

## Auxin inhibits the outgrowth of tiller buds in rice (*Oryza sativa* L.) by downregulating *OsIPT* expression and cytokinin biosynthesis in nodes

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### Abstract

Auxin and cytokinin (CTK) play important roles in regulating the growth of rice tiller buds. Auxins inhibit bud growth and CTK, which are regulated by auxins, promote growth. However, little is known about the underlying molecular mechanisms. Here, we studied auxin/CTK regulation in Nanjing 44 rice, a japonica cultivar. After full heading, we cut off the roots and removed the panicles. The tiller buds grew rapidly after panicle removal; significantly elevated CTK levels were found in tiller buds and nodes preceding bud growth. However, external application of the auxin indole-3-acetic acid (IAA) inhibited the growth of tiller buds and reversed the increase of CTK levels in tiller buds and nodes induced by panicle removal. According to previous studies, the rice gene adenosine phosphate isopentenyltransferase (*OsIPT*) encodes a key enzyme in CTK biosynthesis. To determine the site of CTK synthesis involved in bud growth, we examined the expression pattern of *OsIPT* in tiller buds and nodes. Our results demonstrated that tiller bud growth is not regulated by CTK derived from roots; rather, CTK is biosynthesised mainly in the nodes and subsequently delivered to tiller buds. Auxin negatively regulates the expression of *OsIPT* and the biosynthesis of CTK in nodes, thus inhibiting the growth of tiller buds in rice.

**Keywords** Tiller bud; Rice (*Oryza sativa* L.); Auxin; Cytokinin; *OsIPT*.

**Abbreviation** IAA: indole-3-acetic acid; CTK: cytokinin; *OsIPT*: rice gene adenosine phosphate isopentenyltransferase; RP: removal of the panicle after full heading; tZ: *trans*-zeatin; tZR: *trans*-zeatin riboside; tZRMP: tZR 5'-monophosphate; iP: isopentenyladenine; iPR: iP riboside; iPRMP: iPR 5'-monophosphate; PCR: polymerase chain reaction.

### Introduction

The tillering of rice (*Oryza sativa* L.), determines the number of panicles per plant, is an important agronomic trait for grain production and serves as a model in the study of branching patterns in monocotyledonous plants (Li et al., 2003). Although moderate tillering greatly contributes to increased rice yield, excessive tillering leads to a high rate of tiller abortion, poor grain setting, small panicle size, and, as a result, a low grain yield (Peng et al., 1994). Understanding the regulatory mechanisms underlying the growth of rice tiller buds could enhance rice yields. Plant branches develop from lateral buds, and auxin and cytokinin (CTK) play important roles in the regulation of their growth. Auxin are synthesised at the shoot apex and inhibit the growth of lateral buds. Decapitation of the apex generally frees lateral buds from growth inhibition, and the addition of auxin to the cut stump restores the dormancy of lateral buds (Cline, 1991). Leopold (1949) indicated that the shoot growth of grasses is similar to the auxin-induced apical dominance of dicotyledonous plants; removal or suppression of auxin activity releases tillers (lateral buds) from apical control. In contrast to auxin, CTK are either synthesised in the root and move acropetally to the shoot (Bangerth, 1994) or are locally synthesised (Faiss et al., 1997; Schmulling, 2002) and have a stimulatory effect on lateral bud growth. Moreover, external CTK stimulates tiller bud growth in wheat (Langer et al., 1973). In contrast, externally applied auxin inhibits this growth

(Harrison and Kaufman, 1982). It is widely agreed that axillary bud growth depends on the ratio of these two hormones, rather than on their absolute levels (Emery et al., 1998; Shimizu-Sato and Mori, 2001; Wang et al., 2006). These studies provide clear evidence that auxin and CTK are involved in regulating tiller bud growth in rice. Little is known, however, about the underlying molecular mechanism. The results of several studies suggest that basipolar auxin may control CTK production in roots and its possible delivery to lateral buds (Letham, 1994; Li et al., 1995; Bangerth et al., 2000). However, Tanaka et al. (2006) reported that under apical dominance, one role of auxins is to repress the local biosynthesis of CTK in the nodal stems of pea (*Pisum sativum* L.) plants. In the present study, we cut off the roots of Nanjing 44 rice plants after full heading and then removed the panicles to stimulate the growth of dormant tiller buds located at elongated internodes. In one group, we applied indole-3-acetic acid (IAA) externally to inhibit the effect of panicle removal on tiller bud growth. We then analysed changes in CTK levels and the expression pattern of the rice gene adenosine phosphate isopentenyltransferase (*OsIPT*), which encodes a key enzyme in CTK biosynthesis, in both groups. Our objectives were to investigate the relationship between auxins and the biosynthesis of CTK and the role of *OsIPT* in the regulation of tiller bud growth in rice.

**Table 1.** Effect of panicle removal and external IAA on the tiller buds length (cm)

Time after treatments (h)	Treatments		
	Control	RP	IAA
0	0.62a	0.65a	0.68a
12	0.65a	0.66a	0.63a
24	0.64a	0.68a	0.66a
36	0.66b	1.12a	0.69b
48	0.61b	1.59a	0.63b

At 0 h, 12 h, 24 h, 36 h and 48 h after treatment, the length of tiller buds located at the second node from the top was measured. Values within a row followed by different letters were significantly different at  $P = 0.05$ . RP: removal of the panicle after full heading; IAA: application of IAA after panicle removal; control: panicle retained.

## Materials and methods

### Plant growth and treatments

The experiment was conducted in the net house of the Nanjing Agriculture University (Jiangsu Province, China) during the rice-growing season of 2009. Nanjing44, a japonica cultivar, was used for the study. Seedlings were planted in a seedbed on May 22<sup>nd</sup>. Twenty-day-old seedlings were transplanted into plastic pots 30 cm in both diameter and height. Each pot contained four plants and 15 kg of sieved soil; nitrogen (1.6 g plot<sup>-1</sup> as urea), phosphorus (0.8 g plot<sup>-1</sup> as single superphosphate), and potassium (1.2 g plot<sup>-1</sup> as KCl) were applied at the time of transplanting. Surface water was maintained over the entire growth season of the plants. After full heading, we cut off the roots and transplanted the plants into 20 L plastic pots containing quartz sand, each with forty plants. Next, we applied one of three different treatments: RP (removal of the panicle after full heading), IAA (panicle removed plus the application of IAA), and control (retention of the panicle). The concentration of the IAA was 50 mg L<sup>-1</sup>, and we used 5 mL per plant. All treatments received the same quantity of water. Three repetitions were performed for each treatment. IAA was purchased from Sigma (USA).

### Sampling and measurements

At 0 h, 3 h, 6 h, 9 h, 12 h and 24 h after the start of the treatments, the flag leaf blade, the second leaf blade from the top, the first and the second leaf sheathes from the top, the second nodes from the top and the tiller buds located at the nodes were sampled. The samples were frozen in liquid nitrogen for 30 min and then stored at -70 °C.

### Measurement of bud length

At 0 h, 3 h, 6 h, 9 h, 12 h and 24 h after the start of the treatments, forty tiller buds located at the second node from the top were sampled and their lengths were measured.

### Measurement of endogenous CTKs

The extraction and purification of *trans*-zeatin (tZ), *trans*-zeatin riboside (tZR), tZR 5'-monophosphate (tZRMP), isopentenyladenine (iP), iP riboside (iPR) and iP 5'-monophosphate (iPRMP) were performed using methods

essentially identical to those described by Dobrev and Kaminek (2002). The determination of tZ, tZR, tZRMP, iP, iP riboside and iPRMP was performed using a high-performance liquid chromatography system (P680 Pump/UV170U UV-VIS Detector, DIONEX, USA) as previously described (Nakagawa et al., 2005).

### Expression analyses

Total RNA was prepared using an E.Z.N.A.® Plant RNA Kit (Omega Bio-tek, Inc., USA). The reverse transcription reaction was performed with a PrimScript™ RT reagent Kit (Takara, Kyoto, Japan), oligo-dT and random hexamer primers according to the manufacturer's protocol. The resulting cDNA sample was used as a template for a PCR reaction with 40 cycles of 0.5 min each at 94 and 55-57°C and 1 min at 72°C. The PCR products were separated by agarose gel electrophoresis (1.5 % agarose gel). A quantitative real-time PCR (qRT-PCR) was performed using an ABI 7300 sequencer and SYBR Premix Ex Taq™ (Takara, Kyoto, Japan) according to the manufacturer's protocol. The PCR and the quantitative real-time PCR primers were the same as those used by Sakamoto et al. (2006).

### Statistical analysis

The results were analysed using SPSS 16.0 for Windows. The data from each sampling event were analysed separately. The means were tested with the least significant difference test and the significance level was set at  $P < 0.05$ .

## Results

### Growth of tiller bud

The removal of the panicle broke the dormancy of the tiller buds and stimulated their growth. Thirty-six hours after the removal of the panicle, the length of the tiller buds was significantly higher in the RP treatment than in the control. The application of IAA completely reversed the effect of panicle removal on the tiller bud growth. Forty-eight hours after the IAA treatment, there was no significant difference in tiller bud length between the IAA and the control treatments (Table 1).

### Endogenous CTK levels in several organs

The removal of the panicle significantly increased the CTK levels in nodes and tiller buds, and the difference between RP and control treatments was already significant at 3 h after the start of the treatments. External IAA completely inhibited the increase of CTK levels in nodes and tiller buds, and there was no significant difference in CTK levels in nodes and tiller buds between the control and IAA treatments during the experimental period (Table 2). Contrary to the treatment effects on nodes and tiller buds, the CTK levels in leaves and sheathes experienced no significant changes after panicle removal, and external IAA had no obvious effect. During the experimental period, there was no significant difference in the CTK levels of leaves and sheathes among the three treatments (Table 2).

### Analyses of the *OsIPT* gene expression in nodes and tiller buds

To determine whether the removal of the panicle affected the

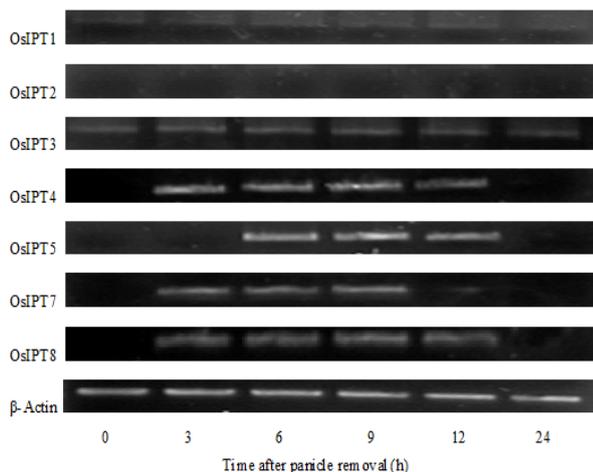
**Table 2.** Effect of panicle removal and external IAA on CTK contents in various organs

CTK (ng g <sup>-1</sup> FW)	Treatments	Node			Tiller bud			Leaf blade			Leaf sheath		
		0h	3h	6h	0h	3h	6h	0h	3h	6h	0h	3h	6h
iZ	CK	1.72a	1.83b	1.76b	2.46a	2.20b	2.64b	3.82a	3.92a	3.65a	5.67a	5.86a	5.14a
	RP	1.67a	3.97a	8.58a	2.53a	8.45a	31.28a	3.97a	4.41a	4.38a	5.68a	5.96a	5.99a
	IAA	1.65a	1.73b	1.80b	2.70a	2.54b	2.61b	3.66a	3.85a	3.99a	5.21a	5.33a	5.68a
iZR	CK	2.32a	2.37b	2.26b	3.11a	3.54b	3.61b	6.92a	6.82a	7.06a	8.95a	9.11a	8.47a
	RP	2.21a	12.82a	53.17a	3.01a	31.21a	94.24a	7.41a	8.15a	8.03a	9.13a	9.56a	9.35a
	IAA	2.28a	2.26b	2.71b	3.06a	3.35b	3.18b	6.58a	7.78a	7.33a	8.86a	9.03a	9.22a
iZRMP	CK	9.48a	8.57b	11.01b	6.81a	7.59b	6.37b	23.96a	24.58a	24.10a	36.82a	37.51a	33.84a
	RP	9.52a	38.14a	152.9a	7.03a	49.84a	105.44a	24.21a	25.17a	26.12a	38.15a	38.15a	39.19a
	IAA	9.45a	10.08b	9.03b	7.11a	7.03b	6.82b	23.82a	22.31a	24.84a	36.99a	38.16a	37.56a
iP	CK	1.33a	1.47b	1.46b	4.01a	3.77b	3.69b	4.13a	4.46a	4.53a	5.84a	5.39a	5.67a
	RP	1.28a	4.56a	7.84a	3.84a	13.51a	38.18a	5.11a	4.81a	4.88a	5.66a	5.71a	5.76a
	IAA	1.40a	1.32b	1.31b	3.76a	3.67b	3.59b	4.93a	4.99a	4.76a	5.74a	5.84a	5.85a
iPR	CK	3.31a	3.54b	3.48b	7.95a	7.96b	8.12b	7.92a	7.86a	7.89a	9.06a	9.68a	9.81a
	RP	3.61a	28.97a	86.45a	7.76a	68.35a	128.41a	8.34a	7.99a	7.84a	9.15a	9.26a	9.33a
	IAA	3.51a	3.38b	3.46b	7.67a	7.88b	7.93b	7.38a	7.64a	7.81a	9.11a	9.35a	9.41a
iPRMP	CK	21.30a	19.48b	20.84b	9.45a	11.26b	11.96b	36.85a	36.84a	37.15a	41.09a	42.13a	41.57a
	RP	20.41a	79.59a	276.84a	9.61a	79.89a	169.69a	36.59a	38.18a	38.33a	40.99a	45.28a	44.16a
	IAA	21.60a	26.12b	20.96b	9.56a	10.11b	9.98b	33.62a	34.84a	34.66a	41.64a	42.45a	41.35a

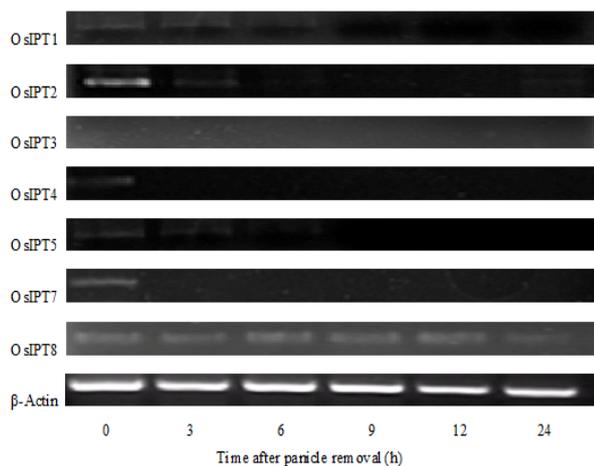
expression of *OsIPT* in nodes and tiller buds, we studied *OsIPT* expression patterns in the second node from the top and the tiller buds located there before and after panicle removal. The analysis of the expression profiles on a 1.5% agarose gel showed that, except for *OsIPT1* and *OsIPT3*, expression of the other *OsIPT* genes was not detected before removing the panicle. After panicle removal, the expression levels of *OsIPT4*, *OsIPT5*, *OsIPT7* and *OsIPT8* increased markedly. The expression of *OsIPT5* was detected at 6 h after panicle removal, and that of the other three genes was detected at 3 h after panicle removal. At 24 h after panicle removal, the expression of these four genes was no longer detectable. In contrast to *OsIPT4*, *OsIPT5*, *OsIPT7* and *OsIPT8*, the expression of *OsIPT1* and *OsIPT3* showed no obvious changes during the 24 h period after panicle removal; the expression of *OsIPT2* was not detected during this period (Fig. 1). In contrast to the node results, the expression of the *OsIPT* genes in the tiller buds located at the second node from the top was markedly reduced after the removal of the panicle, except for *OsIPT3* (which remained unchanged) and *OsIPT8* (which was not detected; Fig. 2). Our results suggest that CTKs, which promote the growth of tiller buds, were mainly synthesised in nodes and then delivered to the tiller buds. Moreover, the high CTK levels repressed the expression of *OsIPT* in tiller buds. To examine whether the external application of IAA affected the expression of *OsIPT* in nodes, we used qRT-PCR to quantitate the expression levels of *OsIPT4*, *OsIPT5*, *OsIPT7* and *OsIPT8*, which were upregulated as a result of panicle removal. The results indicated that panicle removal significantly increased the expression level of these four genes, and the difference between the RP and control treatments was already significant at 3 h after treatment. The application of IAA inhibited the promoting effect of panicle removal on the expression of these four genes; at 3 h and 6 h after treatment, there were no significant differences in the expression levels of *OsIPT4*, *OsIPT5*, *OsIPT7* and *OsIPT8* between the IAA and control treatments. Similarly, the expression levels of these four genes in the IAA and control treatments were significantly lower than in the RP treatment (Fig. 3).

## Discussion

Tillers are the grain-bearing branches in monocotyledonous plants. Normally, the tiller buds formed at the elongated upper internodes become dormant when the panicles of the mother stems begin to differentiate (Li, 1979; Wang and Li, 2005). However, these buds exhibit significant elongation after stem decapitation (Arite et al., 2007). In the present study, the roots were cut off after full heading and then the panicle was removed. Interestingly, we found that the length of the dormant tiller buds was notably increased after panicle removal (Table 1). This result suggests that the growth of tiller buds is not regulated by a substance derived from roots. A number of previous studies have reported that CTK was produced in the roots and delivered to the lateral buds, promoting their growth (Letham, 1994; Li et al., 1995; Bangerth et al., 2000). However, our results indicate that the CTKs that promote the growth of rice tiller buds are not exclusively synthesised in roots. Prior to tiller bud growth, CTK levels in the nodes and tiller buds significantly increased, even when the roots were cut off. However, CTK levels in the leaves and sheaths showed no significant changes before and after tiller bud growth (see Tables 2 and 3). These results indicate that CTKs are synthesised in nodes or buds. The adenosine phosphate isopentenyltransferase (IPT) gene encodes a key enzyme for CTK biosynthesis (Kakimoto, 2001; Takei et al., 2001). Sakamoto et al. (2006) suggested that the products of *OsIPT1-8*, except for *OsIPT6*, are involved in CTK biosynthesis in rice. Therefore, we examined the expression levels of *OsIPT1*, *OsIPT2*, *OsIPT3*, *OsIPT4*, *OsIPT5*, *OsIPT7* and *OsIPT8* in tiller buds and nodes to determine the site of CTK synthesis. Our results revealed that the expression levels of *OsIPT* genes in the nodes significantly increased after the removal of the panicle; however, the *OsIPT* expression level in tiller buds was inhibited by panicle removal (Figs. 1 and 2). These results suggest that the removal of the panicle enhanced the

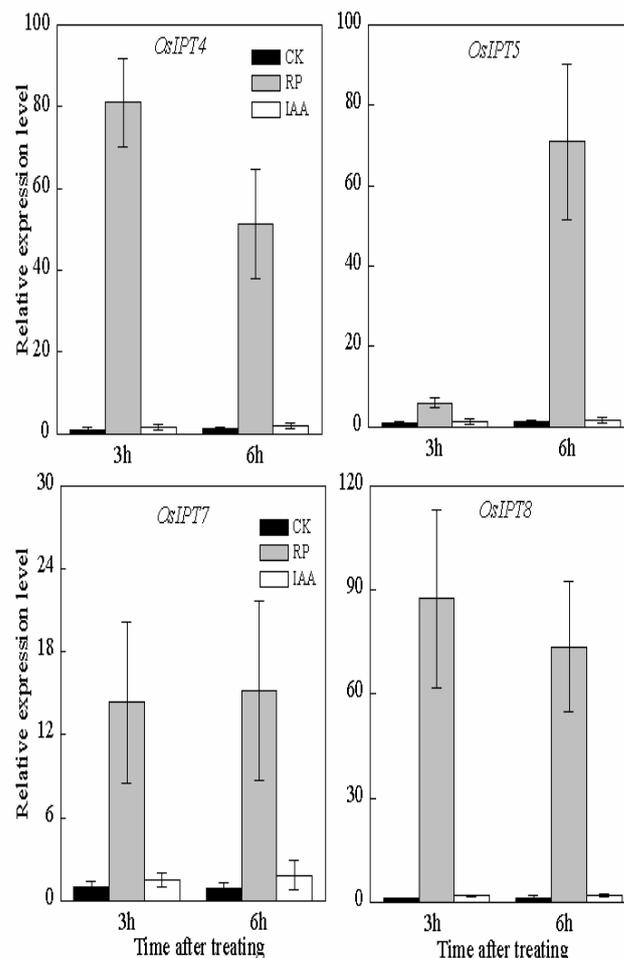


**Fig 1.** RT-PCR analysis of *OsIPT* expression in the second node from the top. Total RNA was prepared from nodes and then subjected to RT-PCR analysis. The results were visualised by agarose gel electrophoresis.  $\beta$ -actin was used as a loading control.



**Fig 2.** RT-PCR analysis of *OsIPT* expression in tiller buds located at the second node from the top. Total RNA was prepared from tiller buds and then subjected to RT-PCR analysis. The results were visualised by agarose gel electrophoresis.  $\beta$ -actin was used as a loading control.

expression of *OsIPT* and so stimulated CTK synthesis in the nodes. Subsequently, CTKs were delivered to the tiller buds and thus promoted their growth. This result is similar to that reported by Tanaka et al. (2006). Auxins significantly inhibit lateral bud growth after decapitation when applied to the stem stump (Cline, 1991; Cline, 1996; Leyser, 2003). In the present study, the external IAA completely reversed the promoting effect of the panicle removal on tiller bud growth (Table 1). Several studies have suggested that auxins may control CTK production in roots and possibly its delivery to lateral buds and, therefore, regulate their growth (Li et al., 1995; Bangerth et al., 2000). Here, we found that external IAA inhibited the decapitation-induced CTK increase in tiller buds and nodes, even when the roots were cut off. The qRT-PCR analysis indicated that IAA significantly repressed the expression of *OsIPT4*, *OsIPT5*, *OsIPT7* and *OsIPT8* in the nodes; in the absence of IAA, their expression was significantly induced by panicle removal (Fig. 3). This may



**Fig 3.** Expression patterns of *OsIPT4*, *OsIPT5*, *OsIPT7* and *OsIPT8* in the second node from the top in the different treatments. Total RNA was isolated from nodes.  $\beta$ -actin was used as a loading control. The value obtained from the control treatment at 3 h after treatment was arbitrarily set at 1.0. qRT-PCR was performed in triplicate and mean values with SD are shown. RP: removal of the panicle after full heading; IAA: application of IAA after panicle removal; control: panicle retained.

be the main reason why auxin application inhibited the increase of CTK levels in tiller nodes and buds otherwise induced by panicle removal. Based on these findings, we concluded that CTKs, which promote tiller bud growth, are mainly biosynthesised in the nodes and that there is little or no contribution from the root. Auxin repressed the expression of *OsIPT* and inhibited the biosynthesis of CTK in nodes and thus controlled the growth of tiller buds. This conclusion is in agreement with Tanaka et al. (2006) and contributes to the understanding of the mechanism of apical dominance in rice plants. In this study, we found that there were differences in the expression patterns of several *OsIPT* genes. The expression of *OsIPT4*, *OsIPT5*, *OsIPT7* and *OsIPT8* in nodes was markedly induced by panicle removal and the application of IAA, but the expression of *OsIPT1*, *OsIPT2* and *OsIPT3* was not affected by panicle removal (Figs. 2 and 3). Miyawaki et al. (2004) found that auxin positively regulated the expression of *AtIPT5* and *AtIPT7* in *Arabidopsis* roots. In contrast, IAA significantly repressed the expression of

*OsIPT4*, *OsIPT5*, *OsIPT7* and *OsIPT8* in rice nodes. These results suggest that auxins are both negatively and positively involved in CTK biosynthesis in a complicated manner perhaps unique to each species. Recent studies have suggested the involvement of a novel hormone in the inhibition of the outgrowth of axillary buds using a series of mutants including the *ramosus (rms)* pea (*P. sativum*) gene, *more axillary growth (max)* in *Arabidopsis*, and *dwarf (d)* in rice (Arite et al., 2007; Ishikawa et al., 2005; Sorefan et al., 2003; Stirnberg et al., 2002). In the proposed biosynthetic pathway, *MAX4*, *RMS1* and *D10* encode carotenoid cleavage dioxygenase 8 (CCD8), which may catalyse sequential carotenoid-cleavage reactions. Moreover, the expression of *RMS1* requires auxins (Foo et al., 2005; Sorefan et al., 2003). These results demonstrate that auxins also control the outgrowth of tiller buds in rice through the synthesis of a branch inhibitor. Similarly, the various signals that regulate rice tiller bud growth do not operate in isolation but rather as parts of a network or a matrix of interacting pathways. Further research on the mechanism of single signals and the cross-talk among them would help to clarify the mechanism of tiller bud growth in rice.

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