously demonstrated that *BUD2* is the *SAMDC4* gene, which encodes an SAMDC. In the *bud2* mutant plant, the homeostasis of polyamines is altered, leading to an obvious increase in putrescine and decreases in spermidine and spermine, which in turn leads to an altered plant morphology [4]. In recent years, studies on the biosynthesis of polyamines have indicated that polyamines are involved in a variety of plant developmental processes [2, 5-8, 11, 71, 72], especially the formation of plant architecture including shoot apical dominance [58]. However, the mechanism underlying

polyamine action needs to be elucidated. In this paper, we provide evidence that polyamines play an important role in controlling the branching of *Arabidopsis* through affecting the homeostasis of cytokinins and sensitivities to auxin and cytokinin.

Quantitative analysis of cytokinins demonstrated that the homeostasis of cytokinins is significantly altered in *bud2* mutant plants (Table 1). The contents of 8 derivatives out of 15 examined cytokinins have significantly changed, among which 6 derivatives are increased. These elevated endogenous cytokinins, especially the active form iP, may explain why the expression levels of three cytokinin-inducible markers, *ARR5*, *ARR6* and *ARR15*, are significantly enhanced in the *bud2* mutant plant. This may also explain why the deficiency in polyamine biosynthesis promotes axillary bud outgrowth and causes a bushy phenotype.

Our study shows that deficiency in polyamine biosynthesis causes hyposensitivity to auxin. Compared with the wild-type plant, the *bud2* mutant plant showed hyposensitivity to exogenous auxin NAA in lateral root formation or axillary bud outgrowth assays, which is reminiscent of previous findings in auxin-signaling mutants, *nph4*, *arf19* or *axr1-3* [22, 73]. Moreover, like most auxin-insensitive mutants [74], the expression levels of auxin-inducible genes are reduced in *bud2*. These findings strongly suggest that the polyamine contents may play an unidentified role in regulating the response to auxin. This hypothesis is further supported by the findings that *BUD2* is inducible by auxin and that the induction by auxin depends on a normal auxin-signaling pathway. In addition to *SAMDCs*, some other genes in the polyamine biosynthetic pathway, for example, *ACL5* and *PAO*, are also regulated by auxin [8, 75], supporting that polyamines are likely involved in the regulation of plant development by auxin.

Our results also demonstrate that *bud2* is hypersensitive to cytokinin. As shown in the root growth inhibition assay, *bud2* seedlings showed hypersensitivity to exogenous cytokinin 6-BA. Taken together, our results showed that the deficiency in polyamine biosynthesis led to hyposensitivity to auxin and hypersensitivity to cytokinin. This suggests that polyamines may play a role in regulating plant morphology by affecting plant response to the ratio of auxin and cytokinin. In fact, this is further confirmed by the callus production experiments. However, a challenge in the future is to understand how polyamines affect the sensitivities of plants to auxins and cytokinins. In addition, a recent study revealed that thermospermine, a structural isomer of spermine, can partially rescue *acl5* mutant phenotype [13]. Further studies will explain whether thermospermine is also involved in

BUD2-regulated stem elongation.

Taken together, our results suggest that polyamines play an important role in regulating plant architecture in *Arabidopsis* and function through the actions of auxin and cytokinin. We propose that polyamines may increase the sensitivity of auxin perception, and thus repress cytokinin biosynthesis or signaling.

Materials and Methods

Plant growth

A. thaliana ecotype Columbia-0 (Col-0) wild-type and mutant plants were grown on vermiculite saturated with $0.3\times$ B5 medium under long photoperiod ($80\text{--}120\ \mu\text{Em}^{-2}\text{sec}^{-1}$) at $23\ ^\circ\text{C}$, as described previously [76]. For root observation, seeds were surface sterilized and vernalized at $4\ ^\circ\text{C}$ for 4 days and seedlings were grown vertically at $23\ ^\circ\text{C}$.

RNA gel blot analysis, quantitative PCR

Total RNA was isolated with the TRIzol reagent (Invitrogen, USA) and the RNA gel blot analysis was performed as previously described [77]. RNA ($20\ \mu\text{g}$ per lane) was separated in a 0.8% agarose gel containing 10% formaldehyde, blotted onto a Hybond N⁺ membrane (Amersham) and probed with the PCR-amplified DNA fragments using a specific primer pair of *SAMDC4* [4]. For the quantitative PCR analysis, $2.5\ \mu\text{g}$ of total RNA was treated with DNaseI (Gibco BRL) and then the first strand cDNA was synthesized using a cDNA synthesis kit (Promega, USA). The real-time PCR was performed with suitable procedures using the SYBR GREEN PCR Master Mix kit (ABI, USA) with specific primers, as described in Supplementary information, Table S1. All manipulations were carried out according to the instructions of manufacturers.

Hormone response assays

To test the sensitivity of the lateral root formation on auxin treatment, wild-type and *bud2* seeds were grown vertically on $0.5\times$ MS agar plates for 3 days after germination under continuous white light. The seedlings were transferred to plates containing various concentrations of NAA. The positions of the primary root tips were marked. Lateral roots were scored under a dissecting microscope and were accounted if visible primordia had formed. The axillary bud growth on auxin treatment in *bud2-2* plants was assayed as described [22]. Seedlings of wild type, *bud2-2* and *axr1-3* were grown on $0.5\times$ MS agar plates, and primary inflorescences appeared 25–30 days after germination. Inflorescence sections with the first cauline node and the axillary bud ($<0.5\ \text{mm}$) were excised and placed over the gap between the two medium blocks in a split plate. The apical ends of the sections were inserted into the medium blocks with $1.0\ \mu\text{M}$ NAA. The split plates were held at 10° off vertically with the node being upright. Growth of the lateral buds was then measured every 24 h.

For the assay of the root elongation on cytokinin treatment, the wild-type and *bud2* seedlings were grown vertically on $0.5\times$ MS agar plates containing various concentrations of 6-BA for 8 days under light and the length of primary roots was measured.

Callus was induced according to the method reported previously [70]. Briefly, the hypocotyls from 5-day-old dark-grown

wild-type and *bud2-1* seedlings were excised into 5-mm segments and cultured on GM medium supplemented with the indicated combinations of 2,4-D and zeatin for 30 days at $23\ ^\circ\text{C}$ in the dark.

To test expressions of cytokinin or auxin-responsive genes, 12-day-old seedlings of wild-type or the *tir1afb2afb3* plants grown on $0.5\times$ MS plates under constant light were transferred to the same liquid medium supplemented with $10\ \mu\text{M}$ of 6-BA, $10\ \mu\text{M}$ of IAA or 0.1% DMSO. Seedlings were harvested at the time indicated and RNA was isolated for RNA blot or real-time PCR analyses.

Cytokinin measurement

Extraction and purification of cytokinin were carried out as described previously [78, 79]. Briefly, 2 g aerial parts of 25-day-old plants grown under long-day conditions were homogenized and 200 pmol of each of the following 12 deuterium-labeled standards were immediately added: [²H₅]Z, [²H₅]ZR, [²H₅]Z7G, [²H₅]Z9G, [²H₅]ZOG, [²H₅]ZROG, [²H₆]iP, [²H₆]iPR, [²H₆]iP7G, [²H₆]iP9G, [²H₅]DHZ and [²H₅]DHZR (Apex Organics, Honiton, Devon, UK). After extraction and SPE purification, cytokinins were analyzed on a liquid chromatography-tandem mass spectrometry system (Finnigan Surveyor LC-LCQ Deca XP MAX, Thermo, San Jose, CA, USA) using positive ion electrospray ionization and quantified as described previously [63].

Generation of the *bud2axr1* double mutant

The double mutant *bud2-2axr1-3* was generated from the cross of homozygous *bud2-2* with *axr1-3* [26], and identified from the F₂ progeny grown on soil by comparing with their parental phenotypes and PCR-based characterization.

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