

Characteristic and Expression Analysis of a Metallothionein Gene, *OsMT2b*, Down-Regulated by Cytokinin Suggests Functions in Root Development and Seed Embryo Germination of Rice^{1[OA]}

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Metallothioneins (MTs) are low molecular mass and cysteine-rich metal-binding proteins known to be mainly involved in maintaining metal homeostasis and stress responses. But, their functions in higher plant development are scarcely studied. Here, we characterized rice (*Oryza sativa*) *METALLOTHIONEIN2b* (*OsMT2b*) molecularly and found that its expression was down-regulated by cytokinins. *OsMT2b* was preferentially expressed in rice immature panicles, scutellum of germinating embryos, and primordium of lateral roots. In contrast with wild-type plants, *OsMT2b*-RNA interference (RNAi) transgenic plants had serious handicap in plant growth and root formation, whereas *OsMT2b*-overexpressing transformants were dwarfed and presented more adventitious roots and big lateral roots. The increased cytokinin levels in RNAi plants and decreased cytokinin levels in overexpressing plants were confirmed by high-performance liquid chromatography quantitative analysis in the roots of wild-type and transgenic plants. In RNAi plants, localization of isopentenyladenosine, a kind of endogenous cytokinin, in roots and germinating embryos expanded to the whole tissues, whereas in overexpressing plants, the isopentenyladenosine signals were very faint in the vascular tissues of roots and scutellum cells of germinating embryos. In vitro culture of embryos could largely resume the reduced germination frequency in RNAi plants but had no obvious change in overexpressing plants. Taken together, these results indicate a possible feedback regulation mechanism of *OsMT2b* to the level of endogenous cytokinins that is involved in root development and seed embryo germination of rice.

Metallothioneins (MTs) were discovered in 1957 by Margoshes and Vallee and identified as low molecular mass (4–8 kD) proteins that contain Cys-rich N and C termini (Vallee, 1991). MT genes have been widely found in animals, plants, and prokaryotes. Not like the primary mammalian MTs that contain 20 highly conserved Cys residues (Klassen et al., 1999), most plant MTs have distinct arrangements of Cys residues (Robinson et al., 1993), which suggests that they may be different not only in sequences but also in functions. According to the arrangement of Cys residues, the MTs of higher plant are further classified into four types that have diverse patterns of expression (Cobbett and Goldsbrough, 2002).

Recently, the studies of MTs in animals and plants were mainly focused on the roles in maintaining homeostasis of essential metals and metal detoxification. For plant, MTs can efficiently bind metals (Murphy

et al., 1997; Leszczyszyn et al., 2007) and some of them are transcriptionally regulated by metals; they are also thought to play important roles in metal tolerance, detoxification, and homeostasis in plants (Cobbett and Goldsbrough, 2002; Hall, 2002; Hassinen et al., 2007). On the other hand, there were also intensive studies for the responses of MTs to stress conditions, such as draft and reactive oxidant species (Akashi et al., 2004; Wong et al., 2004).

In many species of higher plants, MTs were reported to express specifically in organs. For example, the rice (*Oryza sativa*) *ricMT* gene was highly expressed in stem nodes (Yu et al., 1998); in pineapple (*Ananas comosus*), the expression of *PaMT* genes was confined to specific stages of fruit development (Moyle et al., 2005). In those cases, the differential expressions of MT genes strongly imply that MTs may have functions during plant development. However, the studies of MT functions in these reports were largely involved in metal homeostasis. Therefore, the functions of MTs in plant development are still unclear. In this article, we reported that *O. sativa* *METALLOTHIONEIN2b* (*OsMT2b*) has roles in the development of lateral roots and germination of seed embryos by having an effect on the endogenous cytokinin level.

Cytokinin is a vital phytohormone controlling various events of plant growth and development such as cell division, seed germination, root elongation, leaf senescence, and the transition from vegetative growth to reproductive development. But compared with

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research on other plant hormones, such as ethylene and abscisic acid (ABA), the studies on cytokinin were delayed (Eckardt, 2003). The delay might be partially due to the broad responses of whole plant to cytokinin, which frequently occur in complex associations with other hormones, making it difficult to elicit the results that are due specifically to cytokinins (Oka et al., 2002). In higher plants, cytokinins in active forms are isopentenyladenine and trans-zeatin (TZ; Yamada et al., 2001). When Rib is attached at the N⁹ atom of the A ring in isopentenyladenine, an inactive intermediate, isopentenyladenosine (iPA), is formed. In our studies, TZ was used as exogenous active cytokinin in treatments, and iPA was analyzed as endogenous cytokinin in organs and tissues of rice.

As the most important agronomical plant, rice is recognized as a useful experimental model of monocot to study the mechanism of gene expression (Kurata et al., 2005). In rice, 23 A-type and six B-type response regulator (RR) genes had been identified (Jain et al., 2006; Ito and Kurata, 2006). Among them, the *OsRR6*-overexpression transformants had an increased content of TZ-type cytokinins and showed dwarf phenotypes with poorly developed root systems (Hirose et al., 2007). And in the search for cytokinin synthesis enzymes, eight genes for adenosine phosphate isopentenyltransferase, which catalyzes the rate-limiting step of cytokinin biosynthesis, were identified in rice (Sakamoto et al., 2006). Besides that, *Gn1a* was found as a gene for cytokinin oxidase (*OsCKX2*), which catalyzes the irreversible degradation of cytokinin (Ashikari et al., 2005). Compared with the researches on cytokinin receptors and synthesis, much less is known about the genes regulated by cytokinin. Up to now, only the gene *NtMT-L2* was reported to be up-regulated by cytokinin in copper stress (Thomas et al., 2005). Except for that, other reports about the function of MTs in relation to cytokinins were not seen.

Here, we reported the characterization of a type-2 MT, *OsMT2b*, and its expression patterns and functions in rice. The results showed that *OsMT2b* transcript levels were regulated by cytokinin, and abnormal expression of *OsMT2b* affected the endogenous cytokinin levels in organs and tissues by the analysis of *OsMT2b*-RNAi and *OsMT2b*-overexpressing transgenic plants of rice. Considering the interrelation of *OsMT2b* and cytokinin as well as the phenotypes of roots and germinating embryos in transgenic plants, it indicates that *OsMT2b* plays important roles in initiation of lateral root and seed embryo germination.

RESULTS

Analysis of *OsMT2b* in the MT Gene Family of Higher Plants

To study the molecular events of rice early embryo development, suppression subtractive hybridization was performed by using complementary DNA

(cDNA) of rice embryos (*indica* 'Jiayu 948') at 5 to 7 d after pollination (DAP) as tester and cDNA of the embryos at 15 to 17 DAP as driver (J. Yuan, Y. Ren, and J. Zhao, unpublished data). One clone of the subtracted cDNA library showed high similarities to the cDNA of *OsMT2b* (U77294), and the full cDNA clone was denominated to *OsMT2bL* (*O. sativa* METALLOTHIONEIN2b LIKE) and submitted to GenBank with accession number EF584509. The only difference of amino acid sequence between *OsMT2bL* and *OsMT2b* is Ser in *OsMT2b* and Gly in *OsMT2bL* at the position of the 21st amino acid. We chose *OsMT2b* for further study.

Multiple sequence alignment was conducted among *OsMT2b*, *OsMT2bL*, and other proteins, including *AtMT2b* (NP195858) from *Arabidopsis thaliana*, *AbMT2b* (CAC40742) from *Atropa belladonna*, *HvMTL* (BAA23628) from *Hordeum vulgare* and *ZeaMTL* (CAA57676) from maize (*Zea mays*; Fig. 1A). Type-2 MTs contain two Cys-rich domains separated by a space of approximately 40 amino acid residues. The sequences of the N-terminal domain of this type of MT are highly conserved (MSCCGGNCGCGS), and the C-terminal domain contains three Cys-Xaa-Cys motifs (Fig. 1A). The analysis shows that homologous genes of *OsMT2b* exist ubiquitously in monocotyledon and dicotyledon. It suggests that this gene family may play important roles in higher plants.

There are seven members of this protein family in *Arabidopsis* and 15 members in rice. By phylogenetic analysis, the protein members are divided into several small subgroups (Fig. 1B). Some subgroups contain both rice and *Arabidopsis* representatives. For instance, *OsMT3a*, *OsMT3b*, and *OsMT3c* are clustered with *AtMT3*. Similarly, *OsMT4a* is clustered with *AtMT4a* and *AtMT4b*.

The 5'-flanking region of *OsMT2b*, including a region of about 961 bp upstream of the translation initiation codon ATG, is derived from the National Center for Biotechnology Information database and analyzed as a promoter (Fig. 1C). There are five ARABIDOPSIS RESPONSE REGULATOR1 (ARR1)-binding elements (ARR1AT) with core sequence 5'-AGATT-3' in the promoter region. One ARR1AT element was found in the promoter of the *O. sativa* NONSYMBIOTIC HAEMOGLOBIN2 (*OsNSHB2*) gene, and the mutation of this element abolished promoter activation in response to cytokinin (Ross et al., 2004). The presence of these elements suggests that the *OsMT2b* gene is probably regulated by cytokinin. The predicted transcription initiation site of *OsMT2b* is located at 88 bp upstream of ATG, and is designated as +1 (Fig. 1C). The comparison of the full-length cDNA sequences with the corresponding genomic DNA sequences showed that the coding sequence (CDS) of the *OsMT2b* gene has three exons that are disrupted by two introns (Fig. 1C).

Temporal and Spatial Expression Patterns of *OsMT2b*

The expression levels of *OsMT2b* were analyzed using the Massively Parallel Signature Sequencing

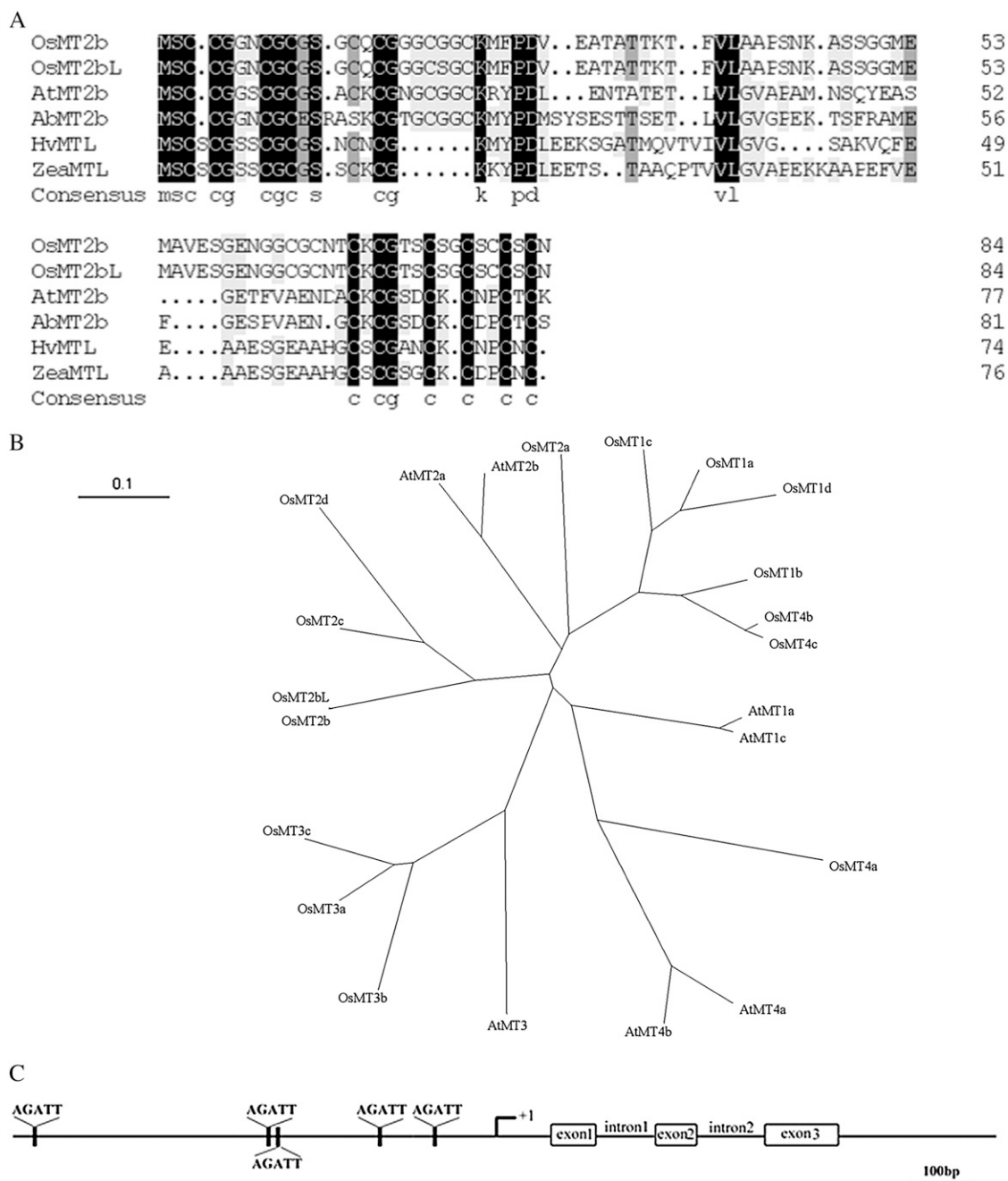


Figure 1. The analysis of the *OsMT2b* gene and its protein sequence. A, Protein sequence multiple alignment of *OsMT2b*. *OsMT2b* (U77294) and *OsMT2bL* (EF584509) are from rice, *AtMT2b* (NP195858) is from Arabidopsis, *AbMT2b* (CAC40742) is from *A. belladonna*, *HvMTL* (BAA23628) is from *H. vulgare*, and *ZeaMTL* (CAA57676) is from *Z. mays*. B, Unrooted dendrogram of MT proteins in Arabidopsis (*AtMT1a*–*AtMT4b*) and rice (*OsMT1a*–*OsMT4c*). Bar represents 0.1 amino acid substitutions per site. C, Promoter structure and exon-intron organization of the *OsMT2b* gene. AGATT is an ARR1-binding element (cytokinin-regulated transcription factor, ARR1); +1 is the predicted transcription initiation site. Boxes indicate three exon regions; lines between two boxes represent the intron regions. Bar = 100 bp.

(MPSS) database that was constructed by Nakano et al., 2006. A practical tag, GATCTCATCATGTAICTC, was available for *OsMT2b* after the BLAST in the MPSS database. The expression levels of *OsMT2b* were measured in transcripts per million of mRNA in the

selected rice organs and tissues (Fig. 2A). The results indicate that the expression level of *OsMT2b* is higher in immature panicles and seeds at 3 d after germination (DAG) than in ovaries and stigmas, young roots, and mature stems, but, hardly detected in leaves and

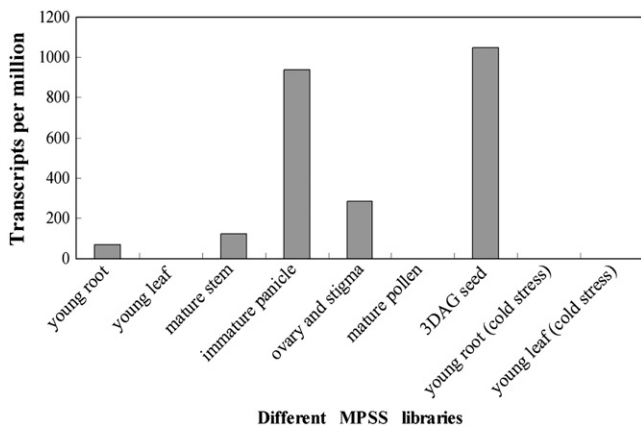


Figure 2. A, MPSS analysis of the *OsMT2b* expression profile in the different developmental stages of rice organs and tissues. The expression level of individual *OsMT2b* is plotted in transcripts per million of mRNA.

mature pollens; its expression level reduces in the roots of 2-week-old seedlings treated at 4°C cold, but still does not change in the leaves of the same treating condition.

To further study the spatial expression pattern of *OsMT2b*, we performed GUS staining in a T-DNA insert mutant 03Z11AN35 that has a GAL4-UAS enhancer trap system located between the promoter and the CDS of *OsMT2b* (Fig. 3A). In this system, the GAL4-UAS is made up of *GAL4/VP16*, a fusion gene of yeast transcriptional activator GAL4 DNA-binding domain with the herpes simplex virus VP16 activation domain, and the upstream activator sequence with six repeats of UAS (6 × UAS). The following part of GAL4-UAS is *GUS*, a β -glucuronidase gene. The enhancer trap system has a higher probability of detecting the expression of gene whose promoter locates near the trap system, and hence is possibly more effective for identifying gene functions in reverse genetic studies (Greco et al., 2001; Wu et al., 2003; Zhang et al., 2006).

The histochemical staining of GUS in different developmental stages of rice embryos showed that the activity of GUS enhanced by the promoter of *OsMT2b* was strong in the top of the scutellum at 7 DAP (Fig. 3C), but almost not detected in the embryos at 5 and 10 DAP (Fig. 3, B and D). In the germinating embryos, GUS staining signal was intense in the scutellum (arrow) and the cap of the radicle at 2 DAG (arrowhead; Fig. 3, E and F), which accorded with the result of MPSS that the *OsMT2b* transcript level was high in seeds at 3 DAG. In the embryos at 4 DAG, with shoots and roots emerging out, the GUS signal still concentrated in rice scutellum, and also appeared in the vascular bundle of shoot (arrowhead; Fig. 3G), which suggests that *OsMT2b* may play a role in the development of rice embryo scutellum.

The GUS signal in immature panicles verified the second expression peak of *OsMT2b* in MPSS analysis.

In florets of a 1-cm-long panicle, the GUS staining located in the pedicel and the basement of glumes (Fig. 3H). In florets of an 8-cm-long panicle, besides the positions described above, GUS expressions were also detected in the anthers and pistils (Fig. 3I). The signal was intense in the basal parts of ovaries before pollination (Fig. 3J), and in the basal parts of stigmas and ovaries after pollination (Fig. 3K).

To locate *OsMT2b* precisely at rice roots as described in MPSS results, GUS staining was performed. The strong signals were detected predominantly in the basal parts of lateral roots and nearby vascular cylinders of roots (Fig. 3, L and M). In the part of the root with lateral root primordium, the GUS staining mainly focused in root primordium, the passage cell of endodermis, and cells of pericycle and phloem, but not in the cells of epidermis, cortex, endodermis, and vessels (Fig. 3, N and O).

Cytokinin Regulates the Transcript Levels of *OsMT2b*

To investigate whether metal ions, hormones, and stress-related factors are involved in the regulation of *OsMT2b*, we harvested rice seedlings treated with various factors and detected the transcriptional level of *OsMT2b* by real-time quantitative reverse transcription (RT)-PCR. The expression levels of *OsMT2b* were markedly increased in the roots treated with iron (Fe), zinc (Zn), and indole-3-acetic acid (IAA), but observably decreased in the treatments of copper (Cu), 6-benzyladenine (6-BA), kinetin (KT), and NaCl (Fig. 4A). In shoots, its expression was elevated in the treatments of manganese (Mn), but remarkably decreased in the treatment of 6-BA and KT (Fig. 4B). Besides that, 4°C cold treatment also obviously reduced the transcript level of *OsMT2b* in roots and shoots, which is consistent with the results of MPSS.

In view of the intensive response of *OsMT2b* transcripts to the treatment of cytokinin, we chose zeatin, a kind of monocotyledonous endogenous cytokinin, to treat rice seedlings and assayed its effect on rice roots by real-time quantitative RT-PCR. The result showed that the *OsMT2b* expression level started to distinctly decrease by the treatment of zeatin from 1 to 20 μ M and reached the lowest value in 10 μ M (Fig. 4C).

To further confirm the down-regulation of *OsMT2b* transcripts by zeatin, we performed GUS activity assay in the roots of GAL4-UAS rice seedlings treated with zeatin. Quantitative analysis showed the alteration of *GUS* translation levels enhanced by the promoter of *OsMT2b* was dependent on the change of zeatin concentration. GUS activities decreased in the treatments of 1 to 20 μ M zeatin, and yet reached the lowest value in 10 μ M zeatin (Fig. 4D), which was accorded with the alteration of *OsMT2b* transcript levels.

Based on the analysis of real-time quantitative RT-PCR in wild-type rice and the assay of GUS activity in GAL4-UAS rice, the results indicate that the expression of *OsMT2b* is down-regulated by cytokinin.

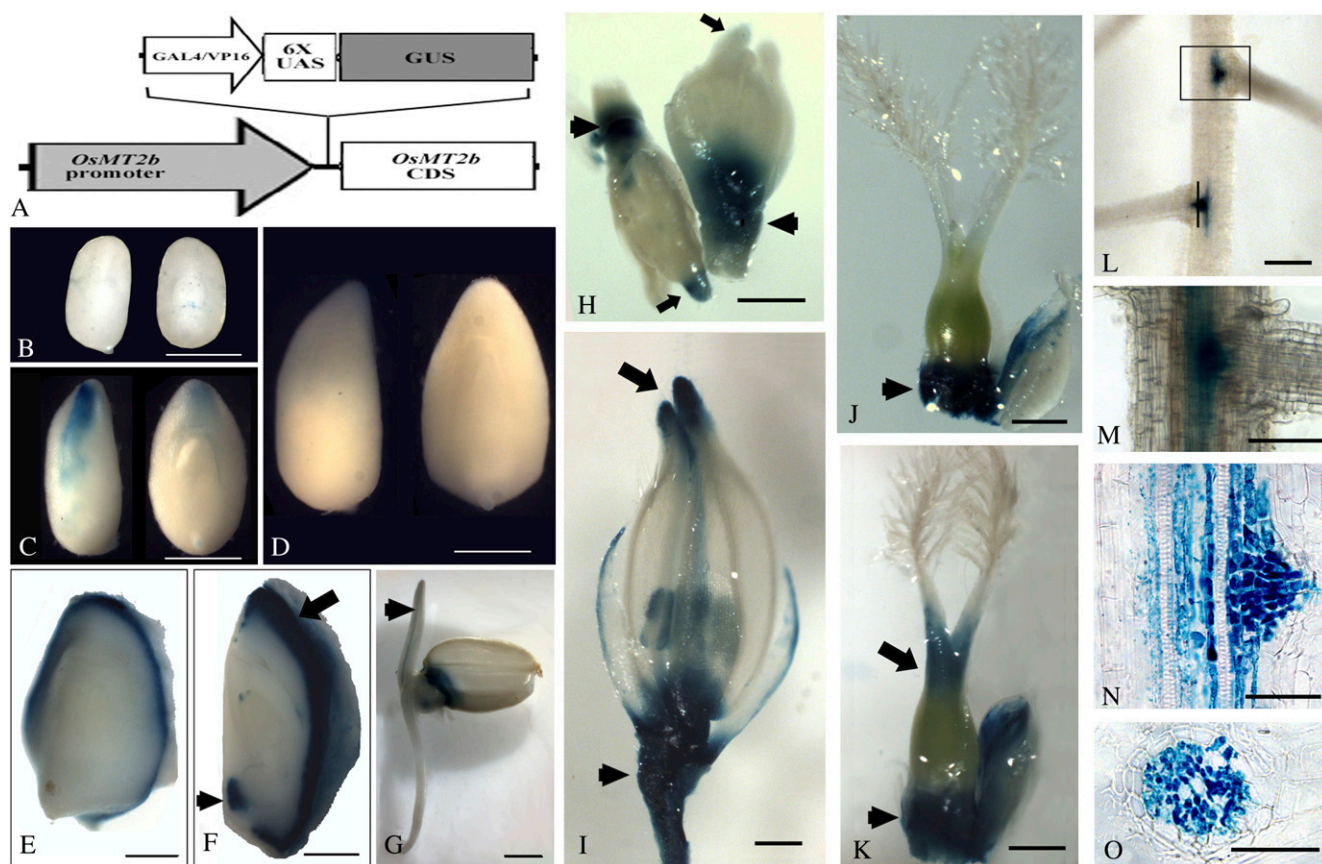


Figure 3. Histochemical localization of *OsMT2b* promoter-enhanced GUS activity in the different organs and tissues of rice GAL4-UAS plants. A, Schematic representation of the *OsMT2b*::GUS construct in GAL4-UAS plants. B to D, *OsMT2b* expression in seed embryos at 5, 7, and 10 DAP, respectively, by histochemical staining of GUS (left, dorsal view of embryo; right, ventral view of embryo). E to K, The GUS stain in seed embryos at 2 DAG (E, ventral view of embryo; F, longitudinal section of embryo) and at 4 DAG (G) of GAL4-UAS plants, in florets of 1-cm-long (H) and 8-cm-long (I) panicles, and in pistils before (J) and after (K) pollination. Arrow and arrowhead indicate the scutellum and root cap in F, nothing and vascular bundle of shoot in G, the top and base ends of glumes in H and I, the basal parts of the stigma and ovary in J and K, respectively. L, GUS expression in rice root. M, A magnified image of the lateral root in a square of Figure 3L. N, The longitudinal section of the lateral root primordium. O, A transection of the lateral root where it is indicated by a vertical line in Figure 3L. Bars are 500 μm in B to F and H to K; 2000 μm in G; 200 μm in L; 100 μm in M; and 50 μm in N and O.

OsMT2b RNAi and Overexpressing Transgenic Plants Exhibit Developmental Alterations

To examine whether the expression change of *OsMT2b* has an effect on the development of rice plants, we analyzed mature transgenic plants of *OsMT2b*-RNAi and *OsMT2b*-overexpressing (Fig. 5A). The RNAi construct was made by cloning a DNA fragment containing two full-length *OsMT2b* cDNAs, in inverse orientation and separated by a GFP sequence linker, into the p2K1+ vector under the control of the maize *Ubg1* promoter (Fig. 5B). In the overexpression construct, a full-length cDNA of *OsMT2b* was subcloned into the p2K1+ vector under the control of the maize *Ubg1* promoter (Fig. 5C). Real-time quantitative RT-PCR analysis confirmed the alterations of *OsMT2b* expression in the leaves and roots of RNAi and overexpressing transgenic plants (Fig. 5D).

As compared with the wild-type plants, the overexpressing plants had more tillers but were a little shorter, whereas the large quantity of RNAi transgenic plants (RL, about 75%) had almost no tillering and were extremely stunted (Fig. 5, A and E). The small quantity of RNAi transformants (RS, about 25%) with slight decline of *OsMT2b* expression could develop into mature plants but were shorter than mature overexpressing plants. The RL plants with significant reduction of *OsMT2b* expression suffered severe developmental defect and eventually died. For this reason, materials for assaying of RNAi plants in the following experiments were carried from the RS transformants. The results of real-time quantitative RT-PCR confirmed that *OsMT2b* transcript levels reduced 67.2% in leaves and 61.9% in roots of the RS plants, and reduced 93.3% in leaves and 93.6% in roots of the

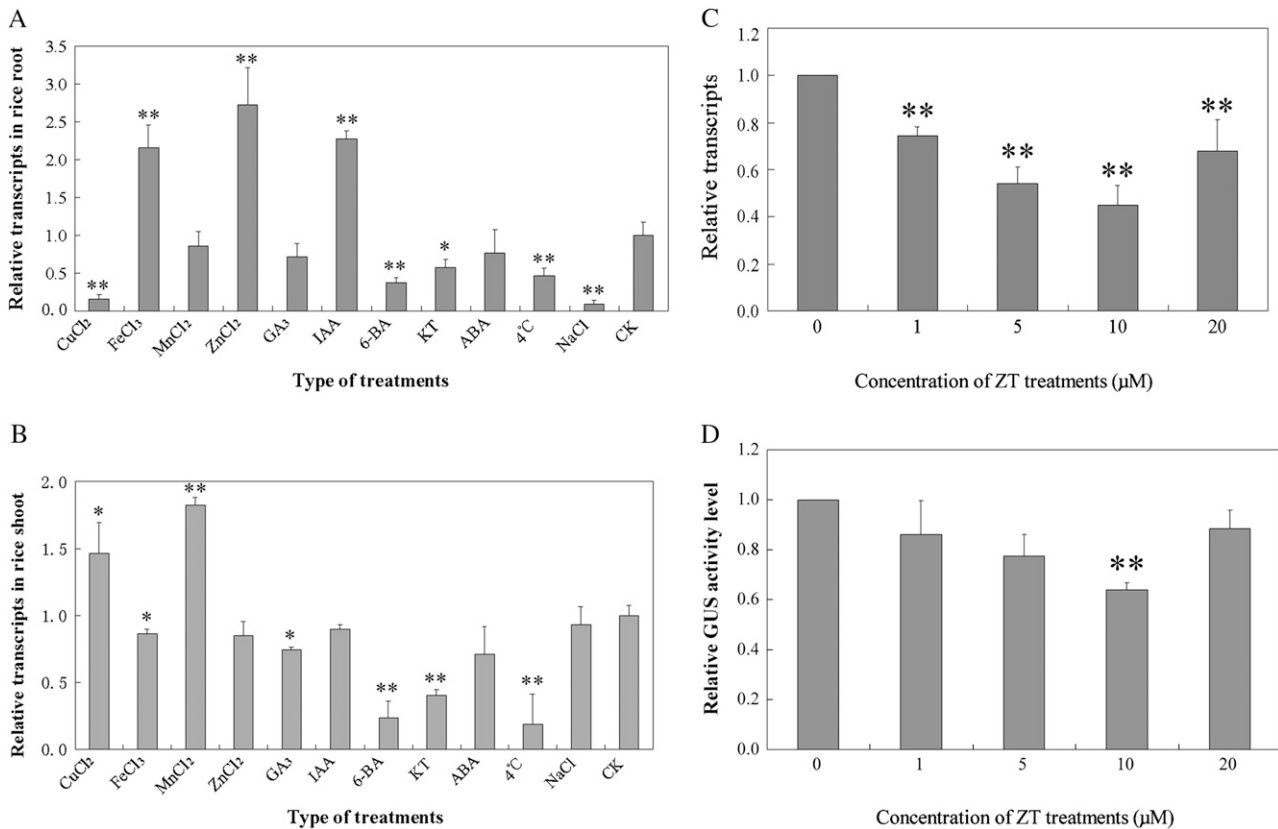


Figure 4. Response of *OsMT2b* transcript to various exogenous factors. A and B, Real-time quantitative RT-PCR analysis of *OsMT2b* expression in rice roots (A) and shoots (B) with the treatments of various factors. The seedlings were treated for 24 h with various exogenous factors, respectively: 0.1 mM CuSO₄, 0.2 mM FeCl₃, 0.1 mM MnCl₂, 0.2 mM ZnSO₄, 5 μM GA₃, 5 μM IAA, 5 μM 6-BA, 5 μM KT, 25 μM ABA, 0.4 M NaCl, and cold treatment at 4°C. The control (CK) was treated with distilled water. C, Dose-dependent effect of zeatin on *OsMT2b* expression in rice seedling roots treated with 0 (control), 1, 5, 10, and 20 μM zeatin for 24 h, respectively, by real-time quantitative RT-PCR analysis. D, By using fluorometric GUS assay, GUS expression of GAL4-UAS rice root in response to zeatin with various concentrations: 0 (control), 1, 5, 10, and 20 μM for 24 h, respectively. One asterisk (*) represents the significance of difference between the control and treatments as determined by repeated-measures ANOVA (two sample *t* tests proceeded by the software Origin 7.5; *P* < 0.05). Two asterisks (**) represent *P* < 0.01. The values are the mean ± SE.

RL transformants compared with that of the wild-type plants. On the other hand, the *OsMT2b* transcript level in overexpressing plants was markedly enhanced up to 12.2 times in leaves and 12.1 times in roots (Fig. 5D).

Abnormal *OsMT2b* Expression in Roots Leads to Defect of Root Development

To analyze the causes for more tillers in *OsMT2b* overexpressing plants and developmental defects in *OsMT2b* RNAi transformants, we first observed the root phenotype of the 3-week-old seedlings (Fig. 6A). Most RNAi transformants (RL) exhibited inhibition of root formation, but the overexpressing plants could form more roots. To describe rice root morphogenesis in detail, we plotted a model of rice seedling with diverse root trait (Fig. 6B). In the RNAi seedlings (RL), the length of the primary root that develops from the radicle of the embryo was dramatically shortened, and

the number of adventitious roots and small lateral roots obviously decreased (Fig. 6C). In the overexpressing seedlings, there were many more adventitious roots and big lateral roots (BLR), which initiate from the adventitious roots and have their own lateral roots (Fig. 6C). However, there were very few BLR in wild-type and RNAi transgenic seedlings. The result suggests a possible function of *OsMT2b* in the development of rice root.

To investigate endogenous cytokinin iPA levels in the roots of wild-type and transgenic plants, we applied HPLC technique to analyze and compare them. The results showed that the retention time of standard iPA was at 7.737 min, and the major peak was clearly detectable when the concentration was 3.2 ng mL⁻¹ (Fig. 6D). At the same retention time, the peak areas of iPA showed differences in the roots of wild-type (Fig. 6E), *OsMT2b*-RNAi (Fig. 6F), and *OsMT2b*-overexpressing plants (Fig. 6G). Compared with the

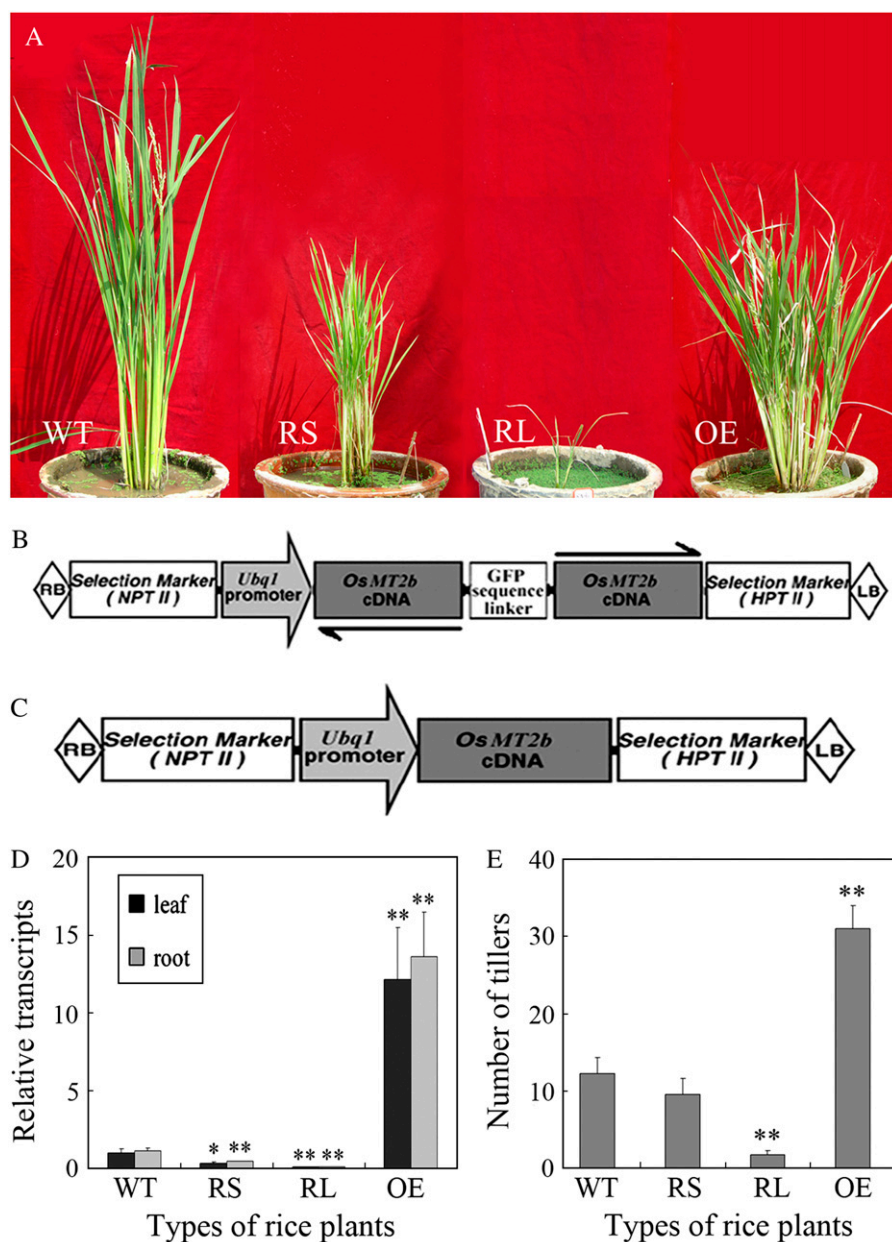


Figure 5. Analyses of *OsMT2b* RNAi and overexpressing transgenic plants in rice. **A**, Wild-type mature plant (WT), the small quantity (RS), and large quantity (RL) of *OsMT2b*-RNAi plants, and the *OsMT2b*-overexpressing plant (OE). **B** and **C**, Maps of the T-DNA portion of the binary vectors used in *OsMT2b*-RNAi and *OsMT2b*-overexpressing constructs, respectively. The right border (RB) and left border (LB) regions are indicated. *NPT II* and *HPT II* genes are used as selection markers. **D**, Real-time quantitative RT-PCR analysis of *OsMT2b* expressions in leaves and roots of wild-type and transgenic plants. **E**, The comparative analysis of tiller numbers in the wild-type and transgenic plants. One asterisk (*) represents the significance of difference between the wild-type and transgenic plant populations as determined by repeated-measures ANOVA (two samples *t* test proceeded by the software Origin 7.5; $P < 0.05$). Two asterisks (**) represent $P < 0.01$. The values are the mean \pm SE.

roots of wild-type plants (2.29 ng g^{-1} fresh weight [FW]), iPA levels doubled in the roots of RNAi plants (4.85 ng g^{-1} FW) and slightly decreased in the roots of overexpressing plants (1.85 ng g^{-1} FW; Fig. 6H).

To detect the spatial change of cytokinin iPA, by using immunohistochemical technique, we localized iPA in the roots of mature wild-type and transgenic plants. In the roots of wild-type plants, iPA signal was mainly in the vascular tissues and epidermis cells, and less in cells of the lateral roots (Fig. 6J). The distribution region of iPA in the roots of RNAi plants was similar to the wild-type plants but its level was higher in the former (Fig. 6L). In the roots of overexpressing plants, the iPA signal was detected only in epidermis cells but not in the vascular tissues (Fig.

6M). There was no signal of iPA in the control root sections (Fig. 6K).

To assess whether the synthesis and metabolism of endogenous cytokinin changed in the transgenic plants, we chose and assayed the expressions of two genes correlated with cytokinin, the isopentenyltransferase gene (*IPT3*, coding a cytokinin synthesis rate-limiting enzyme) and the cytokinin oxidase gene (*CKX2*, coding an enzyme that catalyzes the irreversible degradation of cytokinin). In the overexpressing plants, *OsIPT3* transcript levels decreased 27.7% in leaves and 54.1% in roots compared with wild-type plants, but *OsCKX2* expression had no detectable change. In the RNAi plants, the expressions of both *OsIPT3* and *OsCKX2* in leaves declined 96.6% and 98.1%, respectively, whereas

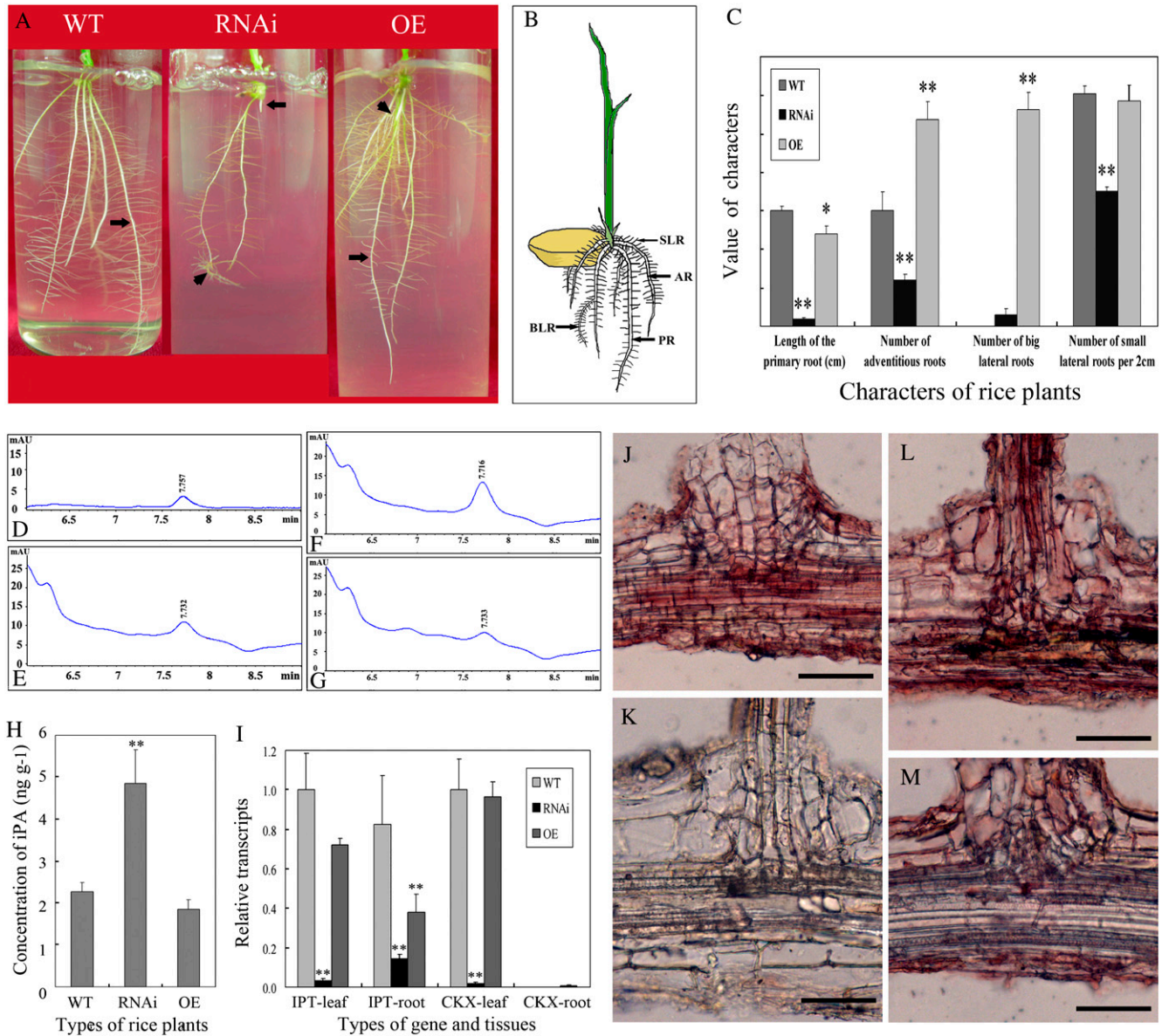


Figure 6. Role of the *OsMT2b* gene in the development of rice roots. A, Phenotype of roots in the 3-week-old seedlings of the wild-type (WT), RNAi, and overexpressing (OE) plants. Arrow and arrowhead indicate the primary root and BLR in the seedling, respectively. B, A model picture of rice seedling for the analysis of root trait. PR, Primary root; AR, adventitious root; SLR: small lateral root. C, Statistic analysis of roots in wild-type, RNAi, and overexpressing seedlings. D to G, Showing the chromatograms of standard iPA (D), wild-type (E), RNAi (F), and overexpressing samples (G) detected by HPLC. The absorbance (mAU) was plotted on the y axis and the retention time (min) on the x axis. H, The iPA concentrations in root samples of wild-type, RNAi, and overexpressing plants. I, Real-time quantitative RT-PCR analysis of *OsIPT3* and *OsCKX2* expressions in the leaves and roots of wild-type, RNAi, and overexpressing transgenic plants. J to M, Immunohistochemical localization of cytokinin iPA in the root longitudinal sections via lateral root of wild-type (J and K), RNAi (L), and overexpressing (M) plants. As a control (K), the section of root in wild-type plant was treated with PBS instead of the primary antibody, and showed no iPA signal. One asterisk (*) represents the significance of difference between the wild-type and transgenic plant populations as determined by repeated-measures ANOVA (two samples *t* test proceeded by the software Origin 7.5; $P < 0.05$). Two asterisks (**) represent $P < 0.01$. The values are the mean \pm se. Bars in J to M are 50 μ m.

in roots, *OsIPT3* transcript levels decreased 82.5% but that of *OsCKX2* had no visible change (Fig. 6I).

All these results indicate that the endogenous cytokinin level was slightly reduced in roots of the overexpressing plants, whereas distinctly increased in

roots of the RNAi plants (Fig. 6H). Therefore, the abnormal expressions of *OsMT2b* have an effect on the levels of cytokinin in roots of the transgenic plants, which hampers the development of rice plants, especially in root growth.

Abnormal *OsMT2b* Expression in Embryos Affected Scutellum Development and Seed Embryo Germination

The analysis of morphology showed that the structures of mature seed embryos were similar in *OsMT2b*-RNAi, overexpressing transgenic plants and wild-type plants, but the former two were smaller than the latter in size (Fig. 7A). In addition, in the transgenic plants the scutellum of embryos evidently diminished and its cell thickness lessened 39.2% in RNAi plants and 27.7% in overexpressing plants compared with wild-type plants, but its layers had no obvious change (Fig. 7B). This kind of phenotype was more obvious in seed embryos of RNAi plants than in those of overexpressing plants. In *OsMT2b*-RNAi and overexpressing plants, the frequency of seed embryo germination declined dramatically (in vivo), but resumed largely in *OsMT2b*-RNAi plants and a little in *OsMT2b*-overexpressing plants when the seed embryos were cultured in N6 medium without hormone (in vitro; Fig. 7C).

By fluorescein diacetate (FDA) staining to detect the viability of germinating embryos in vivo, the result showed that the FDA fluorescence of embryos was much stronger in the wild-type plants than in the RNAi and overexpressing plants (pictures not shown). The result confirmed the decline of germination frequencies in the seed embryos of transgenic plants.

Furthermore, immunohistochemical localization was used to assay whether the distribution of iPA changed in the seed embryos of transgenic plants. The result showed that iPA signal was mainly located in the coleoptile and scutellum of wild-type plants (Fig. 8A). The signal in the seed embryos of RNAi plants was obviously stronger than that in wild-type plants, and expanded in the whole embryos (Fig. 8C). However, the iPA signal slightly decreased in the embryos of overexpressing plants and was mainly presented in the coleoptiles (Fig. 8D). There was no detectable signal of iPA in the control embryo sections (Fig. 8B). The results indicate that the alteration of cytokinin distribution in the seed embryos of transgenic plants was similar to that in the roots of transgenic plants, and its change was likely to be one of the reasons for the reduction of the germination frequency in the seed embryos of rice transgenic plants.

DISCUSSION

There Is Correlation between *OsMT2b* Gene Expression and Cytokinin Levels

In this article, most *OsMT2b*-RNAi transformants exhibited serious obstacles to plant growth and root development. It was reported that the same typical

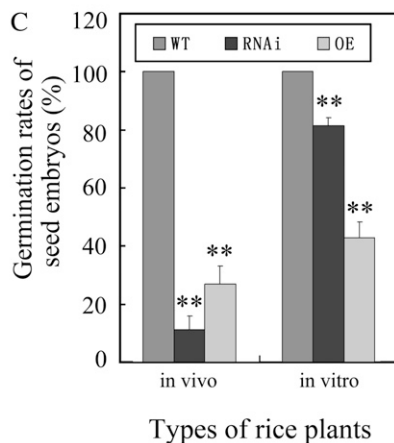
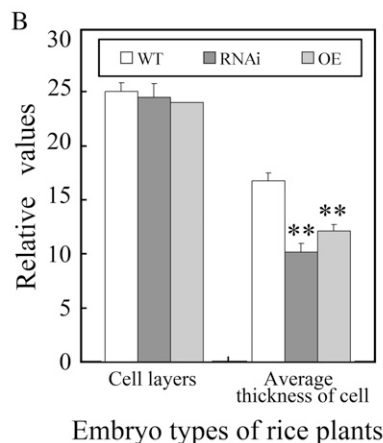
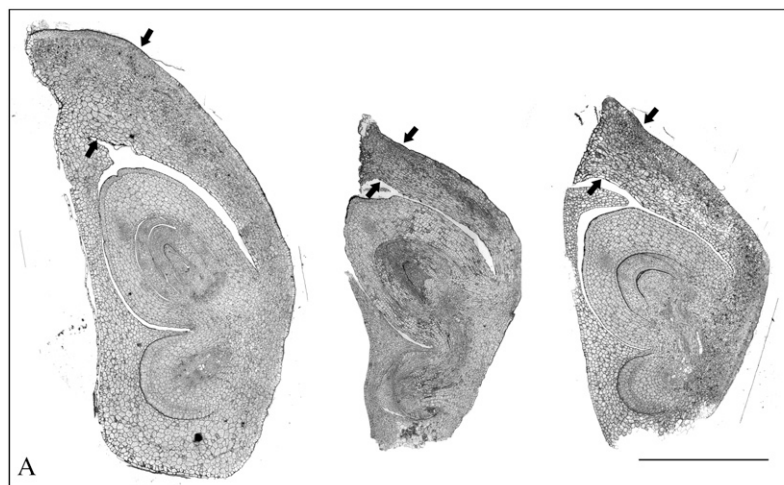
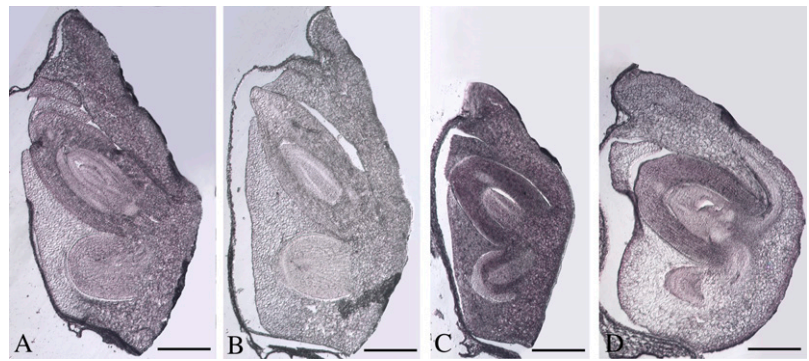


Figure 7. Roles of the *OsMT2b* gene in the scutellum cell size and the germination of rice seed embryos. A, The longitudinal semithin sections of the seed embryos at 2 DAG in rice wild-type (WT), RNAi, and overexpressing (OE) plants. Bar is 500 μ m. In the embryos, the cells between the two arrows were analyzed in Figure 7B. B, Analysis of cell layers and thickness in the scutellum top of seed embryos in rice wild-type and transgenic plants. C, Germination frequency of the seed embryos in vivo and in vitro in the wild-type and transgenic plants. Two asterisks (**) represent the intensive significance of difference between the wild-type and transgenic plant populations as determined by repeated-measures ANOVA (two samples *t* test preceded by the software Origin 7.5; $P < 0.01$). The values are the mean \pm SE.

Figure 8. The iPA distribution of germinating embryos in the wild-type and transgenic plants of rice. A to D, Immunohistochemical localization of iPA in the seed embryos at 2 DAG in the wild-type (A), RNAi (C), and overexpressing (D) plants. As a control (B), the section of embryo in wild-type plant was treated with PBS instead of the primary antibody, and showed no iPA signal. Bars are 300 μ m.



phenotype was caused by cytokinin overproduction in rice (Sakamoto et al., 2006). The handicap of root formation in the RNAi transgenic plants led to inefficient absorbability of nutrition, which resulted in an obstacle to plant growth. On the other hand, *OsMT2b*-overexpressing plants were shorter than the wild-type plants when they reached maturity, and the number of their adventitious roots was obviously increased. This was similar to the major effects of cytokinin deficiency in *Arabidopsis*, such as restrained shoot development and enhanced root growth, leading to dwarfed plants (Eckardt, 2003). Therefore, the relation between abnormal *OsMT2b* expression and cytokinin level indicates that the alteration of *OsMT2b* gene expression affected the cytokinin levels in transgenic plants.

In our studies, the quantitative analysis confirms the alteration of cytokinin levels in the roots of rice transgenic plants. Compared with the roots of wild-type plants, the cytokinin iPA level was much higher in *OsMT2b*-RNAi plants but a little lower in *OsMT2b*-overexpressing plants (Fig. 6H). Besides that, the immunohistochemical localization of iPA in rice roots (Fig. 6, J–M) and germinating embryos (Fig. 8, A–D) further validates the abnormal levels of iPA in the transgenic plants. In *OsMT2b*-RNAi plants, iPA signals were the strongest and expanded to a majority of tissues, but in *OsMT2b*-overexpressing plants, distinctly weakened in the scutellum of embryos and the vascular tissue of roots. It makes clear that *OsMT2b* plays an important role in regulating the cytokinin levels of rice plants.

The results of real-time quantitative RT-PCR analysis and GUS activity assay in the treated roots of rice seedlings showed that *OsMT2b* expression kept declining when the exogenous zeatin level gradually increased. It indicates that *OsMT2b* is down-regulated by cytokinin and involved in the cytokinin signaling pathway. In the promoter region of *OsMT2b*, there are five binding motifs (AGATT) for the type-B ARR1, which is involved in early responses to cytokinins (Oka et al., 2002). It was reported that the expression of *OsNSHB2*, which has one AGATT motif in its promoter, is regulated by cytokinin (Ross et al., 2004). In the cytokinin signaling pathway of rice, cytokinin

signals are received by His kinases and then transferred to B-type RRs by His phosphotransfer proteins (Ito and Kurata, 2006). In the following, B-type RR possibly down-regulates *OsMT2b* expression. However, when zeatin concentration reached an exceedingly high level, the *OsMT2b* expression was clearly enhanced (Fig. 4, C and D). This indicates that *OsMT2b* expression has a feedback to cytokinin level, and the gene can regulate it when cytokinin exceeds the normal physiological level. In view of indications that the increase of *OsMT2b* transcripts results in lower cytokinin levels in the *OsMT2b*-overexpressing plants, we presume that the expression of the *OsMT2b* gene maintains the homeostasis of cytokinin in rice plants.

The Possible Mechanism of *OsMT2b* Gene Regulating Cytokinin Levels

Cytokinin levels of plant tissues are mainly determined by the rate of biosynthesis and catabolism (Eckardt, 2003). Overexpression of the *OsiPT3* gene, catalyzing the rate-limiting step of cytokinin biosynthesis, led to the accumulation of high levels of cytokinin in transgenic rice (Sakamoto et al., 2006). Reduced expression of *OsCKX2*, catalyzing the irreversible degradation of cytokinin, caused cytokinin accumulation in inflorescence meristems of rice (Ashikari et al., 2005). In our studies, the expression of *OsiPT3* was decreased in both transgenic plants, and the expression of *OsCKX2* had no obvious change in overexpressing plants but was reduced in RNAi plants (Fig. 6I). Therefore, we deduce that the decrease of cytokinin levels in *OsMT2b*-overexpressing plants was mainly caused by the effect of *OsiPT3* reduction, and the increase of cytokinin in RNAi plants was because the expression of *OsCKX2* declined more severely than that of *OsiPT3*.

Another possibility is that the activity of CKXs is altered in transgenic plants. It was reported that the addition of Cu ions markedly enhanced cytokinin oxidase activity in *Phaseolus vulgaris* (Chatfield and Armstrong, 1987). Furthermore, MTs can efficiently bind metals, such as Cu and Zn, and then donate these metals to higher-affinity ligands on other proteins

(Coyle et al., 2002). If *OsMT2b* protein can bind Cu ions and donate them to CKXs, the protein level of *OsMT2b* will affect the utilization of Cu ions. Therefore, it is suggested that the activity of CKX could be regulated by *OsMT2b* expression in transgenic plants.

Besides altering the biosynthesis and metabolism of cytokinin by regulating the related genes, *OsMT2b* may have other pathways to affect endogenous cytokinin levels. We found that the *OsMT2b* gene predominantly expressed in the scutellum of rice germinating seed embryo and the primordium of lateral root. The expression pattern was similar to that of *OsENT2*, which was involved in the long-distance transport of nucleoside-type cytokinins by loading and unloading from the phloem, respectively (Hirose et al., 2005). Maybe, the *OsMT2b* protein is related to the *OsENT2* protein and is involved in cytokinin transport. This needs to be further studied.

OsMT2b Gene Has Functions in the Development of Roots and the Germination of Seed Embryos

Organ specificity has been reported for MT genes in many species of plants (Zhou et al., 2006), which strongly suggests that MTs are critical for plant development. In this article, *OsMT2b* as a type-2 MT was found to have a temporal and spatial expression pattern in the development of rice roots and embryos.

OsMT2b expresses predominantly in the primordium of lateral roots, and alters the cytokinin level in the roots of the transgenic plants, which was confirmed by the HPLC quantitative analysis. In rice, it was reported that both the KT and zeatin impeded lateral root formation by inhibiting the initiation of lateral root primordia (Debi et al., 2005). In our study, the number of lateral roots and adventitious roots remarkably decreased in *OsMT2b*-RNAi transgenic plants, but observably increased in *OsMT2b*-overexpressing transformants, which was possibly caused by the alteration of cytokinin content. The results of immunohistochemical localization further bear out that the signal of iPA was strong in the vascular cylinder of *OsMT2b*-RNAi root, which led to hampering lateral root formation. On the other hand, in *OsMT2b*-overexpressing root, the cytokinin iPA signal was faint at the vascular cylinder where lateral roots more easily initiated. Taken together, it is suggested that the accumulation of cytokinin that was caused by reduction of *OsMT2b* expression inhibited the formation of lateral roots in *OsMT2b*-RNAi plants, whereas the decline of cytokinin levels, which was resulted by *OsMT2b* overexpression, promoted the development of lateral roots and led to lots of BLR formation in the overexpressing plants.

GUS assay showed that *OsMT2b* was located in the scutellum top of embryos at 7 DAP but not at 5 and 10 DAP (Fig. 3, B–D). Generally, the scutellum formation of rice is at about 6 to 8 DAP, and the differentiation of scutellum procambium is at 6 DAP. In view of indications that scutellum cell thickness narrowed in the

germinating embryos of transgenic plants (Fig. 7, A and B), this suggests that the *OsMT2b* gene is involved in the development and function of scutellum. In addition, *OsMT2b* was predominantly expressed in the scutellum of rice germinating embryos (Fig. 3, E and F). It is well known that the epithelium layer in the dorsal portion of the scutellum elongates and acts as an absorptive tissue of storage reserves from endosperm during germination (Hirose et al., 2005). By this reason, the developmental defects of embryo scutellums in *OsMT2b*-RNAi and *OsMT2b*-overexpressing transformants lead to the decline of embryo germination frequencies.

To investigate whether the insufficiency of nourishment is the main reason for germination decline in transgenic embryos, we cultured the embryos in medium with enough nutrition. Due to all of the embryos having contact with the medium and absorbing nutrition, the germination frequency of *OsMT2b*-RNAi embryos rose and largely resumed. But the situation in *OsMT2b*-overexpressing plants was not obviously changed, which indicates that there are other factors having effects on embryo germination in the transgenic plants. In the aleuronic layer of wheat dormant seed, GA could not induce α -amylase activity until treated with exogenous cytokinin (Eastwood et al., 1969). As a crucial factor for embryo germination in our tests, cytokinin had its distribution difference in both embryos of *OsMT2b*-RNAi and overexpressing plants. Therefore, when the isolated mature embryos were cultured in nutrition medium, high levels of cytokinin in the scutellum of *OsMT2b*-RNAi transformants can largely resume germination ability, but low levels of cytokinin in the scutellum of *OsMT2b*-overexpressing plants cannot.

In conclusion, the expression of the *OsMT2b* gene is down-regulated by cytokinin and there is a positive feedback regulation mechanism of *OsMT2b* to cytokinin levels. Considering the phenotypes of two transgenic plants that have ectopic expression of *OsMT2b*, we conclude that the *OsMT2b* gene has a crucial role during the development of roots and the germination of seed embryos in rice by acting as a regulator to control cytokinin at an appropriate level.

MATERIALS AND METHODS

Plant Material

The wild-type plants and 03Z11AN35 mutant (GAL4-UAS plant) of rice (*Oryza sativa japonica* 'Zhonghua 11'), the *OsMT2b*-overexpressing and *OsMT2b*-RNAi transgenic plants of rice (*japonica* 'Kinmaze'), and the plants of rice (*indica* 'Jiayu 948') were grown in a greenhouse at Wuhan University. There is a GAL4-UAS enhancer trap system that includes GUS as the reporter gene and locates between the promoter and CDS of *OsMT2b* in the 03Z11AN35 mutant. The temperature for plant growth was 30°C/25°C under a photoperiod of 16-h light and 8-h dark.

Gene Expression Analysis in Rice through MPSS

As a sequencing-based technology, MPSS is used to quantify gene expression levels by generating millions of short sequence tags per library (Zhang

et al., 2005; Woll et al., 2006). In our experiments, the rice *OsMT2b* transcript expression levels were measured using the MPSS database constructed by Nakano et al. (2006). The cDNA sequence of *OsMT2b* was used for BLAST in the MPSS database and the short sequence signature of *OsMT2b* was obtained for analysis. In this database, the MPSS signatures can uniquely identify >95% of all genes in rice (*japonica* 'Nipponbare'). Out of 20 MPSS libraries that include the samples of different developmental stages and several biological replicates, we chose several libraries for analysis, such as 14-d-old root (young root) and leaf (young leaf), 60-d-old stem (mature stem), immature panicle, ovary and stigma, mature pollen, 3 DAG seed, 14-d-old root (young root in cold stress), and leaf (young leaf in cold stress) cold stressed in 4°C for 24 h.

Histochemical Localization of GUS Activity

GUS assay was conducted according to Jefferson et al. (1987). For GUS staining, embryos and flowers at different developmental stages, and the mature roots of GAL4-UAS rice plants (*japonica* 'Zhonghua 11') were vacuumed for 1 h and then incubated in X-gluc solution (1 mM X-gluc, 50 mM sodium phosphate buffer [pH 7.2], 5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% Triton X-100) for 8 h at 37°C. After that, the samples were observed and photographed under a stereomicroscope (SZX12; Olympus) equipped with a cool SNAP digital camera system (RS Photometrics). More than 10 individual samples were stained and analyzed for each organ.

To localize the GUS signal in the primordium of lateral roots in detail, paraffin section analysis was performed after GUS staining. In each type plant, five separate roots (every one was longer than 6 cm) were observed and then cut into 0.3-cm segments for GUS assay as described below.

Treatments of Exogenous Factors

To characterize the effects of different metal ions, hormones, and abiotic stresses on *OsMT2b* expression, the 2-week-old seedlings of rice (*japonica* 'Zhonghua 11') were cultured in aqueous solutions containing 0.1 mM CuCl₂, 0.2 mM FeCl₃, 0.1 mM MnCl₂, 0.2 mM ZnCl₂, 5 μM GA₃, 5 μM IAA, 5 μM 6-BA, 5 μM KT, 25 μM ABA, and 0.4 M NaCl as high salinity treatment, respectively, in 26 °C for 24 h. The treatment of low temperature was at 4 °C; the culture of seedlings with distilled water in 26°C was used as control. All the treatments were repeated three times, and the results represented the means (± SD) of those three independent experiments. After the treatments, the shoots and roots were immediately frozen in liquid nitrogen for RNA extraction and real-time quantitative RT-PCR analysis.

To analyze zeatin regulation of *OsMT2b* transcription, the 2-week-old seedlings of rice (*japonica* 'Zhonghua 11') were also submerged separately in aqueous solutions containing 0, 1, 5, 10, and 20 μM zeatin, respectively, for 24 h. Following that, the roots of treated seedlings were immediately frozen in liquid nitrogen for RNA extraction and real-time quantitative RT-PCR to detect *OsMT2b* expression. The treatments of zeatin were repeated three times.

The 3-week-old GAL4-UAS seedlings of rice (*japonica* 'Zhonghua 11') were grown in aqueous solutions containing 0, 1, 5, 10, and 20 μM zeatin, respectively, for 24 h to measure GUS activity. The total protein was extracted from the roots of treated seedlings and assayed with 4-methyl umbelliferyl glucuronide substrate using a spectrofluorophotometer (RF-5301PC; Shimadzu) at the excitation/emission wavelengths of 365/455nm, as described by Jefferson et al. (1987). The protein concentrations were quantified according to the method of Bradford (1976), and GUS enzyme activity obtained without zeatin treatment was set at 1.0 value. GUS activity measurement was repeated two times.

Real-Time Quantitative RT-PCR

The real-time quantitative RT-PCR was performed on equal amounts of cDNA prepared from the various materials by SYBR-green fluorescence using a Rotor-Gene 6000 real-time PCR machine (Corbett Research).

For assaying the expressions of the *OsMT2b*, *OsIPT3*, and *OsCKX2* genes in RNAi and overexpressing transgenic and wild-type plants of rice (*japonica* 'Kinmaze'), the leaves and roots from 6-week-old plantlets were, respectively, collected and immediately frozen in liquid nitrogen for RNA extraction and real-time quantitative RT-PCR analysis.

The expressions of those genes in different tissues were standardized with the gene *rac1* as an internal control. The PCR protocol contained an initial 8-min incubation step at 95°C for complete denaturation, followed by 45 cycles consisting of 95°C for 20 s, 56°C for 30 s, and 72°C for 45 s. The specificity of the PCR amplification was checked with a heat dissociation curve (65°C–95°C) following the final cycle of the PCR.

Primers of *OsMT2b* (GenBank accession no. U77294) are 5' AAGAAG-CCTGGCACGCATGAG 3' and 5' TCGGTGTGTCGATCAATGTTGGA 3'. Primers for *OsIPT3* (GenBank accession no. AB239799) are 5' CGGGAG-GTGGGGATGTTTCTGC 3' and 5' CCGCCGTCGTCTCCAGCAACC 3'. Primers for *OsCKX2* (GenBank accession no. AB205193) are 5' GCACC-CATGGCTGAACCTGTT 3' and 5' GCAGGATCCCCACCGTGTAGAA 3'. Primers of *rac1* (the gene of GTP binding protein; GenBank accession no. X16280) are 5' GGAGCGTGGTTACTCATTC 3' and 5' AAAGGCGACGGG-ACTCCA 3'. The constitutively expressed gene *rac1* was used as an internal standard. Data represent the means (± SD) of three independent experiments, each performed in triplicate.

Extraction and Quantification of Cytokinin iPA

Extraction and HPLC analysis of the N6-iPA, a kind of cytokinin, were performed as reported by Yang et al. (2001) and Mwangi et al. (2005), respectively, with some modifications. Four grams (FW) of roots from rice mature transgenic and wild-type plants were, respectively, cut and immediately frozen in liquid nitrogen. The samples were extracted in cold 80% (v/v) methanol with butylated hydroxytoluene (1 mM) overnight at 4°C. The supernatant was collected after centrifugation at 10,000 rpm for 15 min, and passed through a C18 Sep-Pak cartridge (Waters). The effluent was concentrated to dryness in a rotary evaporator (Heto). The dried extracts were dissolved in 1 mL of the mobile phase (consisting of 60% [v/v] methanol/0.1% [v/v] phosphoric acid) and used for HPLC analysis.

HPLC analysis was done using a computer-assisted HP1100 (Agilent Technologies) and a ZORBAX SB-C18 (Agilent Technologies) column (4.6 × 250 mm; 5 μm). The mobile phase was described above and used in HPLC analysis after filtration through a 0.25-μm filter. Flow rate was 0.5 mL min⁻¹ and the temperature of column was 45°C. Detection wavelength was 270 nm. Peak identification was based on retention time, main absorption maxima, and spectral shape as compared with the corresponding standards under the same separation conditions. The grade concentrations of iPA (Sigma) were used for peak identification and constructing the external standard curve. Three independent HPLC experiments were performed and the samples tested in each experiment were separate.

Light Microscopic Observation

Paraffin Section of Roots

For fine localization of GUS signals in roots, paraffin section analysis was used after GUS staining. Before GUS staining, the roots were fixed with 90% cold acetone for 20 min. The GUS-stained roots of GAL4-UAS rice plants (*japonica* 'Zhonghua 11') were immersed in an ethanol series (15%, 30%, 50%, 70%, 85%, 95%, and 100% for 30 min per step), and a xylene series (25%, 50%, 75%, and 100% for 60 min per step, using ethanol as solvent). After two series, paraffin chips continued to be added at 42°C until the solution was saturated. The xylene/wax mixture was replaced with 100% molten wax to embed the tissues. The root sections were cut at 10-μm thickness under a rotary microtome (Leica) mounted on poly-Lys-coated glass slides and observed.

Semithin Section of Germinating Embryos

The isolated embryos at 2 DAG in rice transgenic and wild-type plants were fixed for 1 h with 4% paraformaldehyde and 0.5% glutaraldehyde in 100 mM phosphate-buffered saline (PBS) under vacuum, and then in fresh fixatives for 4 h. After the samples were rinsed in PBS buffer five times for 20 min, they were dehydrated in a graded ethanol series and embedded in Epon812 resin. Semithin sections of 0.5-μm thickness were cut longitudinally under an ultramicrotome (MT-X; Sorvall) and stained with 0.5% (w/v) aniline blue. The sections were observed under an inverted microscope (DM IRB; Leica) and photographed with a CCD camera (Photometrics). For semithin section assay of the embryo in every type plant, two to four embryos were used.

In Vitro Culture of Rice Embryos

To study the effects of nutrition condition on the seed embryo germination, the mature seeds of transgenic and wild-type plants were germinated in water for 2 d. After that, some of the seed embryos were isolated and incubated in a 0.01% FDA solution for 10 min to observe their live status under a DMIRE2 invert microscope (Leica) with UV. The other seed embryos were isolated and cultivated in N6 medium containing 3% Suc and 0.4 g L⁻¹ casein hydrolysate for 3 d at 26°C in the dark. Following that, the germination percentages of the seed embryos were counted. FDA staining was repeated three times. Embryo number of each type was more than 50 and 20 in germination test of in vivo and in vitro, respectively. The germination assay of each type embryo was repeated more than three times.

Immunohistochemical Localization of Cytokinin iPA in Roots and Germinating Embryos

Using the immunohistochemical technique, iPA was localized in the roots and germinating embryos of transgenic and wild-type rice (*japonica* 'Kinmaze'). To strengthen the signals of cytokinin, the samples were treated with the method described by Karkonen and Simola (1999) before fixation. The immunohistochemical localization was performed as Qin et al. (2007) reported with some modification. The used anti-iPA mouse monoclonal antibody came from the iPA ELISA kit produced at the Nanjing Agricultural University (China), and has been commercially available for more than a decade. The specificity of the monoclonal antibody were checked previously and proved reliable by a dot-blot technique (data not shown). This primary antibody was diluted 1:500 in diluent (0.8% bovine serum albumin in 10 mM PBS, pH 7.0). When the sections presented color signal, they were rinsed with stop buffer, dehydrated, observed under an inverted microscope (DM IRB; Leica), and photographed with a CCD camera (Photometrics). In the experiment of the immunohistochemical localization, 20 sections from more than five individual samples were examined, and repeated three times.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number U77294.

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