Rpp16 and Rpp17, from a Common Origin, have Different Protein Characteristics but Both Genes are Predominantly Expressed in Rice Phloem Tissues

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The genes for two types of rice phloem protein (RPP16 and RPP17) were isolated and characterized. Conservation of five exon sizes as well as splicing positions between the two genes suggest that either RPP16 or RPP17 is a result of gene duplication. By protein blot analysis, RPP16 and RPP17 proteins were specifically detected in soluble and insoluble fractions of a crude extract of rice plants, respectively, suggesting that these proteins play different roles in individual cells. The expression of Rpp16 and Rpp17 was monitored by the β-glucuronidase (gusA) reporter-gene method. Rpp16-gusA and Rpp17-gusA were expressed preferentially in the phloem tissues from different parts of the plant, but almost no GUS staining was observed in the rest of the tissues. In roots of both constructs, interestingly, stronger GUS-accumulation was detected in younger vascular tissues than in aged vascular tissues. In situ hybridization also showed that Rpp17 was more strongly expressed in vascular tissues of tiller buds. These results suggest that transcript of these genes was more abundant in young tissues. The presence of two copies of the gene in higher plants, from a common origin, which have different protein characteristics, indicates that evolutionary diversification might have occurred in the gene function.

Keywords: GUS staining — In situ hybridization — Phloem tissue — Rice — Transgenic plant.

Abbreviations: CC, companion cell; EST, expressed sequence tag; GUS, β-glucuronidase; MP, movement protein; ORF, open reading frame; SE, sieve element.

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Introduction

Land plants have vascular tissue that is continuous throughout the plant. The vascular tissue is composed of two kinds of complementary conducting tissues for long-distance transport of micro- and macromolecules (Mezitt and Lucas 1996, Golecki et al. 1999, Ishiwatari et al. 1998, Jorgensen et al. 1998, Thompson and Schulz 1999). The phloem plays a central role in trafficking organic materials, and the xylem is the conduit for water and soil-derived nutrients as well as providing structural support (Aloni 1987, Turgeon 1996).

In angiosperms, the phloem is mainly composed of two types of cells: the sieve elements (SEs) and their associated companion cells (CCs). SEs are highly specialized for the translocation of assimilate, with most intracellular structures and organelles such as nuclei, vacuoles, ribosomes and Golgi bodies being degraded during the development of SEs. In contrast, CCs have strong viability to supply enough substances required in SEs. Mitochondria and free ribosomes are responsible for an extraordinary density of the cytoplasm in CCs, distinguishing them from all other cells of the phloem (Sjölund 1997, Opara and Turgeon 1999).

A number of morphological and physiological studies of the phloem tissue have been reported (e.g. Aloni 1987). Several genes involved in the phloem function have been reported to date (Kühn et al. 1997, Thompson and Schulz 1999). However, molecular studies of phloem function are still very limited.

Recently, CmPP16 from pumpkin has been cloned as phloem specific protein, and the protein is shown to possess properties similar to those of viral movement proteins (MPs) (Xoonostle-Càzares et al. 1999). It is reported that CmPP16 moves from cell to cell, mediates the transport of sense and antisense RNA, and moves together with its mRNA into the SEs. However, the function of CmPP16 in plants and the presence of homologous protein in other plants are still unclear.

In order to understand the function of viral MP-like protein encoded by plant genome, we intended to identify and characterize new protein gene(s) related to viral MP in different plant species. Rice (Oryza sativa), a monocot plant, was used as the plant material.

In this study, two different types of genes homologous to the viral MP gene were identified, and these genes having different protein characteristics seem to exist throughout the higher plants. Expression of these genes is examined and discussed.
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Fig. 1 Comparison of RPP16, RPP17 and their related sequences. (a) Schematic representation of genomic sequence structures of Rpp16 and Rpp17. Exons (e1 to e5) are shown by black boxes. Introns are indicated by broken lines. A dotted broken line indicates an intron between exon 1 and exon 2 in Rpp17-1. An open box shows an additional sequence of three amino acids in the exon 1 which is found in RPP17-1 but not in RPP17-2. (b) Alignment of predicted amino acid sequences deduced from rice RPP16, RPP17-1, RPP17-2 cDNAs (this study), pumpkin CmPP16-1 and CmPP16-2 (Xoconostle-Cázares et al. 1999), and movement protein of red clover necrotic mosaic virus (RCNMV-MP) (accession number P10838). Identical amino acids are indicated by black boxes and similar amino acids are shown by shaded boxes. A protein kinase C-like Ca\(^{2+}\)/phospholipid-binding (C2) domain is indicated by thick lines in the upper sides. Three sub-domains of the C2 domain (A, B, C) are also indicated by open rectangles. Black and open triangles indicate the splicing sites of Rpp16 and Rpp17, respectively. The numbers to the left and right of the sequence indicate the amino acid positions in each protein.

Results

Identification of Rpp16 and Rpp17

To examine whether a viral MP-like protein gene is present in the rice genome or not, a homologous sequence was searched from the rice expressed sequence tag (EST) database using a BLAST algorithm. Two clones R0374 and E61187 (DDBJ accession numbers AU031663 and C74955, respectively) were found and were subsequently used as probes for the isolation of full-length cDNAs and corresponding genomic DNA sequences. Three different clones were obtained from the cDNA library. One clone has an open reading frame (ORF) capable of encoding 144 amino acids with a predicted molecular mass of 15.9 kDa, named 16-kDa rice phloem protein gene (Rpp16) (Fig. 1b). The other two clones were almost similar to each other, differing only by one insertion/deletion of nine nucleotides within the ORF sequence (data not shown but available in database). They are capable of encoding 159 and 156 amino acids with predicted molecular masses of 17.7 kDa and 17.4 kDa, respectively, and both are designated 17-kDa rice phloem protein genes (Rpp17-1, Rpp17-2) (Fig. 1b).

The deduced amino acid sequences of Rpp16 and Rpp17-2 cDNAs shared 57% similarity in the entire region and both proteins showed a high degree of sequence similarity to the MP of red clover necrotic mosaic virus (RCNMV-MP) in specific regions (Fig. 1b). Both proteins contain conserved regions that are similar to the consensus sequences for the Ca\(^{2+}\)/phospholipid-binding (C2) domain (Kopka et al. 1998) (Fig. 1b). The isoelectric point of RPP16 is estimated to be 4.06, suggesting that it is an acidic protein. On the other hand, the isoelectric points of RPP17-1 and RPP17-2 are deduced to be 6.05 and 6.26, respectively, indicating that these proteins are neutral proteins.

To examine the copy number of these genes, genomic DNA was digested with BambHI, EcoRI, EcoRV and HindIII; and, DNA blot analysis was subsequently carried out using the Rpp17 cDNAs as probes. A single band was observed in all cases except for EcoRI digestion. There is one EcoRI recognition site inside the Rpp17 genomic clone. These results suggest that the Rpp17 gene is a single-copy gene in the rice genome (data not shown). On the other hand, the difference of nine nucleotides between Rpp17-1 and Rpp17-2 cDNAs seems to result from an alternative splicing event. DNA blot analysis was also carried out using the Rpp16 cDNA as a probe and a single band was observed, suggesting Rpp16 gene is also a single-copy gene in rice genome (data not shown).

In order to know the genomic sequence structure of Rpp16 and Rpp17 in detail, we isolated and sequenced their corresponding genomic sequences. Comparison of the cDNA and its corresponding genomic sequences revealed that both of the Rpp16 and the Rpp17 genes consist of five exons (Fig. 1a). It is of great interest that three out of four splicing sites from the Rpp16 and Rpp17 are exactly the same (Fig. 1). The above results suggest that these genes were evolved by gene duplication as proposed in c-type lysozyme and α-lactalbumin (Kumagai et al. 1992).

A similarity search was conducted using the BLAST program in order to find RPP16- and RPP17-related proteins in the database. Sequences from pumpkin CmPP16, Arabidopsis thaliana [accession numbers CAB75905 and AAF34860], chickpea [accession number AJ012692] and maize [accession numbers U64437 and AF152601] showed 52–83% homology to RPP16 and RPP17, although their functions have not yet been reported (except for pumpkin CmPP16). Similarities of the two genes to pumpkin CmPP16 are 56% and 58%, respectively (Fig. 1b). The protein phylogeny was analyzed by Neighbor-Joining Method clustering strategy, suggesting that RPP17 is...
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more closely related to pumpkin CmPP16 than RPP16 (Fig. 2). These results together with the deduced isoelectric point of the proteins suggest that RPP17 is an evolutional counterpart of CmPP16. Moreover, RPP16 seems to be a novel protein, although it is also related to CmPP16 to some extent. The characteristics of RPP16 protein are different from those of RPP17.

The expression of the RPP16 and RPP17 proteins was examined by immunoblotting. Antisera against either RPP16 or RPP17 protein were raised separately in rabbits. A 17 kDa protein was specifically detected by using anti-RPP17 antibody in the insoluble fraction of an extract prepared from rice leaves (Fig. 3a). The size of the detected polypeptide coincided with that deduced from the Rpp17 cDNA sequence, suggesting that it corresponds to the product of RPP17.

In contrast, when the anti-RPP16 antibody was used for protein blot analysis, a polypeptide with a molecular mass of approximately 27 kDa was detected in the soluble fraction of rice total proteins (Fig. 3b). Therefore, RPP16 seems to be a soluble protein and present in the cytoplasm, because the protein cross-reacted only in the soluble fraction.

When the anti-RPP17 antibody was used, the proteins with a similar molecular mass to the RPP17 were also detected in the insoluble fraction of the extract prepared from leaves of sugarcane, barley, tobacco, melon and *Arabidopsis* (Fig. 3c). Proteins with similar molecular masses were also detected in the soluble fraction of barley (28 kDa), sugarcane (26 kDa), melon (28 kDa), Azuki bean (25 kDa) and *Arabidopsis* plants (25 kDa) when the anti-RPP16 antibody was used (Fig. 3d). These results suggest that proteins homologous with RPP16 and RPP17 are widespread throughout the higher plants.

Tissue-type specific expression of Rpp16 and Rpp17 genes in rice

Transcription of Rpp16 and Rpp17 genes was investigated by RNA blot analysis. In leaves, 0.7-kb and 0.8-kb transcripts were detected using Rpp16 and Rpp17 cDNAs as

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**Fig. 2** Phylogenetic analysis of rice RPP16, RPP17-1, RPP17-2 and their related proteins. A phylogenetic tree for rice RPP16, RPP17-1 and RPP17-2 (this study), pumpkin CmPP16-1 and CmPP16-2 (Xoconostle-Cazares et al. 1999), *Arabidopsis thaliana* (accession numbers CAB75905 and AAF34860), *Cicer arietinum* (accession number AJ012692) and *Zea mays* (accession numbers U64437 and AF152601) was generated according to the Neighbor-Joining Method clustering strategy using PAUP* 4.0b4a. The numbers on the branches are percentages of 10,000 bootstrap samples supporting the branch. The rice proteins are indicated in box.

**Fig. 3** Protein blot analyses of RPP16 and RPP17. Proteins in each fraction of rice leaves (S1, soluble; S2, treated with tritonX-100; M, insoluble) were separated by SDS-PAGE, and immunodetection of peptide was performed. A 17-kDa polypeptide and a 27-kDa polypeptide cross-reacted with anti-RPP17 antisera (a) and anti-RPP16 antisera (b) are indicated by arrowheads, respectively. Leaf proteins [rice (lane 1); barley (lane 2); sugarcane (lane 3); *Arabidopsis* (lane 4); melon (lane 5); Azuki bean (lane 6) and tobacco (lane 7)] from insoluble fraction (c) or soluble fraction (d) were separated by SDS-PAGE. Immunodetection was performed using anti-RPP17 (c) and anti-RPP16 (d) antisera. The numbers at the left of the figure indicate the position of molecular size markers.
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probes, respectively (data not shown). The sizes of the transcripts are in good agreement with those of Rpp16 (750 nucleotides) and Rpp17 (850 nucleotides) cDNAs, respectively.

In order to characterize the tissue- or cell-type-specific expression of Rpp16 and Rpp17, we individually fused each promoter region upstream of the β-glucuronidase (gusA) reporter gene. The chimeric genes were introduced into rice (named Rpp16-gusA and Rpp17-gusA plants, respectively) and transgenic plants were produced.

In roots of both Rpp16-gusA and Rpp17-gusA plants, β-glucuronidase (GUS) staining was specifically observed in vascular tissues (Fig. 4a–d). It is noteworthy, however, that GUS staining was not observed in other cell types (Fig. 4a–d). In leaves of both Rpp16-gusA and Rpp17-gusA plants, GUS staining was clearly confined to vascular tissues. In vascular tissue, GUS staining was more strongly detected in the phloem tissues (companion cells and phloem parenchyma cells) (Fig. 4e, f). The above results strongly indicate that Rpp16 and Rpp17 genes are predominantly expressed in the phloem tissues.

In roots, interestingly, GUS staining was more strongly

Fig. 4  Histochemical localization of GUS staining in Rpp16-gusA and RPP17-gusA plants. Upper and lower row panels show tissues from Rpp16-gusA and Rpp17-gusA transgenic rice plants, respectively. (a–d) Roots; (e, f) leaves. VC, vascular cylinder; BR, branched root; CR, crown root; LVB, large vascular bundle; CC, companion cell; PP, phloem parenchyma cell. Size bars in the figure represent 50 µm (a, b) and 10 µm (c–f), respectively.

Fig. 5  Localization of RPP17 mRNA in nodes of stems by in situ hybridization. For in situ hybridization of mRNA, digoxigenin-labeled sense and antisense riboprobes were synthesized by in vitro transcription using T7 or T3 RNA polymerase. (a) Antisense Rpp17 riboprobe, stem cross-section. The presence of Rpp17 transcripts is shown in purple. (b) Sense riboprobe, stem cross-section. An arrow indicates one of the young vascular tissues. Size bars represent 50 µm.
detected in the branched roots as well as in the division zones of crown roots, whereas weak staining was observed in root hair zones of crown roots of the both Rpp16-gusA and Rpp17-gusA plants (Fig. 4a–d). It seems that stronger GUS staining is observed in the early stage of tissue development in roots. This may indicate that the gene expression is affected by the stage of tissue development.

In situ hybridization of RPP17 transcripts

In situ hybridization was carried out on a section of stems to examine the accumulation of endogenous Rpp17 transcripts. Signals were observed in vascular tissues of tiller buds by using the antisense RNA probe (Fig. 5a). In contrast, no signal was observed by using the sense RNA probe (Fig 5b). It is noteworthy that less accumulation of Rpp17 transcripts was observed in mature vascular tissues (data not shown). Therefore, Rpp17 transcripts specifically accumulated more abundantly in young vascular tissues.

Discussion

The presence and distribution of viral MP-like protein in higher plants were not well known. This study clearly demonstrates that two kinds of gene homologous to the viral MP-like proteins are present in rice: one is a novel viral MP-like gene, Rpp16, and the other is the pumpkin CmPP16-homologous gene, Rpp17. Protein blot analyses also showed that specific protein related to Rpp16 was found in all six examined plants, suggesting that both protein homologues are conserved throughout the higher plants.

To investigate the function of the RPP16 in rice, we characterized the expression pattern of Rpp16 by GUS histochemical analysis of transgenic plants. Histochemical analysis of the Rpp16-gusA plants demonstrated that the 5’-flanking sequence of Rpp16 was sufficient to direct gene expression in vascular tissues. In detail, the GUS staining was preferentially found in phloem tissues of leaves, indicating that Rpp16 is predominantly expressed in phloem tissues and is likely to be involved in phloem development and/or function.

Histochemical analysis in roots showed that Rpp16 was more strongly expressed in branch roots compared to crown roots. It is known that the branch roots grow from the maturation zone of crown roots. In crown roots, Rpp16 was more strongly expressed in the division zone than the root hair zone (data not shown). The evidence of GUS staining that is localized in the early stage of tissue development supports that the gene expression of Rpp16 is more abundant in younger vascular cells than in aged vascular cells, and that the spatial gene-expression is affected by the stage of tissue development.

The Rpp17-gusA plants showed that the gene expression of Rpp17 was almost the same as that of Rpp16, although almost no DNA sequence similarity is observed in the 5’-flanking sequence between the Rpp16 and the Rpp17. In short, Rpp17 is also predominantly expressed in the phloem tissues. In roots, Rpp17 was also more strongly expressed in branch roots than in crown roots. The observation is in good agreement with the result of in situ hybridization in stems. In situ hybridization revealed that Rpp17 was strongly expressed in vascular tissues of tiller buds, which is the early stage of leaf development. These results show that expressions of the two genes are predominantly expressed in phloem tissues and seem to be affected by the stage of tissue development.

The in situ hybridization signal by Rpp17 antisense probe was only visible after a long incubation (two times longer than Rpp17 incubation), suggesting that the level of transcription of Rpp16 is much lower than that of Rpp17 (data not shown). This observation is in good agreement with the result of GUS assays. When we analyzed GUS enzyme activity in both Rpp16-gusA and Rpp17-gusA plants, 8-fold higher enzyme activity was observed in the Rpp17-gusA plants compared to that of Rpp16-gusA plants (data not shown).

By protein blot analysis using the anti-RPP16 antibody, a polypeptide with a molecular mass of approximately 27-kDa was detected in the soluble fraction of the rice leaf sample. It is quite strange that the peptide detected by anti-RPP16 in rice is 27 kDa, which is 11 kDa larger than the size deduced from the Rpp16 cDNA. Remarkable overestimations of the molecular size by SDS-PAGE have been reported for several other proteins that have high acidic amino acid content (Burton et al. 1981, Kaufmann et al. 1984, Kleinschmidt et al. 1986, Takano et al. 1988). They report that high content of acidic amino acids may restrict the binding of SDS to protein molecules, resulting in slower migration. In fact, part of the RPP16 protein expressed in E. coli (amino acid position 39–144; Asp: 9.5%; Glu: 11.1%) also migrated as the 11.5 kDa-protein, which was 5.5 kDa larger than the predicted size (data not shown). The full size RPP16 contains many acidic amino acid residues (Asp: 8.3%; Glu: 11.1%). Therefore, we conclude that the polypeptide detected by protein blot analysis is the transatlational product of the Rpp16 gene. Since the protein cross-reacted only in the soluble fraction, RPP16 seems to be a soluble protein. In contrast, the results of immunoblotting indicate that RPP17 was specifically detected in the insoluble fraction; RPP17 seems to be a membrane-bound or membrane-associated protein. Despite similar gene structure and GUS expression behavior, RPP16 and RPP17 proteins have different protein characteristics. The structural divergence between RPP16 and RPP17 suggests RPP16 and RPP17 proteins are located in different places in the cell and may have different functional roles in phloem tissues.

We created two types of transgenic rice plants containing the antisense of Rpp16 and Rpp17 gene, respectively. However, they showed no obvious change in morphology compared with the wild-type rice (data not shown). The function of RPP16 and RPP17 is still unclear, although it is apparent that both genes are conserved among the higher plants. The phylogeny analysis and the localization of the RPP17 in the insoluble
fraction suggest that rice RPP17 is functionally a counterpart of the pumpkin CmPP16, which has been reported to be associated with the plasma membrane of SE (Xocconostle-Cazares et al. 1999). The function of RPP16 is not understood clearly yet, although the expression pattern of RPP16 suggests that it may play a role in younger phloem tissues. Some of these discrepancies may be attributable to the complex nature of gene expression system.

Materials and Methods

Plant material and nucleic acid isolation

Rice plants (Oryza sativa L. cv. Nipponbare) were grown at 28°C in a greenhouse. Genomic DNA and total RNA were extracted from the young leaves by the procedure described in Kadowaki et al. (1996).

Screening of rice cDNA and genomic DNA libraries

Rice cDNA and genomic DNA libraries were constructed as described previously (Kadowaki et al. 1996). For screening of cDNA and DNA, insert DNAs from two rice EST clones R0374 and E61187 (DDBJ accession numbers AU031663 and C74955, respectively) were used as probes. The probes were labeled by using the enhanced-chemiluminescence direct nucleic acid labeling system (Amersham Pharmacia) according to the manufacturer’s instructions. Subsequently, hybridization was performed by the method of Kadowaki et al. (1996).

DNA sequencing analysis

DNA sequence was determined by the dideoxy chain termination method using fluorescent dye primer (PE Biosystems). Nucleotide and deduced amino acid sequences were analyzed as described previously (Kadowaki et al. 1996). A phylogenetic tree was generated according to the Neighbor-Joining Method clustering strategy (Saitou and Nei 1987) using PAUP* 4.0b4a (Swofford 1998) and bootstrap values were obtained by 10,000 bootstrap replicates. The deduced isoelectric points were analyzed by using GENETYX software (Software Development, Tokyo, Japan).

DNA and RNA gel blot analysis

Rice genomic DNA (5 μg) was digested with BamHI, EcoRI, EcoRV and HindIII, subjected to electrophoresis through a 0.8% agarose gel, and blotted onto Hybond-N+ membrane (Amersham Pharmacia) by the conventional capillary method (Ausubel et al. 1987). Poly(A)+ RNA (1 μg) was fractionated through a 1% agarose/formaldehyde gel and blotted onto positive-charge membrane (Roche). The whole region of Rpp16 or Rpp17 cDNAs was used as a probe, respectively. Probe labeling was performed according to the manufacturer’s instructions (Roche). Hybridization and washing were carried out as described by Ausubel et al. (1987) and subsequent reaction was performed according to the manufacturer’s instructions (Roche). The final washing was carried out using 0.1× SSC and 0.1% SDS at 42°C.

Protein blot analysis

The leaves from rice (Oryza sativa L.), sugarcane (Saccharum officinarum L.), barley (Hordeum vulgare L.), Azuki bean (Vigna angularis L.), Arabidopsis thaliana, tobacco (Nicotiana tabacum L.) and melon (Cucumis melo L.) plants were ground into a fine powder in five volumes of extraction buffer (100 mM Tris-HCL, pH 7.5; 2 mM EDTA; 5 mM 2-mercaptoethanol; 10% glycerol; 50 mM KCl; 0.2% tritonX-100). The tube was rotated at 4°C for 30 min, subsequently centrifuged at 20,400×g for 30 min, and the supernatant was collected (S1 fraction). The pellet fraction was resuspended in an equal volume of buffer (50 mM Tris-HCl, pH 7.5; 2 mM EDTA; 5 mM 2-mercaptoethanol; 10% glycerol; 50 mM KCl; 0.2% tritonX-100). The tube was rotated at 4°C for 30 min, subsequently centrifuged at 20,400×g for 30 min, and the supernatant was collected (S2 fraction). Pellet fraction was also resuspended in SDS-PAGE running buffer and saved (insoluble fraction). Proteins were separated using a 12% polyacrylamide gel and transferred to Immobilon PVDF membrane (Millipore).

Antisera against rice RPP16 and RPP17 proteins were prepared as follows: cDNAs of a portion of the RPP16 (amino acid position 39–144) and RPP17 (amino acid position 43–159) cDNAs were amplified by PCR, ligated into pGEX 4T-3 vector (Amersham Pharmacia Biotech) and transformed into E. coli JM109. Fusion protein from GST/ RPP16 and GST/RPP17 was induced by the addition of 1 mM IPTG and was purified by the method of Asano et al. (2002). The purified fusion protein was injected into a rabbit six times at intervals of 2 weeks. The antisera were purified by affinity purification procedure using CNBr-activated sepharose 4B column.

Production of transgenic plants

Genomic sequences containing putative promoter regions of Rpp16 (−1,346 bp to −1 bp from the translational initiation codon) and Rpp17 (−2,000 bp to −1 bp from the translational initiation codon) were ligated in frame at the upstream of the gusA gene in a pCAMBIA1301 plasmid. The resultant plasmid was introduced into Agrobacterium tumefaciens strain EHA105. Transgenic rice plants (based on O. sativa cv Nipponbare) were produced by Agrobacterium-mediated transformation as described by Toki (1997). Transgenic rice plants were selected on media containing hygromycin (50 mg liter−1), and the presence of gusA gene in the rice genome was confirmed by PCR.

Analysis of GUS gene expression

GUS staining was conducted according to Jefferson (1987). For histochemical analysis, leaf, stem and root segments were sectioned into 30 μM-thick pieces by a microslicer and incubated in 50 mM sodium phosphate buffer (pH 7.0) containing 1.0 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide and 5% methanol at 37°C for 2–24 h. The reaction was stopped by adding ethanol.

In situ RNA hybridization analysis

Plant materials were fixed by overnight incubation at 4°C in 50 mM phosphate buffer (pH 7.2) containing 4% (w/v) paraformaldehyde and 0.25% glutaraldehyde. Tissues were then dehydrated through 50 mM phosphate buffer (pH 7.2) containing 1.0 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide and 5% methanol at 37°C for 2–24 h. The reaction was stopped by adding ethanol.

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