RESEARCH ARTICLE

Cloning, characterization and expression of *OsFMO*$_{(t)}$ in rice encoding a flavin monooxygenase

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Abstract

Flavin monooxygenases (FMO) play a key role in tryptophan (Trp)-dependent indole-acetic acid (IAA) biosynthesis in plants and regulate plant growth and development. In this study, the full-length genomic DNA and cDNA of *OsFMO*$_{(t)}$, a FMO gene that was originally identified from a rolled-leaf mutant in rice, was isolated and cloned from wild type of the rolled-leaf mutant. *OsFMO*$_{(t)}$ was found to have four exons and three introns, and encode a protein with 422 amino acid residues that contains two basic conserved motifs, with a 'G×G××G' characteristic structure. OsFMO$_{(t)}$ showed high amino acid sequence identity with FMO proteins from other plants, in particular with YUCCA from *Arabidopsis*, FLOOZY from *Petunia*, and OsYUCCA1 from rice. Our phylogenetic analysis showed that OsFMO$_{(t)}$ and the homologous FMO proteins belong to the same clade in the evolutionary tree. Overexpression of *OsFMO*$_{(t)}$ in transformed rice calli produced IAA-excessive phenotypes that showed browning and lethal effects when exogenous auxins such as naphthylacetic acid (NAA) were added to the medium. These results suggested that the OsFMO$_{(t)}$ protein is involved in IAA biosynthesis in rice and its overexpression could lead to the malformation of calli. Spatio-temporal expression analysis using RT-PCR and histochemical analysis for GUS activity revealed that expression of *OsFMO*$_{(t)}$ was totally absent in the rolled-leaf mutant. However, in the wild type variety, this gene was expressed at different levels temporally and spatially, with the highest expression observed in tissues with fast growth and cell division such as shoot apexes, tender leaves and root tips. Our results demonstrated that IAA biosynthesis regulated by *OsFMO*$_{(t)}$ is likely localized and might play an essential role in shaping local IAA concentrations which, in turn, is critical for regulating normal growth and development in rice.

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Introduction

As the endogenous auxin in plants, indole-acetic acid (IAA) is known to regulate diverse processes in plant growth and development (Palme *et al.* 1991). Recent findings have shown that local auxin biosynthesis plays a key role in shaping the local auxin gradient and is essential for regulating many developmental processes, such as embryogenesis, seedling growth, vascular patterning, leaf formation, etc. (Cheng *et al.* 2006, 2007; Stepanova *et al.* 2008; Tao *et al.* 2008). Due to the complex pathways involved in endogenous IAA biosynthesis, such as several tryptophan (Trp)-dependent and Trp-independent pathways (see figure 1A), the molecular mechanisms and the physiological roles of *de novo* auxin biosynthesis in plants are still poorly understood (Zhao 2010).

It has been clearly demonstrated that Trp-dependent auxin biosynthesis is essential for plant developmental processes (Cheng *et al.* 2006, 2007; Gallavotti *et al.* 2008; Zhao 2010). Genetic studies so far have undoubtedly revealed that YUC (flavin monooxygenases of *Arabidopsis*) and TAA (tryptophan aminotransferases of *Arabidopsis*) are major players in Trp-dependent IAA biosynthetic pathway (Cheng *et al.* 2006, 2007; Zhao *et al.* 2001; Zhao 2010, 2012). Other enzymes, such as CYP79B2/B3, nitrilases, aldehyde oxidases and pyruvate decarboxylases, are probably not the main contributors to IAA biosynthesis (Normanly *et al.* 1997; Zhao *et al.* 2002; Vande Broek *et al.* 2005; Sugawara *et al.* 2009; Mashiguchi *et al.* 2011).

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Jicai Yi and Lanna Liu contributed equally to this work.

Keywords. cloning; characterization; *OsFMO*$_{(t)}$ gene; flavin monooxygenase; indole-acetic acid; expression analysis.

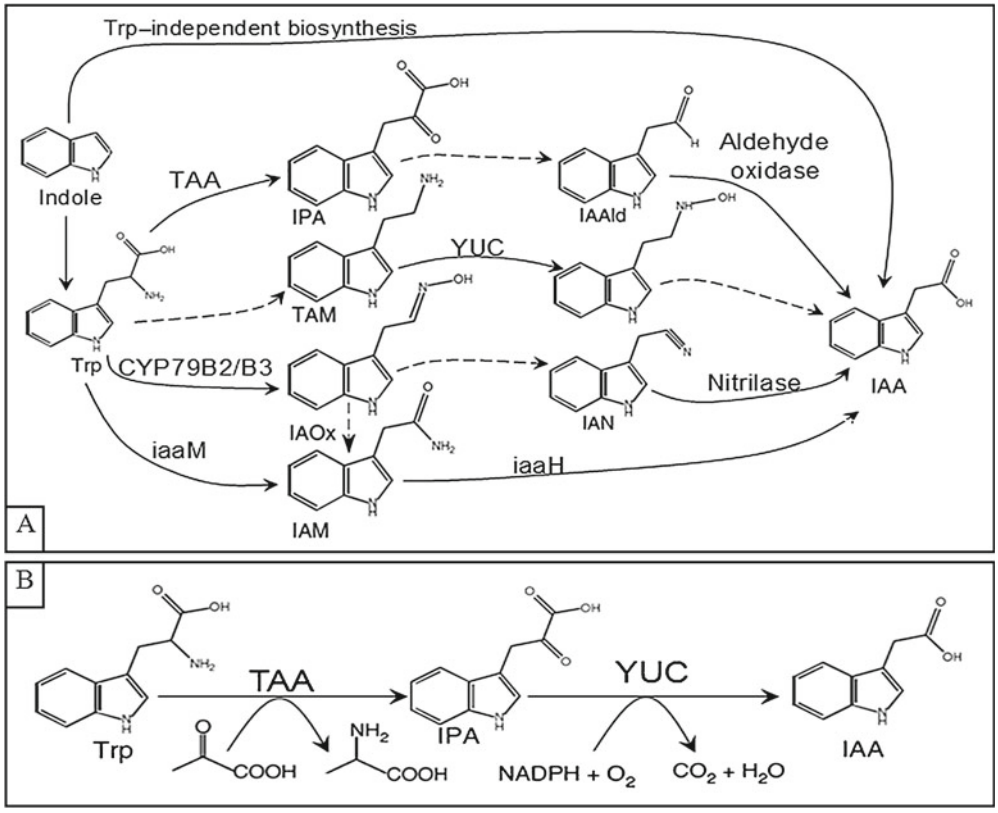

Figure 1. The endogenous IAA biosynthetic pathways. Adapted and revised from Won *et al.* (2011), Zhao *et al.* (2002) and Zhao (2012). (A) Trp-dependent pathways of IAA biosynthesis. (B) A simple two-step pathway for IAA biosynthesis. TAM, tryptamine; IAOx, indole-3-acetaldoxime; IAM, indole-3-acetamide; IAN, indole-3-acetonitrile; IAAld, indole-3-acetaldehyde. TAA, tryptophan aminotransferases of *Arabidopsis*; YUC, flavin monooxygenases of *Arabidopsis*.

YUC flavin monooxygenase was previously proposed to catalyze the conversion of tryptamine to N-hydroxyl tryptamine, which may be utilized for IAA production (see figure 1B) (Zhao *et al.* 2001; Ljung *et al.* 2002) and TAA tryptophan aminotransferase participated in the step from Trp to indole-3-pyruvate (IPA) (Stepanova *et al.* 2008; Tao *et al.* 2008). The enzymes, YUC and TAA, which were previously considered to participate in separate pathways in IAA biosynthesis, are now thought to function in the same auxin biosynthesis pathway (reviewed by Zhao 2010, 2012). Trp is converted into IPA by TAA and IPA is then used as a substrate by YUC to produce IAA in *Arabidopsis* (Mashiguchi *et al.* 2011; Won *et al.* 2011). Genetic studies have shown that this simple two-step pathway is the main mechanism for *de novo* IAA biosynthesis in plants, and the rate-limiting step for IAA biosynthesis is the second step that is catalyzed by YUC (see figure 1B; Zhao 2010, 2012). The mutation of YUC-like FMO genes in *A. thaliana* and *Petunia*, resulted in varied levels of local endogenous IAA (Zhao *et al.* 2001; Santamaria *et al.* 2002) and abnormal phenotypes, such as leaf-rolling in these plants (Cheng *et al.* 2006, 2007).

Rice 'rolled-leaf' related genes are important for high photosynthetic efficiency breeding (Lu *et al.* 2005). Some of these genes encode MYB transcription factor-like proteins (Luo *et al.* 2007), GARP-like proteins of the KANADI family in *A. thaliana* (Yan *et al.* 2006; Zhang *et al.* 2009) and AGO family proteins (Shao *et al.* 2005; Shi *et al.* 2007). In the genetic analysis of a rolled-leaf mutant induced by γ rays from Qinghuazhan (QHZ), a variety of *indica* rice (*Oryza sativa* L.), Yi *et al.* (2007) identified an open reading frame (ORF) encoding flavin monooxygenase (FMO), temporarily named $OsFMO_{(t)}$, as the candidate for the rolled-leaf mutation. In the present study, we isolated $OsFMO_{(t)}$ from QHZ, the corresponding wild type variety, and conducted a sequence analysis based on the sequence of the *FMO* gene in Nipponbare (*O. sativa* L. *japonica*) in the public rice sequence database (http://www.ncbi.nih.gov). We also overexpressed the gene $OsFMO_{(t)}$ to study the role of this gene in auxin biosynthesis. These studies coupled with our spatio-temporal studies suggest an essential role of $OsFMO_{(t)}$ in local *de novo* IAA biosynthesis in rice.

Materials and methods

Plant materials

Qinghuazhan (QHZ, *O. sativa* L. *indica*), the wild type variety (provided by College of Agronomy, South China

Agricultural University, Guangzhou, China) corresponding to *γ-rl*, a rolled-leaf mutant (Yi *et al.* 2007), was used as the plant material for gene cloning and characterization of $OsFMO_{(t)}$. DNA was extracted from the tender leaves of QHZ's seedlings by CTAB method (Murray and Thompson 1980). Total RNA was extracted from the tender leaves of seedlings 10 days after germination using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). The methods of extraction and measurement of IAA were adapted from Wang *et al.* (2002). Rice calli, 100 mg, were weighed accurately and grounded in liquid nitrogen. To these samples, 1 mL of 80% (v/v) precooled (4°C) methanol containing 0.01% butylated hydroxytoluene (BHT) was immediately added, and the samples were then incubated for 4 h at 4°C before centrifuging for 10 min at 10,000 g. The pellets were extracted again in 0.5 mL of 80% (v/v) precooled (4°C) methanol containing 0.01% BHT and recentrifuged in the same manner. Supernatants of these first and second extractions were pooled together and then cleaned-up by the Sep-Pak C18 Cartridge (Waters, Milford, USA). The Sep-Pak C18 Catridge was preconditioned with 5 mL of methanol and 5 mL of 70% (v/v) methanol. The sample solution was passed through the preconditioned C18 column. The column was then rinsed with 5 mL methanol. The elution was dealt with a nitrogen drying step and redissolved in 100 μL of 0.05 mol/L phosphate buffer (pH 7.0). The extract was filtered through a 0.22-μm filter and a portion of 20 μL was injected into a Waters 2695 Series HPLC system equipped with fluorescence detector. Separation was performed on a C18 column (150 mm × 2.1 mm ID, 5 μm) from Elite (Dalian, China). A 5.0 mm-C18 security guard column from Phenomenex (Torrance, USA) was attached to the analytical column. A solvent system consisting of acetonitrile and formic acid-water was used. The gradient profile of the mobile phase was from 5 to 40% (v/v) of acetonitrile and 95 to 60% (v/v) of formic acid over 20 min. The flow rate was 0.4 mL/min.

cDNA cloning and sequence analysis of $OsFMO_{(t)}$

Primers (table 1) were designed using the public rice sequence database (http://www.ncbi.nih.gov) for Nipponbare (*O. sativa* L. *japonica*) to amplify full-length genomic DNA and cDNA from $OsFMO_{(t)}$. Using a reverse-transcription kit (Invitrogen, Carlsbad, USA), single-stranded cDNA of the $OsFMO_{(t)}$ gene was first synthesized and used as a template for amplification of the full-length cDNA. PCR was conducted in 50 μL reaction volumes that included 2.0 μL templates, 5.0 μL 10× buffer, 3.0 μL 25 mM $MgSO_4$, 5.0 μL 2 mM dNTPs, 0.5 μL of each primer (5 μM) and 1.0 μL KOD-plus *Taq* DNA polymerase; distilled water was added to a total volume of 50 μL. In the amplification of cDNA, *actin* primers (0.5 μL each) were also added as internal standard. The PCR programme was as follows: 94°C for 2 min; 30 cycles of 94°C for 30 s, 55°C for 60 s, 68°C for 2 min; and 68°C for 10 min. The PCR products were examined by electrophoresis using a 1% agarose gel.

The generated nucleotide sequences were verified using BLAST tools (http://blast.ncbi.nlm.nih.gov/). The MEGA 4.0 program (http://www.megasoftware.net/mega4/mega.html) was used for phylogenetic analysis and a phylogenetic tree was constructed using the neighbour-joining (NJ) method (bootstrapping with 1000 replicates). The resulting nucleotide sequences were deposited in GenBank under accession numbers HQ443270 and HQ443271.

Construction of the $OsFMO_{(t)}$ overexpression vector

To directly investigate the differential effect of overexpression, we constructed a transgene driven by the maize *pUbi* promoter. First, the full-length cDNA of $OsFMO_{(t)}$ was amplified from QHZ plants by RT-PCR and digested with *Bam*HI and *Spe*I, then inserted into a *pUbi* promoter-driven-pCAMBIA1380, which was used as the binary vector for *Agrobacterium*-mediated transformation of Zhonghua 11 (*O. sativa* L. *japonica*). The *pUbi::OsFMOt-Ov*, the overexpression fusion vector with the promoter *Ubi* and $OsFMO_{(t)}$ gene, was verified by sequencing.

Rice transformation and growth conditions

Calli derived from seeds of Zhonghua 11 (*O. sativa* L. *japonica*) were used for genetic transformation. GUS-fusion constructs and $OsFMO_{(t)}$ overexpression recombinant vectors were stably transferred into rice using the *Agrobacterium*-mediated transformation according to the procedures of Hiei *et al.* (1994). All transformants were confirmed for the presence of the foreign *GUS* or hygromycin-resistant gene (*Hpt*) by PCR and Southern blotting. Transgenic plants were transplanted into fields and planted under routine growth conditions.

Expression analysis of $OsFMO_{(t)}$

To understand the expression pattern of $OsFMO_{(t)}$ in QHZ, appropriate primers (table 1) were designed according to the sequences in the region close to $OsFMO_{(t)}$, so as to amplify the promoter region. The amplified fragment was then double digested with *Bam*HI and *Hin*dIII and inserted into pCAMBIA1300G to construct *pOsFMOt::GUS*, the fusion vector with an $OsFMO_{(t)}$ promoter and a GUS reporter gene. The fusion vector was transferred into Zhonghua 11 (*O. sativa* L. *japonica*) by *Agrobacterium*-mediated transformation, and the empty vector pCAMBIA1300G was used as a negative control. Histochemical staining of transformants and observations were conducted according to the methods described by Jefferson *et al.* (1987). The histochemical GUS assay was performed in a staining solution containing 1.0 mg/mL 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide in 0.1 M sodium phosphate buffer (pH 7.0), 10 mM Na_2 EDTA,

Table 1. PCR primers used in present study.

Primer	Primer sequences (underlined endonucleases)	Primer purpose
OsFMOt-g	F: 5′-atgcaggggcagcagaagc R: 5′-ctacaccgaggagatgttgg	Primers used for full-length genomic DNA cloning
OsFMOt-c	F: 5′-catcgatcgatcgaccgatc R: 5′-cggcctgtggacgactaca	Primers used for full-length cDNA cloning
RT	F: 5′-gggaaacatcaagatagtaccg R: 5′-agaatcccacgcagtagagc	Primers used for expression analysis
pOsFMOt	F: 5′-aaaaaaggatccaagggctcgcattgtcacc (*Bam*HI) R: 5′-aaaaaaaagcttcccgaggaagctctgttctcgta (*Hin*dIII)	Primers used for promoter cloning
OsFMOt-Ov	F: 5′-aaaaaaggatccagtgcagcgtgac (*Bam*HI) R: 5′-aaaaaaactagtatgcgactccggccatg (*Spe*I)	Primers used for overexpression vector construction
Hpt	F: 5′-gtctccgacctgatgcagctctcgg R: 5′-gtccgtcaggacattgttggag	Primers used for *HPT* gene detection of transformants
Gus	F: 5′-gtcacgccgtatgttattgc R: 5′-catctcttcagcgtaagggt	Primers used for *GUS* gene detection of transformants
Act	F: 5′-gacattcagcgttccagccatgtat R: 5′-tggagcttccatgccgatgagagaa	Primers used for *Actin* gene control in RT-PCR

0.5 mM potassium ferricyanide/ferrocyanide and 0.1% Triton X-100. Samples were infiltrated under vacuum for 10 min and then incubated overnight at 37°C. The staining solution was removed, and the samples were cleared in 70% ethanol.

RT-PCR analysis was performed similar to the procedure described above in the 'cDNA cloning and sequence analysis of *OsFMO*$_{(t)}$' section, except that the number of cycles was increased to 35.

Results and discussion

Isolation of OsFMO$_{(t)}$ *and gene sequence analysis*

The full-length genomic DNA of *OsFMO*$_{(t)}$ was found to be 2504 bp (GenBank accession no. HQ443270). Its alignment with the *FMO* gene (Os03g0162000) from Nipponbare (*O. sativa* L. *japonica*) from the rice database (http://www.ncbi.nih.gov) showed that they were completely identical in base sequence. The full-length cDNA sequence of *OsFMO*$_{(t)}$ (1266 bp) (GenBank accession no. HQ443271) alignment with the cDNA clone (accession no. AK072466) of the *FMO* gene in Nipponbare showed that these two sequences were also identical. Comparison of the genomic and cDNA sequences of the *OsFMO*$_{(t)}$ gene, revealed that the gene consisted of four exons and three introns (figure 2A).

Previous studies (Schlenk 1998; Krueger and Williams 2005) have indicated that almost all FMO proteins are highly conserved in different kinds of organisms and share a highly conserved domain. Homologic search of *FMO* genes (http://www.ncbi.nlm.nih.gov/) showed that they are widely distributed in nematodes, bacteria, mammals (including humans) and higher plants. The deduced amino acid sequence of OsFMO$_{(t)}$ containing 422 residues was found to be similar to OsYUCCA1 in rice (Yamamoto *et al.* 2007), YUCCA in *A. thaliana* (Zhao *et al.* 2001) and FLOOZY in *Petunia* (Santamaria *et al.* 2002) with a sequence identity of 71, 71 and 70%, respectively. Kubo *et al.* (1997) and Hou *et al.* (2011) demonstrated that the conserved domain of FMO contained two basic conserved motifs i.e., a FAD-binding motif and a NADP-binding motif, both of which have the same characteristic structure of 'G×G××G' in their amino acid sequences which is essential for binding with flavin-adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NADP), respectively, and hence plays a key role in FMO-catalyzed processes. The results of multiple sequence alignment (see figure 2B) among OsFMO$_{(t)}$ and its homologous proteins YUCCA (Zhao *et al.* 2001), FLOOZY (Santamaria *et al.* 2002) and OsYUCCA1 (Yamamoto *et al.* 2007) showed that these two basic motifs are also highly conserved among these proteins, possessing the same characteristics in base sequence as reported (Kubo *et al.* 1997; Hou *et al.* 2011).

By searching the rice genome database (http://www.ncbi.nlm.nih.gov/) with the amino acid sequence of OsFMO$_{(t)}$ as query, 32 other putative *FMO* genes located on different rice chromosomes were found. Results (figure 3) from phylogenetic analysis of these FMO proteins in rice and other homologous FMO proteins identified in *A. thaliana* and *Petunia* indicated that the FMO family members in the rice genome were divided into two clades designated as clade I and clade II. In clade I, 19 members of the rice FMO family, excluding OsFMO$_{(t)}$, were clustered with some FMO-like proteins from *A. thaliana* indicating that clade I was more distant to OsFMO$_{(t)}$ in evolution and probably had different functions relative to OsFMO$_{(t)}$ in rice. In clade II, OsFMO$_{(t)}$ which was investigated in the present study and OsYUCCA1, identified by Yamamoto *et al.* (2007), were found to be clustered together with YUCCA, YUCCA2 and YUCCA3 from *A. thaliana* and FLOOZY from *Petunia*, suggesting that these proteins are closer in evolutionary distance.

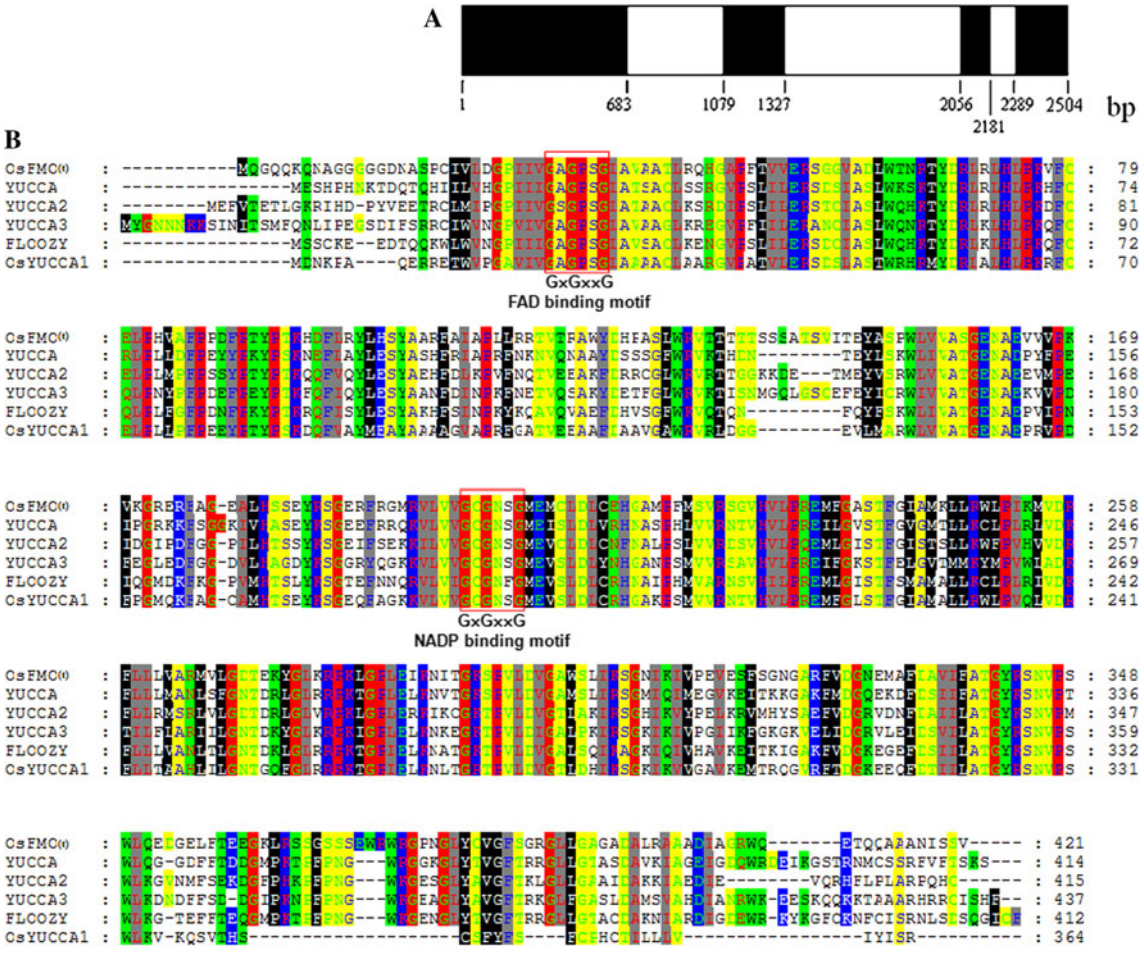

Figure 2. (A) Exons and introns of the *OsFMO$_{(t)}$* gene. The black and white regions indicate exons and introns, respectively. (B) Comparison of amino acid sequences among OsFMO$_{(t)}$ and the homologous FMO proteins in plants. Two basic conserved motifs are indicated by boxes. YUCCA, YUCCA2 and YUCCA3, FMO-like proteins in *A. thaliana*; FLOOZY, FMO-like protein in *Petunia*; OsYUCCA1, FMO-like protein in *O. sativa*.

FMO usually participates in xenobiotic metabolism in animals (Krueger and Williams 2005; Hou *et al.* 2011). In plants, FMO proteins play an important role in biosynthesis of endogenous IAA, as demonstrated for YUCCA (Zhao *et al.* 2001). FMO catalyzes the biochemical reaction that converts IPA to IAA, which is the limiting step of the major pathways for Trp-dependent IAA biosynthesis in plants (Zhao 2012). In our study, *OsFMO$_{(t)}$*, a *FMO* gene encoding flavin monooxygenase, was cloned and characterized from the wild type variety (QHZ) of *γ-rl*, a rice rolled-leaf mutant. The sequence analysis and phylogenetic tree demonstrated that *OsFMO$_{(t)}$* has high sequence identity with *FMO* genes such as *YUCCA* (Zhao *et al.* 2001), *FLOOZY* (Santamaria *et al.* 2002) and *OsYUCCA1* (Yamamoto *et al.* 2007); and also showed a closer evolutionary relationship with these genes compared to other rice FMO proteins. Therefore, *OsFMO$_{(t)}$* may exert functions similar to those of *FMO* genes in different plants and might play critical roles in auxin biosynthesis and regulating growth and development of rice.

Phenotypes and gene expression analysis of rice transformants with pUbi::OsFMOt-Ov

To investigate the possible role(s) of *OsFMO$_{(t)}$*, we transformed *pUbi::OsFMOt-Ov* into calli of rice (supplementary data in electronic supplementary material at http://www.ias.ac.in/jgenet/). As shown in figure 4C, all hygromycin-resistant (*Hpt*$^+$) calli (45 total) that were transformed with the construct would not proliferate and differentiate normally in the auxin-supplemented differentiation medium (NAA added at 1.0 mg/L), and large numbers of hairy roots were observed. These calli ultimately browned and died (figure 4C). On the other hand, when vector pCAMBIA1380 without *OsFMO$_{(t)}$* (control) was transformed, the calli (*Hpt*$^+$, 33 total) regenerated normal shoots in the same medium (figure 4A). Further, when calli overexpressing *OsFMO$_{(t)}$* were grown in the medium without NAA (figure 4D), around 42% of them sustained for around two months, though eventually still unable to regenerate into shoots. Additionally, this type of *Hpt*$^+$ calli showed significant phenotypes, such as large numbers of adventitious

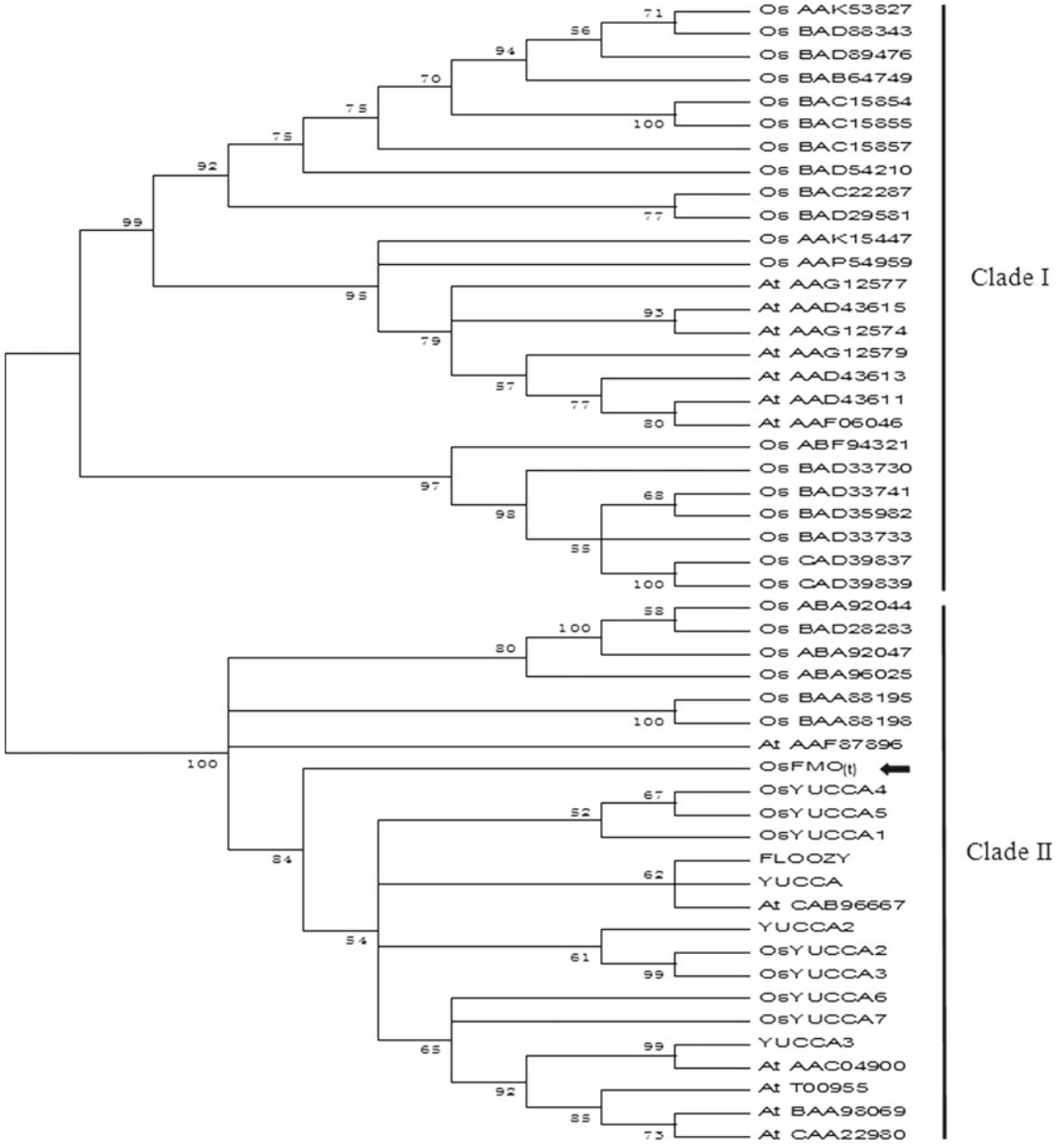

Figure 3. Phylogenetic tree of the homologous FMO-like proteins from *A. thaliana*, *Petunia* and *O. sativa*. At, *A. thaliana*; Os, *O. sativa*; YUCCA, YUCCA2 and YUCCA3, FMO-like proteins in *A. thaliana*; FLOOZY, FMO-like protein in *Petunia*; OsYUCCA1∼7, FMO-like proteins in *O. sativa*. Bootstrap values (1000 replicates) are displayed near the nodes. The scale indicates the number of amino acid residue substitutions per site.

roots growing (figure 4a, red solid arrow), abundant root hairs (figure 4, e&f), malformed leaves and stems (figure 4, a,b&c, red solid arrow) and adventitious roots on the malformed stems (figure 4, c&d), which were associated with IAA overproduction phenotypes reported previously (Zhao *et al.* 2001; Yamamoto *et al.* 2007).

Using RT-PCR (35 cycles), expression of $OsFMO_{(t)}$ was analysed within two of these survived calli (Hpt^{+}) transformed by *pUbi::OsFMOt-Ov*. The expression level of $OsFMO_{(t)}$ was different between them (shown in figure 4E), suggesting an expression variation caused by different transformation events. Expression product was not detected in the Hpt^{+} calli transformed with the control vector, indicating that the expression level of this gene is low in normal tissues. In addition, the IAA contents in the two survived calli increased with different levels as compared to the control (figure 4F). These results indicated that transformation of *pUbi::OsFMOt-Ov* did increase $OsFMO_{(t)}$ transcription level in the calli, and the excessive auxin produced in these transformants due to the overexpression of $OsFMO_{(t)}$ (which encodes FMO associated with IAA biosynthesis *in vivo*), could seriously affect shoot regeneration

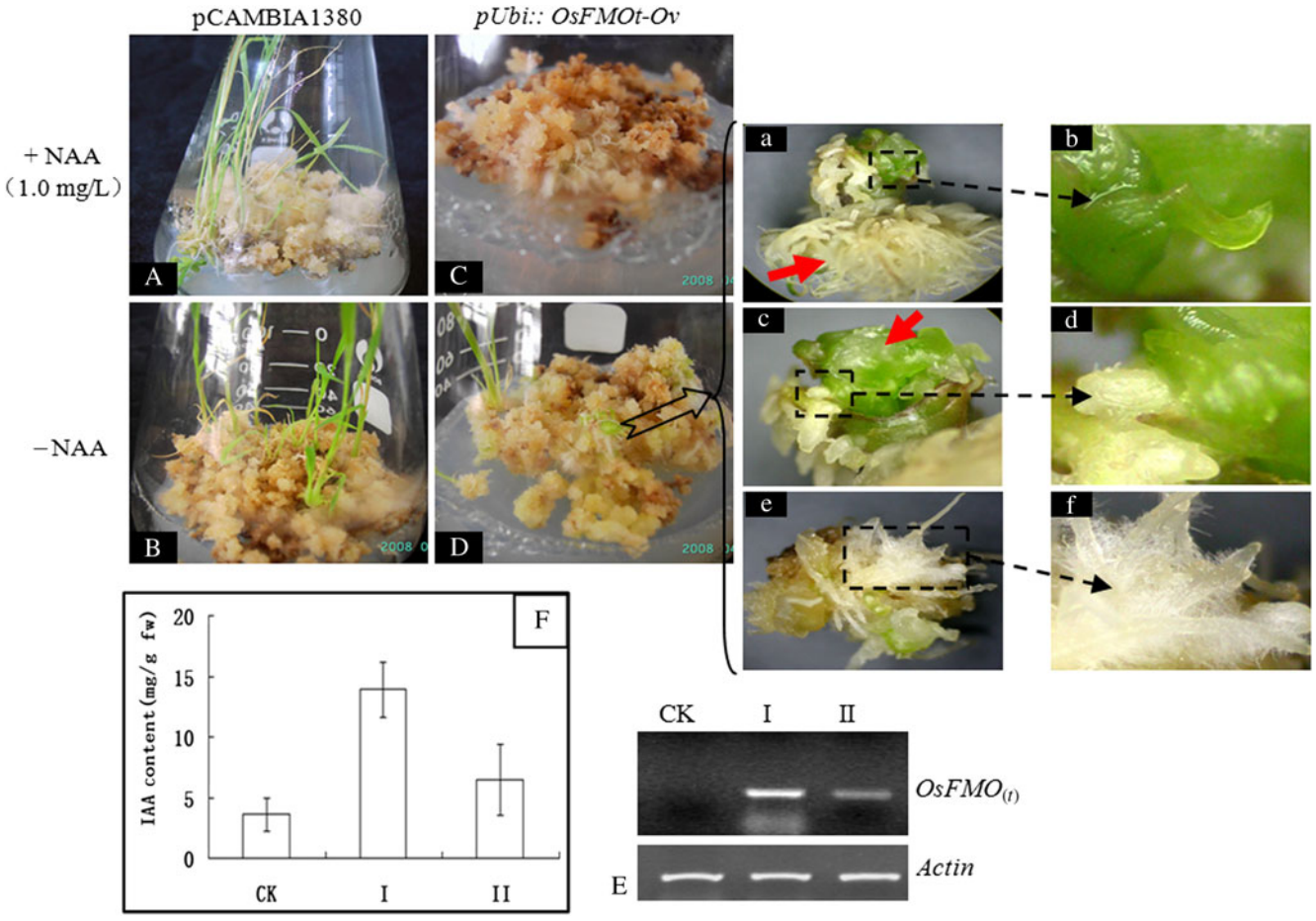

Figure 4. Phenotypes of hygromycin-resistant (*Hpt*$^+$) calli transformed by *pUbi::OsFMOt-Ov*. (A–D) Differentiation cultivation of the *Hpt*$^+$ calli (A & B, transformed by pCAMBIA1380, the negative control; (C & D), transformed by *pUbi::OsFMOt-Ov*); a–f, IAA-excessive phenotypes from *OsFMO*$_{(t)}$ overexpression (a, red solid arrow shows adventitious roots; b, abnormal leaf; c, red solid arrow shows abnormal stem; d, adventitious roots on abnormal stem; e & f, adventitious roots with abundant root hairs); (A & C), medium with exogenous naphthylacetic acid (NAA) at a final concentration of 1.0 mg/L; (B & D), medium without exogenous NAA; (E), RT-PCR analysis (35 cycles) of *OsFMO*$_{(t)}$; (F), IAA analysis of rice calli. CK, the *Hpt*$^+$ callus transformed by pCAMBIA1380; I & II, two *Hpt*$^+$ calli transformed by *pUbi::OsFMOt-Ov*.

which, in turn, might inhibit normal growth of the calli that ultimately led to the death of the transformants.

Genetic analyses with overexpression and loss of function mutations revealed that *FMO* plays an important role in *de novo* IAA synthesis, both in dicots (*A. thaliana*) and monocots (rice) (Cheng *et al.* 2006, 2007; Zhao 2010). Overexpression of *FMO* genes is usually accompanied by IAA-excessive phenotypes in plants. For example, overexpression of *YUCCA* in *A. thaliana* (Zhao *et al.* 2001) led to vigorous growth of explants and emergence of large numbers of root hairs in MS medium without IAA. *OsYUCCA1*-overexpression and *NAL7*-overexpression in rice also led to the emergence of identical auxin-excessive phenotypes, such as the appearance of large numbers of hairy roots and difficulty in regenerating transgenic seedlings (Yamamoto *et al.* 2007; Fujino *et al.* 2008). Our study reiterates this fact, as seen from figure 4C, where calli from transformants overexpressing *OsFMO*$_{(t)}$ showed browning and eventually died. These calli showed the IAA overproduction phenotypes even in the absence of NAA from the medium (figure 4D, a–f), suggesting that *OsFMO*$_{(t)}$ plays a key role in biosynthesis of endogenous IAA in rice.

Spatio-temporal study using histochemical staining and RT-PCR

By using rice plants developed from calli transformed with *pOsFMOt::GUS* (supplementary data in electronic supplementary material), expression of *OsFMO*$_{(t)}$ in various tissues was observed. As shown in figure 5A, GUS activity was detected in roots and tender leaves of seedlings five days after germination, and was high in root tips and the prophyll leaf tip (figure 5a′). The intensity of GUS staining was also strongly enhanced in shoot apex (figure 5b′) and lateral root tips (figure 5c′) at 15-day-old seedling stage. Intense staining was also observed in some parts of the adult plant, e.g. tender auricles (figure 5e′), filaments (figure 5f′), paleae

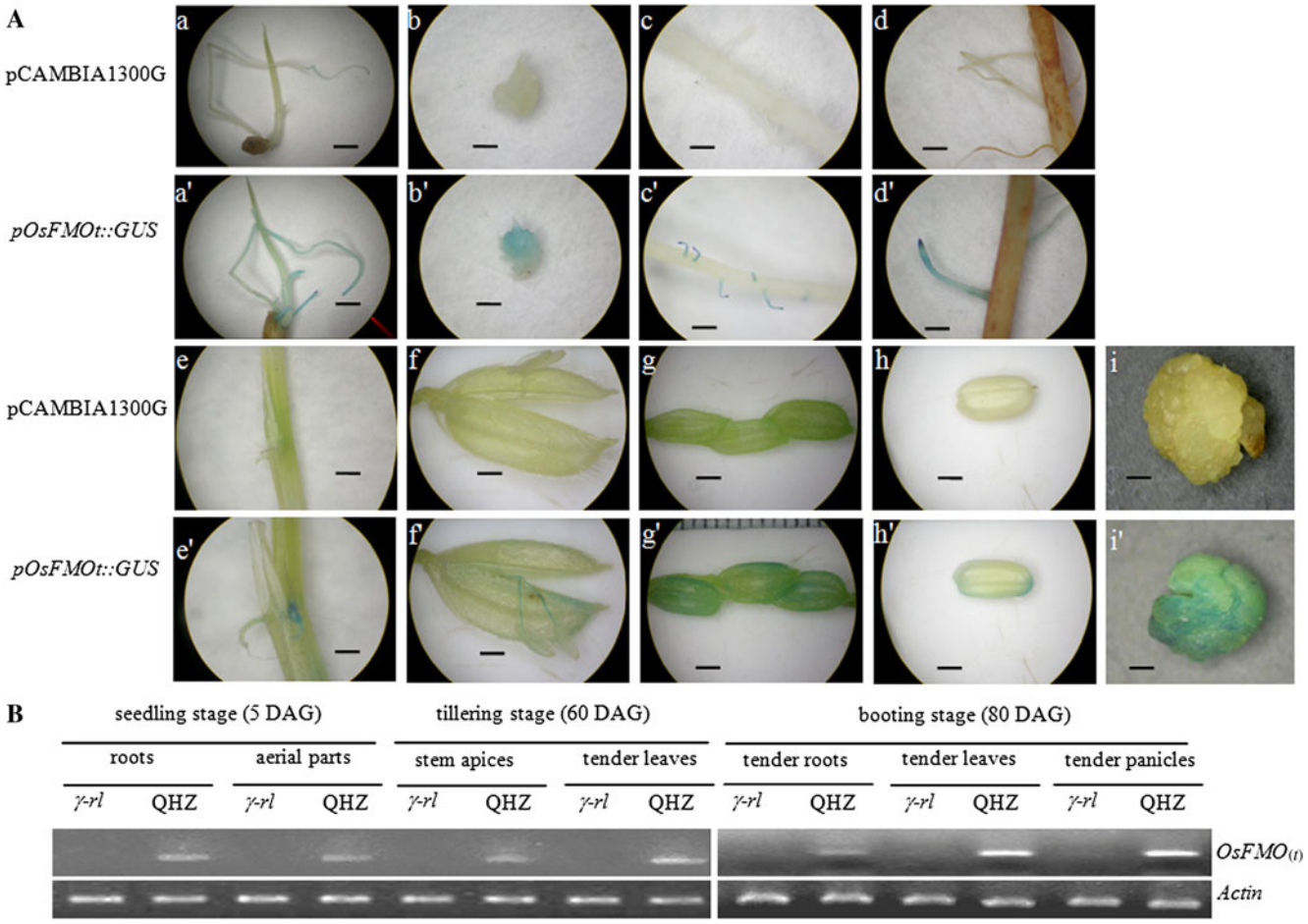

Figure 5. (A) The results of histochemical staining in rice plants transformed with *pOsFMOt::GUS*. (a–i) Negative control; (a′–i′) various tissues of transgenic rice plants harbouring *pOsFMOt::GUS*; (a & a′) seedling at five days after germination; (b & b′) stem apex at 15-day-old seedling stage; (c & c′) lateral roots at 15-day-old seedling stage; (d & d′) lateral roots on older root at tillering stage; (e & e′) auricles at 30-day-old seedling stage; (f & f′) spikelet and filaments at heading stage; (g & g′) spikelets at grain filling stage; (h & h′) seed coat at grain filling stage; (i & i′) rice calli at preliminary differentiation stage. Scale bar = 2 mm. (B) Reverse transcription pattern of $OsFMO_{(t)}$ in different organs (35 cycles). *γ-rl*, the rolled-leaf mutant; QHZ, the wild type variety; DAG: day after germination.

and lemmas (figure 5g′), as well as episperm (figure 5h′) of seeds during the grain filling stage and lateral roots on the matured roots (figure 5d′); but not in other matured parts such as the matured leaves. In summary, GUS staining was mainly observed in the tender parts of plants undergoing active growth and cell division and became undetectable in the old tissues. The results indicated that $OsFMO_{(t)}$ expression was closely associated with the development of tender organs during rice growth and development.

RT-PCR (35 cycles) was also performed to further analyse the $OsFMO_{(t)}$ gene expression in tender organs at different stages, including roots and aerial parts at the seedling stage (five days after germination, DAG); stem apices and tender leaves at the active tillering stage (60 DAG); and tender roots, leaves and panicles at the booting stage (80 DAG). The rice housekeeping gene *Actin* was amplified at the same time as an internal standard control as shown in figure 5B. $OsFMO_{(t)}$ gene expression products were amplified in tender roots, leaves and panicles of QHZ (wild type variety), indicating that $OsFMO_{(t)}$ was expressed in the young parts of the seedlings and plants, with a higher expression in tender leaves and panicles at the booting stage. However, amplifications of the specific products failed in more mature tissues with slow cell growth such as in matured leaves and old roots (data not shown). This might be due to the fact that the expression of this gene in these mature tissues was too low to be detected by RT-PCR. Our previous study (Yi *et al.* 2007) showed that a large deletion might occur around the $OsFMO_{(t)}$ locus in the genome of *γ-rl*, the rolled-leaf mutant. Consistent with our previous findings, we did not observe expression of this gene in any of the organs of *γ-rl*. (figure 5B).

For a long time, the predominant view has been that the shoots were the only source of auxin biosynthesis. It has been postulated that polar auxin transport is responsible for auxin distribution to other parts of the plant and auxin gradient maintenance (Tanaka *et al.* 2006; Grieneisen *et al.* 2007). However, recent findings clearly demonstrate that *de novo*

auxin biosynthesis is regulated both temporally and spatially and contributes to local auxin gradient generation and maintenance (Cheng *et al.* 2007; Stepanova *et al.* 2008; Tao *et al.* 2008; Zhao 2010, 2012). FMO genes such as TAA and YUC expressed with temporal and spatial precision in all organs including shoots, roots and flowers and leaves in *Arabidopsis* and each organ appears to be selfsufficient in terms of controlling auxin gradients for growth and development (Zhao 2010, 2012). Our results from histochemical staining and RT-PCR indicate that the expression of $OsFMO_{(t)}$ varies in different organs as well as different developing stages. Generally, its expression level is higher in tender tissues with faster growth, especially in root tips and shoot apices with vigorous cell division; which are the major sources of endogenous IAA in plants. Since FMOs are involved in catalyzing the limiting step of Trp-dependent *de novo* IAA biosynthesis, the higher expression of the $OsFMO_{(t)}$ gene restricted to these small parts suggests that *de novo* auxin biosynthesis regulated by this gene is possibly highly localized and local auxin biosynthesis appears to be an important aspect of shaping local auxin concentrations for regulating organ growth and development. Mutations in this gene might affect normal development of organs such as leaves.

OsYUCCA1 was the first reported FMO gene in rice and is located on chromosome 1 (Yamamoto *et al.* 2007). $OsFMO_{(t)}$, located on chromosome 3 (Yi *et al.* 2007), may play a role that is different from *OsYUCCA1*. *OsYUCCA1* expressed highly in all the investigated tissues; while $OsFMO_{(t)}$ expression were detected only in young and fast growth organs, as demonstrated in this study. Transformed calli with *OsYUCCA1* overexpression could successfully regenerate into seedlings despite of abnormal phenotypes. The calli overexpressing $OsFMO_{(t)}$, however, survived only on medium without NAA and failed to regenerate, indicated that $OsFMO_{(t)}$ and *OsYUCCA1* had similarity as well as differences in tissue expression specificity and functions.

Fujino *et al.* (2008) reported the spontaneous rice narrow-leaf mutant, *nal7*, was resulted from a single base mutation in *NAL7* also located on chromosome 3. Overexpression of both $OsFMO_{(t)}$ and *NAL7* gene could produce same IAA-excessive phenotypes. Preliminary studies indicated that a large deletion in the vicinity of $OsFMO_{(t)}$ occurred in the genome of *γ-rl* mutant (Yi *et al.* 2007). RT-PCR anaylsis could not detect expression of $OsFMO_{(t)}$ in different organs of this mutant. On the other hand, expression of *NAL7* was detected in various organs in the *nal7* mutant. In some organs such as in panicles, its expression in the mutant was even higher than that in the wild type (Fujino *et al.* 2008). In addition, the phenotypes of *γ-rl* and *nal7* mutants were also different, *γ-rl* displayed as rolled leaf in the whole plant, reduced flag-leaf area, increased tiller number, enlarged midrib, reduced lateral veins number, reduced total root length, root number (data not shown), etc.; while *nal7* only observed with narrowed leaf, abnormal bulliform cells, but no changes in its root development and tiller number (Fujino *et al.* 2008). The results indicated that $OsFMO_{(t)}$ and *NAL7* had similarity as well as differences in mutation phenotypes and functions.

In summary, the $OsFMO_{(t)}$ gene was cloned and characterized in the present study. The results from sequence identity and phylogenetics analyses suggested that this gene possessed similar functions to that of other homologous *FMO* genes in plants. The results from overexpression transformation and gene expression analysis indicated that $OsFMO_{(t)}$ is involved in endogenous synthesis of IAA in rice and expressed in young plant tissues with vigorous growth and cell division. Our study suggests a possible essential role for $OsFMO_{(t)}$ in local biosynthesis of IAA and maintenance of local IAA concentrations, which are undoubtedly critical for regulating growth and development of rice.

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References

Cheng Y., Dai X. and Zhao Y. 2006 Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in *Arabidopsis*. *Genes Dev.* **20**, 1790–1799.

Cheng Y., Dai X. and Zhao Y. 2007 Auxin synthesized by the YUCCA flavin monooxygenases is essential for embryogenesis and leaf formation in *Arabidopsis*. *Plant Cell* **19**, 2430–2439.

Fujino K., Matsuda Y., Ozawa K., Nishimura T., Koshiba T., Fraaije M. W. and Sekiguchi H. 2008 NARROW LEAF 7 controls leaf shape mediated by auxin in rice. *Mol. Genet. Genomics* **279**, 499–507.

Gallavotti A., Barazesh S., Malcomber S., Hall D., Jackson D., Schmidt R. J. and McSteen P. 2008 Sparse inflorescence 1 encodes a monocot specific YUCCA-like gene required for vegetative and reproductive development in maize. *Proc. Natl. Acad. Sci. USA* **105**, 15196–15201.

Grieneisen V. A., Xu J., Marée A. F., Hogeweg P. and Scheres B. 2007 Auxin transport is sufficient to generate a maximum and gradient guiding root growth. *Nature* **449**, 1008–1013.

Hiei Y., Ohta S., Komari T. and Kumashiro T. 1994 Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J.* **6**, 271–282.

Hou X., Liu S., Pierri F., Dai X., Qu L. and Zhao Y. 2011 Allelic analyses of the *Arabidopsis* YUC1 locus reveal residues and domains essential for the functions of YUC family of flavin monooxygenases. *J. Integr. Plant Biol.* **53**, 54–62.

Jefferson R., Kavanagh T. and Bevan W. 1987 GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901–3907.

Krueger S. K. and Williams D. E. 2005 Mammalian flavin-containing monooxygenases: structure/function, genetic polymorphisms and role in drug metabolism. *Pharmacol. Ther.* **106**, 357–387.

Kubo A., Itoh S. and Itoh K. 1997 Determination of FAD-binding domain in flavin-containing monooxygenase 1 (FMO1). *Arch. Biochem. Biophys.* **345**, 271–277.

Ljung K., Hul A. K., Kowalczyk M., Marchant A., Celenza J., Cohen J. D. and Sandberg G. 2002 Biosynthesis, conjugation, catabolism and homeostasis of indole-3-acetic acid in *Arabidopsis thaliana*. *Plant Mol. Biol.* **50**, 309–332.

Lu J., Lang Y., Zhang Z. and Zhu Q. 2005 Comparison of plant morphology and population structure and photosynthetic characteristics among a series of near-isogenic lines with different leaf rolling index in rice. *J. Yangzhou Univ.* (*Agri. Life Sci. Edition*) **26**, 56–60.

Luo Z., Yang Z., Zhong B., Li Y., Xie R., Zhao F. *et al.* 2007 Genetic analysis and fine mapping of a dynamic rolled leaf gene, RL10(t), in rice (*Oryza sativa* L.). *Genome* **50**, 811–817.

Mashiguchi K., Tanaka K., Sakai T., Sugawara S., Kawaide H., Natsume M. *et al.* 2011 The main auxin biosynthesis pathway in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **108**, 18512–18517.

Murray M. G. and Thompson W. F. 1980 Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* **8**, 4321–4325.

Normanly J., Grisafi P., Fink G. R. and Bartel B. 1997 *Arabidopsis* mutants resistant to the auxin effects of indole-3-acetonitrile are defective in the nitrilase encoded by the NIT1 gene. *Plant Cell* **9**, 1781–1790.

Palme K., Hesse T., Moore I., Campos N., Feldwisch J., Garbers C. *et al.* 1991 Hormonal modulation of plant growth: the role of auxin perception. *Mech. Dev.* **33**, 97–106.

Santamaria R. T., Bliek M., Ljung K., Sandberg G., Mol J. N., Souer E. and Koes R. 2002 FLOOZY of petunia is a flavin-monooxygenase-like protein required for the specification of leaf and flower architecture. *Genes Dev.* **16**, 753–763.

Schlenk D. 1998 Occurrence of flavin-containing monooxygenases in non-mammalian eukaryotic organisms. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* **121**, 185–195.

Shao Y., Chen Z., Zhang Y., Chen E., Qi D., Miao J. and Pan X. 2005 One major QTL mapping and physical map construction for rolled leaf in rice. *Acta Genet. Sin.* **32**, 501–506.

Shi Z., Wang J., Wan X., Shen G., Wang X. and Zhang J. 2007 Over-expression of rice OsAGO7 gene induces upward curling of the leaf blade that enhanced erect-leaf habit. *Planta* **226**, 99–108.

Stepanova A. N., Robertson-Hoyt J., Yun J., Benavente L. M., Xie D., Dolezal K. *et al.* 2008 TAA1-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. *Cell* **133**, 177–191.

Sugawara S., Hishiyama S., Jikumaru Y., Hanada A., Nishimura T., Koshiba T. *et al.* 2009 Biochemical analyses of indole-3-acetaldoxime-dependent auxin biosynthesis in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **106**, 5430–5435.

Tanaka H., Dhonukshe P., Brewer P. B. and Friml J. 2006 Spatiotemporal asymmetric auxin distribution: a means to coordinate plant development. *Cell Mol. Life Sci.* **63**, 2738–2754.

Tao Y., Ferrer J. L., Ljung K., Pojer F., Hong F., Long J. A. *et al.* 2008 Rapid synthesis of auxin via a new tryptophan dependent pathway is required for shade avoidance in plants. *Cell* **133**, 164–176.

Vande Broek A., Gysegom P., Ona O., Hendrickx N., Prinsen E., Van Impe J. and Vanderleyden J. 2005 Transcriptional analysis of the Azospirillum brasilense indole-3-pyruvate decarboxylase gene and identification of a cis-acting sequence involved in auxin responsive expression. *Mol. Plant Microbe Interact.* **18**, 311–323.

Wang R., Xiao L., Lin W., Cao Y. and Bo X. 2002 High performance liquid chromatographic determination of internal hormones in inter-subspecific hybrid rice. *Chin. J. Chromatography* **20**, 148–150.

Won C., Shen X., Mashiguchi K., Zheng Z., Dai X., Cheng Y. *et al.* 2011 Conversion of tryptophan to indole-3-acetic acid by TRYPTOPHAN AMINOTRANSFERASES OF *ARABIDOPSIS* and YUCCAS in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **108**, 18518–18523.

Yamamoto Y., Kamiya N., Morinaka Y., Matsuoka M. and Sazuka T. 2007 Auxin biosynthesis by the YUCCA genes in rice. *Plant Physiol.* **143**, 1362–1371.

Yan C., Yan S., Zhang Z., Liang G., Lu J. and Gu M. 2006 Genetic analysis and gene fine mapping for a rice novel mutant (rl9(t)) with rolling leaf character. *Chin. Sci. Bull.* **51**, 63–69.

Yi J., Zhuang C., Wang X., Cao Y., Liu Y. and Mei M. 2007 Genetic analysis and molecular mapping of a rolling leaf mutation gene in rice. *J. Integr. Plant Biol.* **49**, 1746–1753.

Zhang G., Xu Q., Zhu X., Qian Q. and Xue H. 2009 SHALLOT-LIKE1 is a KANADI transcription factor that modulates rice leaf rolling by regulating leaf abaxial cell development. *Plant Cell* **21**, 719–735.

Zhao Y. 2010 Auxin biosynthesis and its role in plant development. *Annu. Rev. Plant Biol.* **61**, 49–64.

Zhao Y. 2012 Auxin biosynthesis: a simple two-step pathway converts tryptophan to indole-3-acetic acid in plants. *Mol. Plant* **5**, 334–338.

Zhao Y., Christensen S. K., Fankhauser C., Cashman J. R., Cohen J. D., Weige D. and Chory J. 2001 A role for flavin monooxygenase-like enzymes in auxin biosynthesis. *Science* **291**, 306–309.

Zhao Y., Hull A. K., Gupta N. R., Goss K. A., Alonso J., Ecker J. R., Normanly J., Chory J. and Celenza J. L. 2002 Trp-dependent auxin biosynthesis in *Arabidopsis*: involvement of cytochrome P450s CYP79B2 and CYP79B3. *Genes Dev.* **16**, 3100–3112.

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