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The relationship between nitrogen, auxin and cytokinin in the growth regulation of rice (*Oryza sativa* L.) tiller buds

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Abstract

Nitrogen (N), auxin and cytokinins (CTKs) play important roles in regulating the growth of rice tiller buds. However, little is known about the underlying mechanisms and the relationships between them in the regulation of tiller bud growth in rice. In the present study, N and auxin indole-3-acetic acid (IAA) were used to regulate tiller bud growth in Nanjing 44 rice, a japonica cultivar. The tiller buds grew rapidly after external application of 40 mg L^{-1} N; significantly elevated CTK levels were found in tiller buds and nodes preceding bud growth. However, external application of IAA inhibited the growth of tiller buds and reversed the increase in CTK levels in tiller buds and nodes induced by N. According to previous studies, the rice adenosine phosphate isopentenyltransferase (*OsIPT*) genes encode a key enzyme in CTK biosynthesis. To determine the site of CTK synthesis involved in bud growth, we examined the expression patterns of the *OsIPT* genes in tiller buds, tiller nodes and roots. Our results indicate that the CTKs that promote tiller bud growth are biosynthesised mainly in the tiller nodes, and there is little or no contribution from the roots. N and IAA regulated the expression of the *OsIPT* genes and inhibited the biosynthesis of CTKs in the tiller nodes, thus regulating the growth of rice tiller buds.

Keywords Tiller bud; Rice (*Oryza sativa* L.); Nitrogen; Indole-3-acetic acid; Cytokinin.
Abbreviations: IAA: indole-3-acetic acid; CTK: cytokinin; *OsIPT*: rice gene adenosine phosphate isopentenyltransferase; N: nitrogen; NH: 40 mg L^{-1} N in the nutrient solution; Z: zeatin; ZR: zeatin riboside; iP: isopentenyladenine; iPR: iP riboside; PCR: polymerase chain reaction.

Introduction

Rice (*Oryza sativa* L.) tillering is an important agronomic trait for grain production (Li et al., 2003), and the number of tillers is dynamic and adjustable (Kariali and Mohapatra, 2007). Although moderate tillering contributes greatly to rice yields, excessive tillering leads to high tiller abortion, poor grain setting, and small panicle size and ultimately reduces grain yield (Peng et al., 1994). Elucidating the regulatory mechanisms that underlie rice tillering will facilitate high-yield rice crop production. Rice tillers develop from tiller buds, and cytokinins (CTKs) play an important role in regulating tiller bud growth. External application of kinetin stimulates tiller bud growth in wheat (Langer et al., 1973). Large differences in zeatin (Z) + zeatin riboside (ZR) (Z+ZR is a type of CTK) levels between the main stem and tillers lead to tiller death in wheat (Liang and Ma, 1998). The germination and dormancy of ratooning buds are closely related to variations in endogenous isopentenyl adenosine (iPA; one CTK) levels in rice plants (Li and Tang, 2002). In addition to CTK, auxin also influences tiller bud growth. Shoot growth in grasses is governed by the same type of auxin-induced apical dominance as dicotyledons; the removal or suppression of auxin activity releases the tillers (lateral buds) from apical control (Leopold, 1949). Exogenous application of auxin inhibits the CTK-induced promotion of tiller bud growth (Harrison and Kaufman, 1982). These results provide evidence that auxin and CTKs are involved in regulating rice tiller bud growth. Little is known, however, about the underlying molecular mechanisms. A number of studies have supported that basipolar auxin may control CTK production in roots and possibly its delivery to lateral buds (Letham, 1994; Li et al., 1995; Bangerth et al., 2000), but Tanaka et al. (2006) indicated that in apical dominance, one role of auxin is to repress the local biosynthesis of CTKs in the nodal stem in pea (*Pisum sativum* L.) plants. However, the relationship between auxin and CTKs in the regulation of tiller bud growth in rice is unclear. Nitrogen (N), a major non-carbon mineral nutrient that is essential for plants, displays a significant promoting effect on tiller development (Ding et al., 1995; Sakakibara et al., 2006). To combine the activities of the nitrogen signal at the whole plant level, plants use multiple signalling routes to integrate their internal and external N statuses. One route depends on nitrate itself, and the other uses CTKs as messengers (Sakakibara et al., 2006). Previous studies have shown that there is a close correlation between the N nutritional status and cytokinin levels in tobacco (*Nicotiana tabacum* L.) (Singh et al., 1992), *Urtica dioica* (Wang and Beck, 1993), barley (*Hordeum distichum* L.) (Samuelson and Larsson, 1993), and maize (*Zea mays* L.) (Takei et al., 2001). However, the relationship between N and CTKs in the regulation of tiller bud growth in rice remains unclear. In the present study, N and auxin indole-3-acetic acid (IAA) were used to regulate the growth of tiller buds in rice. We measured the hormonal variations of tiller buds and tiller nodes and the expression levels of the rice adenosine phosphate isopentenyltransferase (*OsIPT*) genes, which encode a key enzyme in CTK biosynthesis, in tiller buds, roots and tiller nodes. The objective of the study was to investigate the relationships between IAA, CTKs and N in the regulation of tiller bud growth in rice.

Results

Growth of tiller buds

Application of 40 mg L^{-1} external N ended the dormancy of the tiller buds and stimulated growth. Three days after the applica-

Table 1. Primer sequences used in this study

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
OsIPT1	ACCAAGCCCAAGGTTATCTTCGTGC	TCGTCGGTGACCTTGTTGGTGATGA
OsIPT2	AGTCACCCAAGCCCAAGGTCGTCTT	CTCCTCGGTGACCTTGTTCGTGATG
OsIPT3	GAGCTGTGCTTCCTGTGGGTGGACT	GCGACCTTGTACTTGTCTCCGTGCG
OsIPT4	TGGATGTGGTGACGAACAAGGTGAC	GATCTACGTCGACCCAGAGGAAGCA
OsIPT5	AGGTGATCAACGCCGACAAGCTGCA	TCGACGAGCTCCTCGATGTAGGAGT
OsIPT7	TGGACGACATGGTGGACGCTGGCAT	GCTTTGATGTCGTCGATCGCCTCGG
OsIPT8	GTCGACGACGATGTTCTCGACGAAT	TGTTGGCCTTGATCTCGTCTATCGC
β-Actin	CAATCGTGAGAAGATGACCC	GTCCATCAGGAAGCTCGTAGC

Table 2. Effects of external nitrogen (N) and IAA application on the N content in plants

Cultivar	Treatment	N content (%) in leaf blade		N content (%) in leaf sheath		N content (%) in tiller node	
		3 d	5 d	3 d	5 d	3 d	5 d
Nanjing 44	control	3.24b	3.21b	1.98b	2.02b	2.18b	2.19b
	NH	3.99a	4.38a	2.48a	2.72a	2.57a	2.67a
	IAA	4.03a	4.51a	2.54a	2.86a	2.63a	2.75a

At 3 d and 5 d post-treatment, the N contents of leaf blades, leaf sheaths and tiller nodes were measured. NH: 40 mg L^{-1} N in the nutrient solution; IAA: 40 mg L^{-1} N in the nutrient solution and 50 mg L^{-1} IAA was sprayed on the plants; control: 10 mg L^{-1} N in the nutrient solution. Values within a column and for the same cultivar followed by different letters are significantly different at $P<0.05$.

tion of 40 mg L^{-1} N, the lengths and fresh weights of the tiller buds were significantly greater in the NH treatment group than in the control group (Fig. 1). The application of IAA completely reversed the promoting effect of the external 40 mg L^{-1} N on tiller bud growth. Similar to the control plants, 7 days after treatment, the tiller buds of the IAA treatment group remained in dormancy, and the lengths and fresh weights were significantly lower than those observed in the NH group 3 days after treatment.

Changes in the IAA and CTK levels in various organs
IAA

N and IAA significantly affected the IAA concentrations in the tiller nodes. NH treatment increased the IAA levels in tiller nodes compared with the control plants, and significant differences in IAA were observed on the first day post-treatment. External IAA further increased the IAA concentrations in the tiller nodes. The IAA levels in the tiller nodes of the IAA treatment group increased notably after treatment, reaching a peak at 1 d post-treatment and then decreasing gradually. The IAA levels in the tiller nodes of the IAA treatment group were significantly higher than in the NH and control treatment groups at 5 d post-treatment (Fig. 2A). The IAA levels in the tiller buds of the control plants remained nearly constant during the entire experiment. However, at 2 d post-treatment, the IAA levels in the tiller buds of both the NH and IAA treatment groups significantly increased compared with the control plants (Fig. 2B).

CTK

N significantly increased the Z+ZR and iP+iPR levels in tiller buds and tiller nodes. The Z+ZR and iP+iPR levels in the tiller buds and tiller nodes of the control plants remained nearly constant during the entire experiment. However, the Z+ZR and iP+iPR levels in the tiller buds and tiller nodes of the NH-treated plants were significantly higher than those observed in the control group on the first day post-treatment (Fig. 3). The external IAA treatment completely reversed the effect of external 40 mg L^{-1} N on the Z+ZR and iP+iPR levels in tiller buds and tiller nodes. At 7 d post-treatment, the Z+ZR and iP+iPR levels in the tiller buds and tiller nodes of the IAA treatment group showed no significant differences compared to the control treatment group and were significantly lower than those observed in the NH group on the first day after treatment.

Expression levels of OsIPT genes in various organs

To examine whether external N and IAA affect expression of the *OsIPT* genes in various organs, we examined the expression patterns of the *OsIPT* genes in the tiller nodes, tiller buds, and roots using real-time PCR. We found that there were differences in the expression patterns of the *OsIPT* genes in various organs (Fig. 4, 5 and 6). The external N treatment significantly increased the expression levels of *OsIPT2*, *OsIPT4* and *OsIPT7* in the tiller nodes, and the expression of these three genes in the tiller nodes was notably repressed at 6 h and 12 h post-treatment with external IAA. The expression levels of these three genes in the tiller nodes of the IAA and control treatment groups showed no significant differences but were significantly lower than those in the NH treatment group. The expression levels of *OsIPT1*, *OsIPT3*, *OsIPT5*, and *OsIPT8* in the tiller nodes were not affected by N, but IAA significantly inhibited the expression of *OsIPT3*, *OsIPT5*, and *OsIPT8* in the tiller nodes (Fig. 4). Contrary to the observed results in tiller nodes, N significantly repressed the expression of *OsIPT2*, *OsIPT4*, *OsIPT5*, *OsIPT7* and *OsIPT8* (but not *OsIPT1* or *OsIPT3)* in the tiller buds, but external IAA treatment notably increased the expression levels of these five genes. At 6 h and 12 h post-treatment, the expression levels of *OsIPT2*, *OsIPT4*, *OsIPT5*, *OsIPT7* and *OsIPT8* in the tiller nodes of the IAA and control treatment groups showed no significant differences but were significantly higher than those in the NH treatment group (Fig. 5). The expression patterns of the *OsIPT* genes in the roots were also affected by N and IAA. N significantly increased the expression levels of *OsIPT1*, *OsIPT3*, *OsIPT4*, *OsIPT7* and *OsIPT8* in the roots. The effects of IAA on the expression of the *OsIPT* genes in the roots were different. Compared to the control plants, external IAA increased the expression levels of *OsIPT1*, *OsIPT3*, *OsIPT5*, *OsIPT7* and *OsIPT8* in the roots, but IAA repressed the expression of *OsIPT4*, which was increased by N (Fig. 6).

Table 3. Correlation coefficients between the CTK contents in tiller nodes and tiller buds with the expression levels of the *OsIPT* genes in tiller nodes, tiller buds and roots

Correlation with		Z+ZR		iP+iPR	
		Tiller node	Tiller bud	Tiller node	Tiller bud
Tiller node	*OsIPT1*	-0.210	-0.333	-0.343	-0.378
	OsIPT2	0.997*	1.000**	1.000**	0.999**
	OsIPT3	0.152	0.278	0.288	0.323
	OsIPT4	0.981	0.998*	0.998*	1.000**
	OsIPT5	-0.082	0.046	0.057	0.094
	OsIPT7	0.997*	0.999**	0.998*	0.998*
	OsIPT8	0.340	0.457	0.467	0.500
Tiller bud	*OsIPT1*	0.796	0.677	0.774	0.765
	OsIPT2	-0.986	-0.999**	-1.000**	-1.000**
	OsIPT3	-0.869	-0.930	-0.926	-0.811
	OsIPT4	-0.952	-0.983	-0.985	-0.991
	OsIPT5	-0.924	-0.965	-0.968	-0.977
	OsIPT7	-0.984	-0.999**	-0.999**	-1.000**
	OsIPT8	-0.991	-1.000**	-1.000**	-0.999**
Roots	*OsIPT1*	0.010	-0.118	-0.129	-0.166
	OsIPT2	0.244	0.117	0.107	0.069
	OsIPT3	0.853	0.779	0.772	0.748
	OsIPT4	0.953	0.884	0.906	0.922
	OsIPT5	-0.283	-0.404	-0.413	-0.447
	OsIPT7	0.183	0.055	0.045	0.007
	OsIPT8	0.750	0.659	0.651	0.622

The expression levels of *OsIPT* genes in tiller nodes, tiller buds and roots at 6 h post-treatment, and the IAA, zeatin (Z) + zeatin reboside (ZR) and iP (isopentenyladenine) + iPR (iP riboside) concentrations in tiller buds and tiller nodes at 1 d post-treatment were used to perform a regression analysis to analyse the relationships between the expression levels of the *OsIPT* genes in various organs and changes in CTK levels in tiller buds and tiller nodes. Correlation coefficients (r) were calculated, and asterisks (* and **) represent statistical significance at the 0.05 and 0.01 probability levels, respectively (n=3).

Discussion

Relationship between N and tiller bud growth

In the present study, the external application of 40 mg L^{-1} N released the tiller buds from dormancy and significantly promoted growth, but external IAA reversed the effects of N on tiller bud growth (Fig. 1). This result was consistent with previous studies (Harrison and Kaufman, 1982; Jiang et al., 1994). Ding et al. (1995) suggest that external N regulates the N levels in rice plants and further regulates the growth of tiller buds. In the present study, we found that, compared with the control group, NH treatment significantly increased the N concentrations in the leaf blades, leaf sheaths, and tiller nodes; however, the N concentrations in the leaf blades, leaf sheaths, and tiller nodes were not significantly different between the NH and IAA treatment groups (Table 2). These results suggest that, at least in this study, the N concentration of plants may not be the key regulator of rice tiller bud growth and dormancy.

Relationship between hormones and tiller bud growth

Tillers are grain-bearing branches in monocotyledonous plants (Wang and Li, 2005). The number of rice tillers is dynamic and adjustable (Kariali and Mohapatra, 2007), and auxin and CTKs play important roles in regulating tiller growth. CTKs have a stimulatory effect on lateral bud growth. The external application of kinetin stimulates tiller bud growth in wheat (*Triticum aestivum* L.) (Langer et al., 1973). The germination and dormancy of ratooning buds are closely related to variations in endogenous isopentenyl adenosine (iPA) levels in rice plants (Li and Tang, 2002). The present study showed that external N increased the Z+ZR and iP+iPR levels in tiller buds and tiller nodes (Fig. 3), and the increase in Z+ZR and iP+iPR levels began before tiller bud germination. This indicates that, similarly to other plant species, CTKs play an important role in promoting rice tiller bud germination. In contrast to CTKs, auxin, which is derived from the shoot apex, inhibited the growth of lateral buds by polar transport (Dun et al., 2006). The removal or suppression of auxin activity released the lateral buds from apical control (Leopold, 1949), while the exogenous application of auxin inhibited the CTK-induced promotion of tiller bud growth (Harrison and Kaufman, 1982). In the present study, external IAA completely inhibited the growth of rice tiller buds, while external N notably promoted tiller bud growth. Compared with the NH group, IAA treatment significantly increased the IAA levels in tiller nodes and notably inhibited the increase in the Z+ZR and iP+iPR levels in tiller nodes and tiller buds. These effects may be the basis of the IAA-induced inhibition of rice tiller bud growth. Contrary to the results observed in tiller nodes, there was no significant difference in the IAA levels of tiller buds between the NH and IAA treatment groups. These results indicated that, similar to other plants (Li et al., 1995; Bangerth et al., 2000), auxin acts in the stem to inhibit tiller bud growth.

Relationship between IAA and CTKs in the regulation of rice tiller bud growth

It has been reported that the growth of lateral buds is controlled by crosstalk between auxin and cytokinins (Tanaka et al., 2006; Wang et al., 2006). A number of studies have supported that basipolar auxin may control CTK production in roots and possibly CTK delivery to lateral buds (Letham, 1994; Li et al., 1995; Bangerth et al., 2000), but Tanaka et al. (2006) indicated that one role of auxin in apical dominance is to repress the local biosynthesis of CTKs in the nodal stem in pea (*Pisum sativum* L.) plants. The adenosine phosphate-isopentenyltransferase (IPT) genes encode a key enzyme in CTK biosynthesis (Kakimoto, 2001; Takei et al., 2001). Sakamoto et al. (2006)

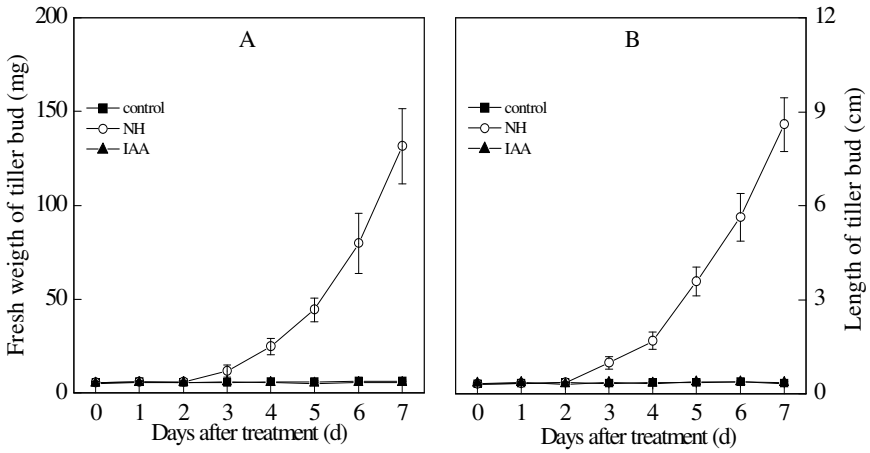

Fig 1. Effects of nitrogen (N) and IAA on the fresh weight (A) and length (B) of tiller buds. NH: 40 mg L^{-1} N in the nutrient solution; IAA: 40 mg L^{-1} N in the nutrient solution and 50 mg L^{-1} IAA was sprayed on the plants; control: 10 mg L^{-1} N in the nutrient solution. Vertical bars represent ± the standard error of the mean (n=120 for length and n=12 for fresh weight).

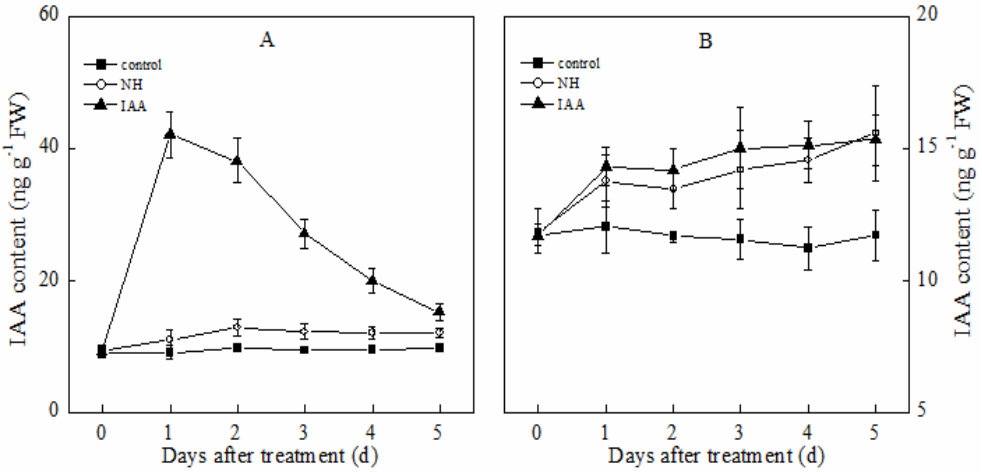

Fig 2. Effects of nitrogen (N) and IAA on IAA content in tiller nodes (A) and tiller buds (B). NH: 40 mg L^{-1} N in the nutrient solution; IAA: 40 mg L^{-1} N in the nutrient solution and 50 mg L^{-1} IAA was sprayed on the plants; control: 10 mg L^{-1} N in the nutrient solution. Vertical bars represent ± the standard error of the mean (n=6).

suggested that the products of *OsIPT1-8*, except *OsIPT6*, are involved in CTK biosynthesis in rice. We therefore determined the expression levels of *OsIPT1*, *OsIPT2*, *OsIPT3*, *OsIPT4*, *OsIPT5*, *OsIPT7* and *OsIPT8* in tiller buds, tiller nodes and roots to determine the main site of CTK synthesis during rice tiller bud growth. External N significantly increased the expression levels of *OsIPT2*, *OsIPT4* and *OsIPT7* in tiller nodes, but external IAA reversed this effect. Regression analysis indicated that the expression levels of *OsIPT2*, *OsIPT4* and *OsIPT7* in tiller nodes were positively and significantly correlated with the Z+ZR and iP+iPR levels in tiller nodes and tiller buds (Table 3). This result suggested that the Z+ZR and iP+iPR levels in tiller nodes and tiller buds may be regulated by *OsIPT2*, *OsIPT4* and *OsIPT7* in the tiller nodes and that the tiller nodes may be the main site of CTK synthesis, which promotes rice tiller bud growth. Contrary to the tiller nodes, IAA treatment significantly decreased Z+ZR and iP+iPR levels in tiller nodes and tiller buds, but the expression levels of *OsIPT1-8* (except *OsIPT6*) in the roots of the IAA treatment group were not significantly lower than those of the NH and control treatment groups. Regression analysis indicated that the expression levels of *OsIPT1-8* (except *OsIPT6*) in roots were not significantly correlated with Z+ZR and iP+iPR levels in tiller nodes and tiller buds. This result suggests that the roots may not be the main site of CTK synthesis for rice tiller bud growth (Table 3). These results are similar to the results of Tanaka et al. (2006) in pea (*Pisum sativum* L.) plants. The expression levels of the *OsIPT* genes in tiller buds were also affected by N and IAA. With the exception of *OsIPT1* and *OsIPT3*, N repressed the expression of the *OsIPT* genes; however, external IAA application inhibited this effect. Miyawaki et al. (2004) found that CTKs repress the expression of the *IPT* gene in *Arabidopsis thaliana* Heynh. Based on these results, we suggest that external N enhances the expression of the *OsIPT* genes and stimulates the synthesis of CTKs in tiller nodes; the CTKs are then delivered to tiller buds, and negative feedback regulates the expression of the *OsIPTs* in tiller buds. Because IAA inhibits the synthesis of CTKs in tiller nodes, little or no CTKs are delivered to tiller buds to negatively regulate the expression of the *OsIPT* genes in tiller buds; therefore, the expression levels of the *OsIPT* genes in tiller buds of the IAA treatment group were significantly higher than

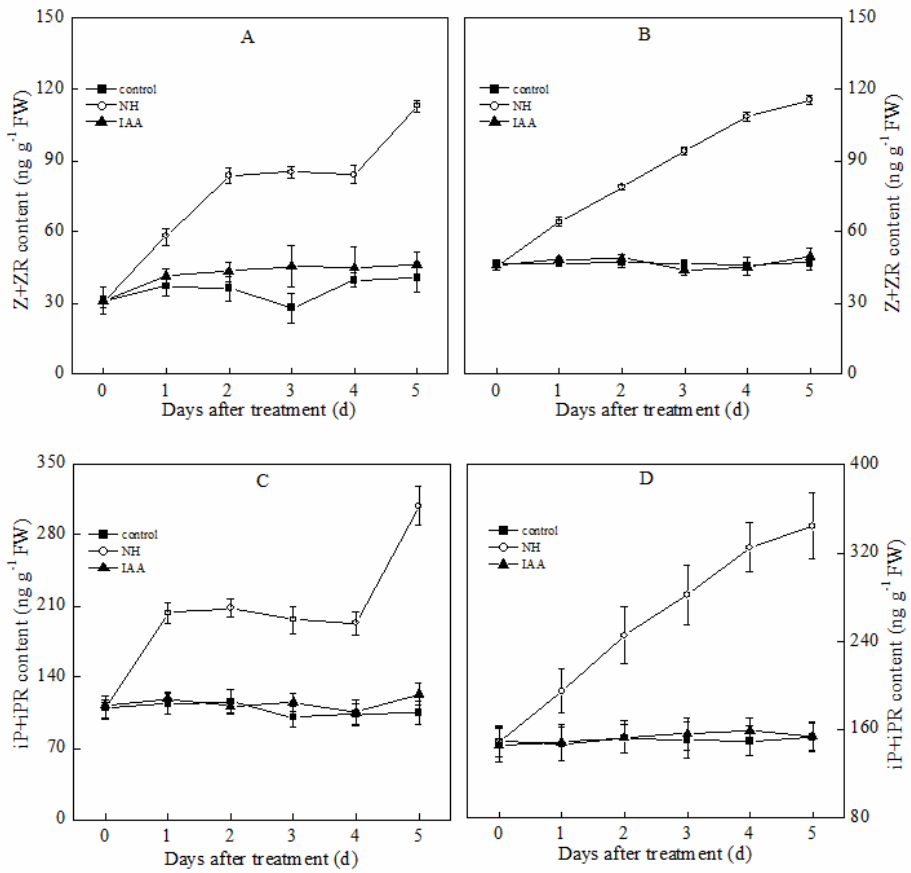

Fig 3. Effects of nitrogen (N) and IAA on Z+ZR and iP+iPR contents in tiller nodes (A and C) and tiller buds (B and D). NH: 40 mg L^{-1} N in the nutrient solution; IAA: 40 mg L^{-1} N in the nutrient solution and 50 mg L^{-1} IAA was sprayed on the plants; control: 10 mg L^{-1} N in the nutrient solution. Z: zeatin; ZR: zeatin riboside; iP: isopentenyladenine; iPR: iP riboside. Vertical bars represent ± the standard error of the mean (n=6).

those of the NH treatment group. However, this change was not observed for the expression of *OsIPT1* or *OsIPT3*, which suggests that these genes are constitutively expressed or regulated by another mechanism (Sakamoto et al., 2006). Based on these findings, we conclude that the CTKs that promote tiller bud growth are biosynthesised mainly in the tiller nodes and that there is little or no contribution from the roots. N and IAA regulate the expression levels of the *OsIPT* genes and inhibit the biosynthesis of CTKs in the tiller nodes, which, as a result, regulates the growth of rice tiller buds. This conclusion agrees with Tanaka et al. (2006) and increases our understanding of the mechanisms of tiller bud growth in rice plants. In this study, we found that there were differences in the expression patterns of the *OsIPT* genes. Miyawaki et al. (2004) found that auxin positively regulates the expression of *AtIPT5* and *AtIPT7* in *Arabidopsis* roots. In the present study, we found that IAA positively regulated the expression of *OsIPT1*, *OsIPT3*, *OsIPT5*, *OsIPT7* and *OsIPT8* in roots. In contrast, IAA notably repressed the expression of *OsIPT2*, *OsIPT3*, *OsIPT4*, *OsIPT5*, *OsIPT7* and *OsIPT8* in nodes. These results suggest that auxin is negatively and positively involved in CTK biosynthesis and that the biosynthesis of CTKs is complicated and different in each organ and species. Recent studies have suggested that a novel hormone is involved in inhibiting the outgrowth of axillary buds using a series of mutants, including *ramosus* (*rms*) of pea (*Pisum sativum*), *more axillary growth* (*max*) of Arabidopsis, and *dwarf* (*d*) of rice (*Oryza sativa*) (Stirnberg et al., 2002; Sorefan et al., 2003; Ishikawa et al., 2005; Arite et al., 2007). In the proposed biosynthetic pathway, *MAX4*, *RMS1* and *D10* encode carotenoid cleavage dioxygenase 8 (CCD8) and might catalyse sequential carotenoid cleavage reactions. Moreover, the expression of *RMS1* requires auxin (Sorefan et al., 2003; Foo et al., 2005). These results demonstrate that auxin also controls the outgrowth of tiller buds in rice through the synthesis of a branch inhibitor and suggest that the various signals that regulate rice tiller bud growth do not operate in isolation but rather as parts of a network or matrix of interacting pathways. Further research on the mechanism of single signals and the cross-talk between signals will be helpful in clarifying the mechanism of tiller bud growth in rice.

Materials and methods

Rice plant growth and treatments

Nanjing 44, a japonica cultivar, was used. The seedlings were planted in seedbeds on May 20, 2009. The organic C concentration in the soil was 13.4 g kg^{-1}, and the available nitrogen, phosphorus and potassium concentrations in the soil were 52.1, 9.07 and 77.3 mg kg^{-1}, respectively. The plants were transplanted at the six-leaf stage into 20 L plastic pots that contained a nutrient solution essentially identical to that described by Yoshida S (1975), which consisted of 10 mg L^{-1}

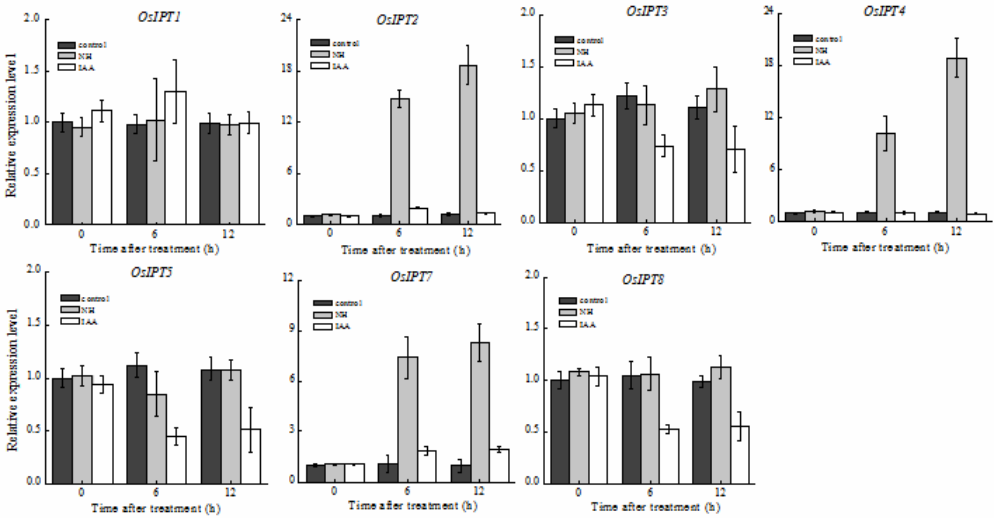

Fig 4. Effects of nitrogen (N) and IAA on the expression levels of the *OsIPT* genes in tiller nodes. Total RNA was isolated from the tiller nodes. β-Actin was used as a loading control. The value obtained from the control treatment at 0 h after treatment was arbitrarily set to 1.0. NH: 40 mg L^{-1} N in the nutrient solution; IAA: 40 mg L^{-1} N in the nutrient solution and 50 mg L^{-1} IAA was sprayed on the plants; control: 10 mg L^{-1} N in the nutrient solution. Vertical bars represent ± the standard error of the mean (n=9).

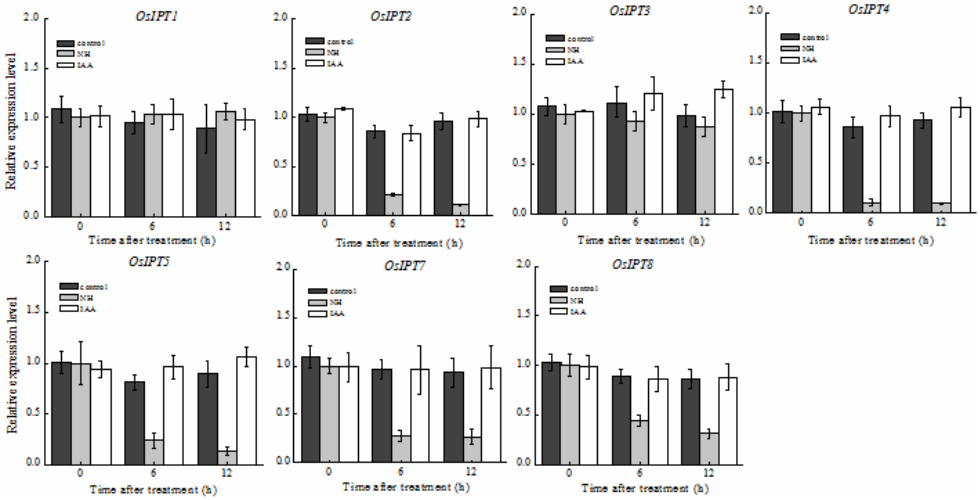

Fig 5. Effects of nitrogen (N) and IAA on the expression levels of the *OsIPT* genes in tiller buds. Total RNA was isolated from the tiller buds. β-Actin was used as a loading control. The value obtained from the control treatment at 0 h after treatment was arbitrarily set to 1.0. NH: 40 mg L^{-1} N in the nutrient solution; IAA: 40 mg L^{-1} N in the nutrient solution and 50 mg L^{-1} IAA was sprayed on the plants; control: 10 mg L^{-1} N in the nutrient solution. Vertical bars represent ± the standard error of the mean (n=9).

$NaH_2PO_4 \cdot 2\ H_2O$, 40 mg L^{-1} K_2SO_4, 40 mg L^{-1} $CaCl_2$, 40 mg L^{-1} $MgSO_4 \cdot 7\ H_2O$, 0.5 mg L^{-1} $MnCl_2 \cdot 4\ H_2O$, 0.05 mg L^{-1} $(NH_4)_6 \cdot Mo_7O_{24} \cdot 2\ H_2O$, 0.2 mg L^{-1} H_3BO_3, 0.01 mg L^{-1} $ZnSO_4 \cdot 7\ H_2O$, 0.01 mg L^{-1} $CuSO_4 \cdot 5\ H_2O$, 2.0 mg L^{-1} $FeCl_2 \cdot 6\ H_2O$ and 10 mg L^{-1} N (the concentration of N was regulated by NH_4NO_3) to sustain the growth of the rice plants. When the rice seedlings developed eight leaves on their main stems, they were divided into the following three treatment groups: (1) 10 mg L^{-1} N in the nutrient solution (control treatment), (2) 40 mg L^{-1} N in the nutrient solution (NH treatment), (3) 40 mg L^{-1} N in the nutrient solution and 50 mg L^{-1} IAA was sprayed on the plants (5 ml per plant; IAA treatment). Twenty pots were used for each treatment with forty plants in each pot. Each treatment consisted of three replicates with a completely randomised design.

Sampling and measurements

Tiller bud growth

Forty tiller buds that were located at the axils of the fifth leaves (from the bottom) on the main stems were sampled every day for 7 days after the treatments, and their lengths were measured. The buds were then divided into groups of 10, and the weight of each group was obtained.

Endogenous hormones in tiller buds and tiller nodes

About 0.2 g of tiller buds and 0.5 g of tiller nodes were sampled, and their endogenous hormone concentrations were measured. The IAA and CTKs were extracted and purified

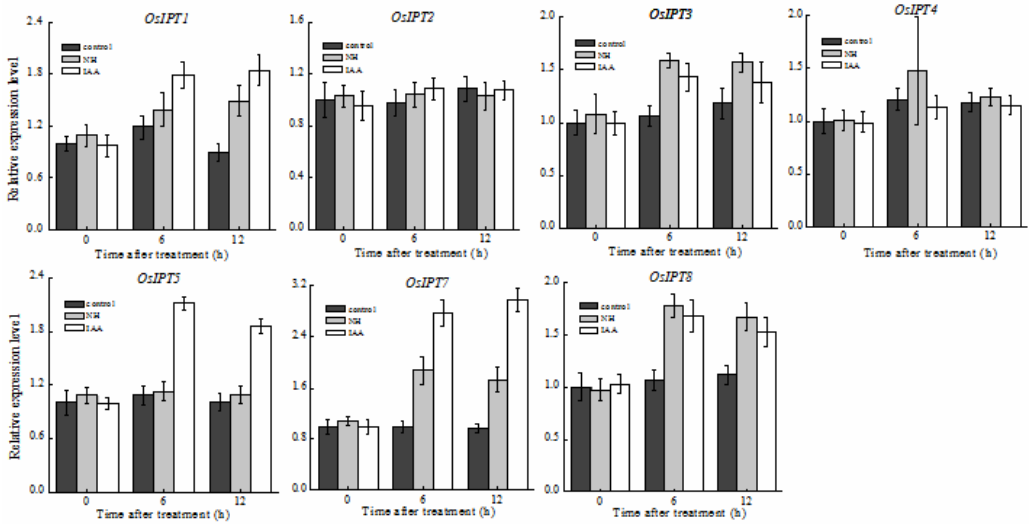

Fig 6. Effects of nitrogen (N) and IAA on the expression levels of the *OsIPT* genes in the roots. Total RNA was isolated from the roots. β-Actin was used as a loading control. The value obtained from the control treatment at 0 h after treatment was arbitrarily set to 1.0. NH: 40 mg L^{-1} N in the nutrient solution; IAA: 40 mg L^{-1} N in the nutrient solution and 50 mg L^{-1} IAA was sprayed on the plants; control: 10 mg L^{-1} N in the nutrient solution. Vertical bars represent ± the standard error of the mean (n=9).

according to Yang et al. (2001). In the present study, two types of CTKs were measured: Z+ZR and isopentenyladenine (iP) + iP riboside (iPR). The samples were ground using a mortar and pestle (on ice) with 5 mL of 80% (v/v) methanol as the extraction medium and 1 mmol L^{-1} of butylated hydroxytoluene (BHT) as an antioxidant. The methanolic extracts were incubated at 4ºC for 4 h and centrifuged at *10,000* × *g* for 15 min at the same temperature. The supernatants were passed through Chromosep C18 columns (C18 Sep-Park Cartridge, Waters Corp., Millford, MA, USA), which were pre-washed with 10 mL of 100% methanol and 5 mL of 80% methanol. The hormone fractions were dried under N_2 and dissolved in 1 mL phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20 and 0.1% (w/v) gelatin (pH 7.5) for an enzyme-linked immunosorbent assay (ELISA). Mouse monoclonal antigens and antibodies against Z+ZR, iP+iPR, and IAA and immunoglobulin G-horseradish peroxidase (IgG-HRP) used with ELISA were manufactured by the Phytohormones Research Institute, China Agricultural University. Quantification of Z+ZR, iP+iPR, and IAA by ELISA was performed as described previously (Yang et al., 2001). The recovery rates of IAA, Z+ZR, and iP+iPR were 83.2±5.1%, 89.9±2.9%, and 84.6±5.1%, respectively.

Expression levels of the OsIPT genes in tiller buds, tiller nodes, and roots

Total RNA was prepared using the E.Z.N.A.® Plant RNA Kit (Omega Bio-Tek, Inc., USA). The reverse transcription reaction was carried out using the PrimeScript™ RT reagent kit (Takara, Kyoto, Japan) with oligo dT and random 6-mer primers. The resulting cDNA sample was used as the template for PCR, with 40 cycles of 30 s at 94°C, 30 s at 55-57°C, and 1 min at 72°C. The PCR products were separated by agarose gel electrophoresis. Quantitative real-time PCR was performed with an ABI 7300 system and SYBR Premix Ex Taq™ (Takara, Kyoto, Japan) according to the manufacturer's protocol. The sequences of the PCR primers were the same as those used by Sakamoto et al. (2006) and are listed in Table 1.

Nitrogen content in plants

Ten plants for each treatment group were sampled at 3 d and 5 d post-treatment. The leaf blades, leaf sheaths, and tiller nodes were removed from the plants and placed into different sample bags. All of the fresh samples were placed in a forced-air oven for 1 hour at 105ºC and then were incubated at 75ºC until they reached a constant weight. The dried samples were milled to pass through a 1 mm screen and stored in plastic bags. The total plant N was determined by the Kjeldahl method; 0.5 g of dry sample was digested with 3 g of a catalyst composed of 3:1 K_2SO_4:$CuSO_4$ for at least 6 hours at 375ºC with 10 ml of H_2SO_4 and 2 ml of H_2O_2. The N content was measured according to Bao (2007).

Statistical analysis

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

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