Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Tissue-specific expression, developmentally and spatially regulated alternative splicing, and protein subcellular localization of *OsLpa1* in rice*

Hai-ping LU[1], Wei-qin PANG[1], Wen-xu LI[1], Yuan-yuan TAN[1], Qing WANG[2], Hai-jun ZHAO[1], Qing-yao SHU[†‡1]

([1]*State Key Laboratory of Rice Biology, Institution of Crop Science, Zhejiang University, Hangzhou 310029, China*)

([2]*Wuxi Hupper Bioseed Technology Academy Ltd., Wuxi 214000, China*)

[†]E-mail: qyshu@zju.edu.cn

Received Aug. 29, 2015; Revision accepted Nov. 30, 2015; Crosschecked Jan. 13, 2016

Abstract: The *OsLpa1* gene (LOC_Os02g57400) was identified to be involved in phytic acid (PA) metabolism because its knockout and missense mutants reduce PA content in rice grain. However, little is known about the molecular characteristics of *OsLpa1* in rice and of its homologues in other plants. In the present study, the spatial pattern of *OsLpa1* expression was revealed using *OsLpa1* promoter::GUS transgenic plants (GUS: β-glucuronidase); GUS histochemical assay showed that *OsLpa1* was strongly expressed in stem, leaf, and root tissues, but in floral organ it is expressed mainly and strongly in filaments. In seeds, GUS staining was concentrated in the aleurone layers; a few blue spots were observed in the outer layers of embryo, but no staining was observed in the endosperm. Three *OsLpa1* transcripts (*OsLpa1.1*, *OsLpa1.2*, *OsLpa1.3*) are produced due to alternative splicing; quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analysis revealed that the abundance of *OsLpa1.3* was negligible compared with *OsLpa1.1* and *OsLpa1.2* in all tissues. *OsLpa1.2* is predominant in germinating seeds (about 5 times that of *OsLpa1.1*), but its abundance decreases quickly with the development of seedlings and plants, whereas the abundance of *OsLpa1.1* rises and falls, reaching its highest level in 45-d-old plants, with abundance greater than that of *OsLpa1.2* in both leaves and roots. In seeds, the abundance of *OsLpa1* continuously increases with seed growth, being 27.5 and 15 times greater in 28-DAF (day after flowering) seeds than in 7-DAF seeds for *OsLpa1.1* and *OsLpa1.2*, respectively. Transient expression of chimeric genes with green fluorescence protein (GFP) in rice protoplasts demonstrated that all proteins encoded by the three *OsLpa1* transcripts are localized to the chloroplast.

Key words: *OsLpa1*, Low phytic acid, Expression pattern, Alternative splicing, Subcellular localization
http://dx.doi.org/10.1631/jzus.B1500205 **CLC number:** Q946.2

1 Introduction

Phytic acid (PA, *myo*-inositol-1,2,3,4,5,6-hexa-*kis*phosphate or $InsP_6$) is the primary storage form of phosphorus (P), accounting for 65%–85% of total P in seeds (Raboy, 2003). Under physiological conditions, $InsP_6$ strongly binds to metallic cations forming a mixed salt known as phytate. During seed germination, phytate is broken down to *myo*-inositol, inorganic phosphate, and minerals for seedling growth. $InsP_6$ can regulate a number of nuclear processes including messenger RNA (mRNA) export, DNA repair, and RNA editing (York, 2006; Monserrate and York, 2010; Lee *et al*., 2015), and, as a signal molecule, adjust membrane dynamics (di Paolo and de Camilli, 2006; Janetopoulos and Devreotes, 2006; Thole and Nielsen, 2008). However, from a nutritional

‡ Corresponding author
* Project supported by the Sino-German Joint Research Project (No. GZ932), the Key Projects in the National Science & Technology Pillar Program during the Twelfth Five-year Plan Period (No. 2014BAA 03B04), and the Wuxi Science and Technology Department Funds (No. CLE01N1408), China
ORCID: Qing-yao SHU, http://orcid.org/0000-0002-9201-0593

and environmental point of view, $InsP_6$ is a major anti-nutrient for livestock and human nutrition because it cannot be digested by monogastric animals, and it also binds to minerals to reduce their bioavailability (Raboy, 2001). These concerns have together provided a strong impetus for developing low phytic acid (*lpa*) crops (Stevenson-Paulik *et al.*, 2005; Raboy, 2007). In rice, several *lpa* mutants have been developed through chemical and physical mutagenesis and the underlying *lpa* genes (or candidate genes) have been identified, i.e. *OsLpa1* (LOC_Os02g57400) (Kim *et al.*, 2008a; Zhao *et al.*, 2008), *OsMRP5* (multidrug resistance protein ATP-binding cassette transporter, LOC_Os03g04920) (Xu *et al.*, 2009), and *OsMIK* (*myo*-inositol kinase, LOC_Os03g52760) (Kim *et al.*, 2008b; Zhao *et al.*, 2013).

While other *lpa* genes have a clear function in either PA biosynthesis or transport, little is known about the biochemical function of *OsLpa1*. Bioinformatics analysis showed that *OsLpa1* has a paralogous gene in rice (LOC_Os09g39870), and one to a few orthologs in other plants, suggesting that *OsLpa1* and its orthologs play active roles in plant biology. However, the only known fact related to its function is that a portion of OsLpa1 has homology to the P-loop kinase domain of 2-phosphoglycerate kinase (2-PGK) in hyperthermophilic methanogens (Aravind *et al.*, 2000; Kim *et al.*, 2008a; Zhao *et al.*, 2008). The possible roles of OsLpa1 in PA metabolism were discussed by Raboy (2009), but none of them has been experimentally confirmed so far. Hence, more investigations are needed to reveal the molecular characteristics and biological functions of the *OsLpa1* gene and its orthologs in plants.

It is well known that alternative splicing (AS) is a form of post- and co-transcriptional regulation in which pre-mRNA transcripts from the same gene are spliced in different ways, which greatly contributes to the enrichment of eukaryotic transcriptomes (Filichkin *et al.*, 2010; Marquez *et al.*, 2012). For example, 92%–94% of human intron-containing genes and approximately 61% of *Arabidopsis* multi-exonic genes can be alternatively spliced (Reddy *et al.*, 2012; Rogers *et al.*, 2012). In the rice transcriptome, AS occurs in 33% of all rice genes (Zhang *et al.*, 2010). However, little is known about the extent and significance of tissue-specific splicing in rice and other plants (Reddy *et al.*, 2013; Staiger and Brown, 2013). Seven AS modes of *OsLpa1* were either previously reported (Kim *et al*., 2008a) or predicted (Zhao *et al*., 2008) through bioinformatics analysis, or annotated in rice genome databases (Fig. 1a).

Aiming to shed light on *OsLpa1* as well as on AS in plants, in this study we investigated the molecular characteristics of *OsLpa1* in terms of its tissue-specific expression, AS, and protein subcellular localization.

2 Materials and methods

2.1 Generation of transgenic plants and histochemical staining

Analysis of the 5' flanking sequence of *OsLpa1* using the tools PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) and PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) indicated that the promoter region of *OsLpa1* encompasses a 2.1-kb fragment. This fragment was polymerase chain reaction (PCR)-amplified using primer pPro1 (Table 1) in a 25-μl volume with 1.0 μl DNA (50 ng/μl), 0.2 mmol/L dNTPs, 1.5 mmol/L $MgSO_4$, 0.3 μmol/L each primer, and 0.5 μl high-fidelity DNA polymerase (TOYOBO, Japan), supplemented with sterile distilled water. The following temperature profile was used: 94 °C 2 min, 40 cycles of 94 °C 10 s, 57 °C 30 s, and 68 °C 60 s, and a final extension at 68 °C for 5 min. The resulting amplicons were cloned into the T vector (TaKaRa, China) after purification. The sequence-verified promoter fragment was then subcloned into the multi-cloning site (MCS) between *Sal*I and *Bam*HI of pCAMBIA1300 and a *ProOsLpa1::GUS* vector (GUS: β-glucuronidase) was constructed. The *ProOsLpa1::GUS* vector was introduced into the *Agrobacterium tumefaciens* strain EHA105 and transferred into the *japonica* cultivar Xidao No. 1 according to Hiei and Komari (2008).

Transgene positive T_0 plants were identified by PCR analysis of the hygromycin resistant gene using primer Hyg^R (Table 1) and T_1 seeds were harvested and grown into plant lines according to Lu *et al.* (2015). Root, stem, leaf, flower tissues and seeds (developing and mature) were histochemically stained for 24 h at 37 °C with GUS dye solution containing 100 mmol/L phosphate buffer (pH 7.0), 20% (v/v) methanol, 0.5% (v/v) Triton X-100, and 0.5 mg/ml X-Gluc, according to Sieburth and Meyerowitz (1997).

Table 1 Primers used for molecular cloning and characterization of *OsLpa1* in rice

Primer*	Forward (F) and reverse (R) sequences (5'→3')	Amplicon size (bp)
pPro1	F: GGATCCCAGTAAACTAACCAACGGAGACG	2100
	R: GTCGACGTCGTCCCCTACCTGCAAAAT	
Hgy^R	F: AGAAGAAGATGTTGGCGACCT	565
	R: GTCCTGCGGGTAAATAGCT	
pV1	F: GCGAAGACCCAAGAGGAA	453 (gDNA)
	R: GACGGCAATGTAGAGGAGC	366 (cDNA)
pV2	F: ACTCTTAGTAGCATACCCTTCG	808 (gDNA)
	R: CTTCTGTGCCTTCCGTTTTGCC	424 (cDNA)
pRT1	F: TCTAGGGAGAAGTTCCTAAGTGTT	192
	R: ACCCAATCTACTACCCAACAAA	
pRT2	F: GCACCAGCCATACAGGAAGTGT	177
	R: CATCAGGAGGATCATCAGCATCT	
pRT3	F: GCACCAGCCATACAGGAAGTGT	143
	R: TCATACTGTAAACACACCAATG	
pRT-*Actin*	F: TGCTATGTACGTCGCCATCCAG	210
	R: AATGAGTAACCACGCTCCGTC	
pSub1.1	F: GGCGCGCCCCATGGCGGAGGAGGCGCC	2058
	R: CCCGGGTGCACACGGCAGTTCTGTGG	
pSub1.2	F: AGATCT TAATGATGAGAGGCTTTACGGA	1488
	R: CCCGGGTGCACACGGCAGTTCTGTGG	
pSub1.3	F: AGATCTTA ATGATGAGAGGCTTTACGGA	1128
	R: CCCGGGTACTGTAAACACACCAATGCATAC	

* Positions of validation PCR (pV1, pV2) and RT-PCR primers (pRT1, pRT2, pRT3) are shown in Fig. 1a

2.2 RNA extraction, reverse transcription, and real-time PCR

To investigate the abundance of the *OsLpa1* transcripts in different tissues during vegetative and reproductive growth, leaf and root tissues of seedlings and plants at different growth stages (3, 14, 45, and 90 d after germination (DAG)), flowers at heading and seeds of different developmental stages (7, 14, 21, and 28 d after flowering (DAF)) were collected for total RNA extraction. RNAs were extracted in 1 ml TRIzol reagent according to the manufacturer's instructions (TaKaRa, China). Total RNAs were treated with DNase, and their concentrations and purities were assessed using a Nano Drop 2000 (Thermo Scientific, Wilmington, Delaware, USA). Complementary DNAs (cDNAs) were reverse-transcribed from 500 ng RNA using the M-MLV reverse transcription kit (TaKaRa, China).

To examine whether transcripts other than *OsLpa1.1*, *OsLpa1.2*, and *OsLpa1.3* exist, two sets of validation primers (pV1 and pV2; Table 1) were designed for PCR of both genomic DNAs (gDNAs) and cDNAs. Because we were not able to detect transcripts other than those confirmed by Kim *et al*. (2008a) (see Section Results), subsequent real-time quantitative reverse transcriptase-PCR (qRT-PCR) was carried out only for *OsLpa1.1*, *OsLpa1.2*, and *OsLpa1.3* using RT primers pRT1, pRT2, and pRT3 (Table 1, Fig. 1a) according to the manufacturer's instructions. Because no part of the *OsLpa1.2* transcript is unique compared with *OsLpa1.1* and *OsLpa1.3*, primer pRT2 was designed to amplify the fragment from both *OsLpa1.1* and *OsLpa1.2* (Fig. 1a). The housekeeping gene *Actin* was used as an internal standard with RT primer pRT-*Actin* (Table 1). qRT-PCRs were performed in 10 μl aliquots, containing 1.0 μl cDNA, 0.2 μl of each primer (10 μmol/L), and 5.0 μl mix buffer, supplemented with sterile distilled water, according to the following schedule: 94 °C 2 min; 40 cycles of 94 °C 15 s and 60 °C 1 min. The relative quantification of gene expression was analyzed using the $2^{-\Delta\Delta C_T}$ method, where C_T value is the cycle number at which the fluorescent signal rises statistically above the background. qRT-PCRs were repeated with three biological replicates.

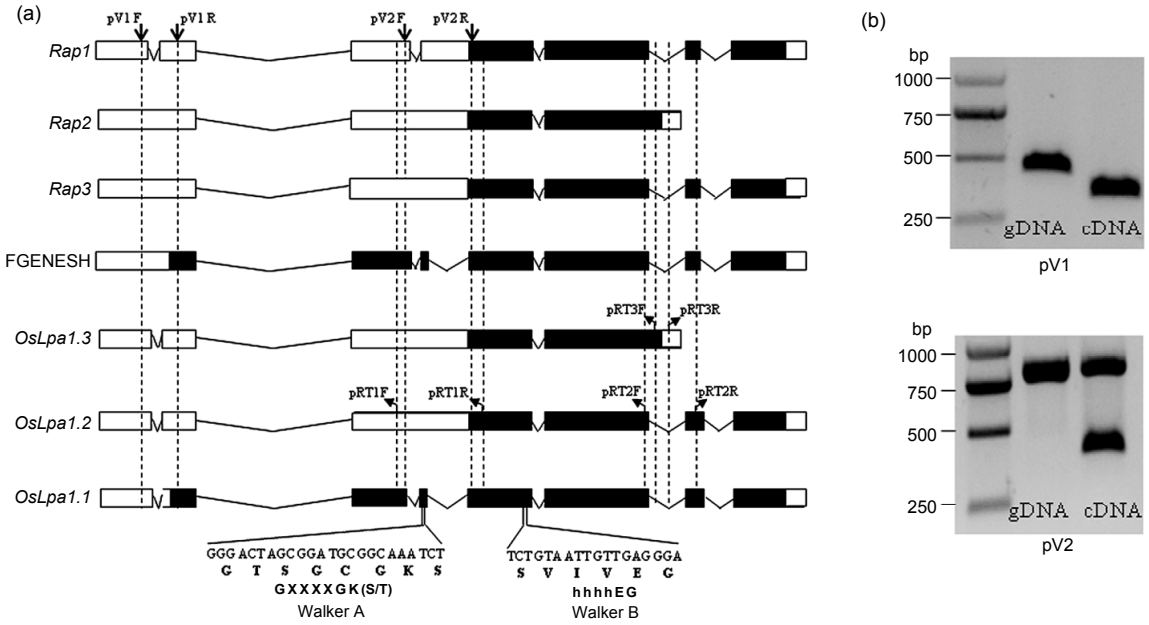

Fig. 1 Predicted *OsLpa1* alternative splicing and translation modes

(a) Three splicing modes (*OsLpa1.1*, *OsLpa1.2*, and *OsLpa1.3*) according to Kim *et al.* (2008a), one predicted using FGENESH by Zhao *et al*. (2008), and three transcripts annotated in the RAP database (http://rapdb.dna.affrc.go.jp/viewer/gbrowse/irgsp1/?name=id:1470481;dbid=annotation:database). Exons (boxes) and introns (lines) are in scales, and empty boxes represent untranslated regions. Primers used for PCRs are shown above with the vertical dotted lines showing their corresponding positions. The characteristic Walker A (GXXXXGK(S/T)) and Walker B (hhhhEG) motifs are indicated in the *OsLpa1.1*; X stands for any amino acid, while h is a hydrophobic residue. (b) Fragments amplified using validation primers from genomic DNA (gDNA) or reverse-transcribed DNA (complementary DNA (cDNA))

2.3 Protein subcellular localization

The full-length coding regions of the three *OsLpa1* transcripts (except for the stop codon) were amplified by PCR using the primers pSub1.1, pSub1.2, and pSub1.3, respectively, from cDNA (Table 1). PCR products were subcloned into the T-vector, and after sequence verification the target fragment was further cloned into the vector pTZM28-GFP to produce a fusion gene with green fluorescence protein (GFP) under the control of the *cauliflower mosaic virus* (CaMV) 35S promoter.

For transient expression, Nipponbare seeds were germinated and cultured for 8 d on 1/2 MS culture medium at 28 °C under continuous lighting. Seedlings were cut into 0.2-cm sections with sterile blades and used for isolation of protoplasts according to Zhang *et al.* (2011). Ten micrograms of *35S::OsLpa::GFP* plasmids were mixed with 220 μl 40% (0.4 g/ml) poly(ethylene glycol) 4000 (PEG-4000) and transformed into 200 μl protoplasts by gentle blending. After incubating for 16 h in the dark, transformed protoplasts were examined under a laser confocal microscope (Leica Microsystems, Bannockburn, IL, USA).

2.4 Data analysis

The mean and standard error were calculated from three independent replicates. Statistical analysis was performed using the one-way analysis of variance (ANOVA) program StaView. The differences are considered to be significant when the probability (P) was less than 0.05.

3 Results

3.1 Expression pattern of *OsLpa1* in rice

The temporal and spatial patterns of *OsLpa1* expression were determined using transgenic plants that contain the GUS reporter gene driven by the *OsLpa1* promoter (*OsLpa1::GUS*). By PCR analysis of the hygromycin resistant gene using primer Hyg^R (Table 1), twenty-eight transgene positive plants were identified and were subsequently used for further

characterization (data not shown). Histochemical staining for GUS activity showed that *OsLpa1* was strongly expressed in stem (Figs. 2a–2c), leaf (Fig. 2d), and root (Fig. 2e). In the floral organ, the filaments were stained dark blue (Figs. 2f and 2g), but little staining was observed in other parts such as anthers (Fig. 2g) and ovaries (Fig. 2h), indicating *OsLpa1* is expressed mainly in the filaments. In seeds, GUS staining was concentrated in the aleurone layers (Fig. 2i); a few blue spots were observed in the outer layers of embryo (Figs. 2i and 2j), but no staining was observed in the endosperm (Fig. 2i). These observations indicate that *OsLpa1* is mainly expressed in aleurone cells.

3.2 Developmental and spatial regulation of *OsLpa1* alternative splicing

Among the seven predicted AS modes of *OsLpa1*, only *OsLpa1.1*, *OsLpa1.2*, and *OsLpa1.3* were experimentally confirmed by RACE analysis (Kim *et al.*, 2008a). We first performed validation experiments on other possible transcripts using mixed cDNAs from 14 DAG seedlings and developing seeds of 14 DAF. If the *Rap2*, *Rap3*, and FGENESH transcripts exist, two fragments would be amplified from cDNAs using the primer pV1, one with length identical to the fragment from gDNA and one shorter (Fig. 1a). Because we amplified only the expected shorter length amplicons from cDNA (Fig. 1b), we deduce that transcripts of *Rap2*, *Rap3*, and FGENESH do not exist. Similarly by using pV2 we excluded the existence of *Rap1* transcripts because we did not observe fragments with the size predicted for *Rap1* transcripts (Fig. 1b).

To reveal whether the AS of *OsLpa1* is regulated spatially and/or developmentally, the relative abundances of its three transcripts were assessed by qRT-PCRs using three sets of transcript-specific primers, i.e. pRT1 for *OsLpa1.1*, pRT2 for both *OsLpa1.1* and *OsLpa1.2*, and pRT3 for *OsLpa1.3* (Fig. 1a). Because very similar amplification efficiencies (0.98 vs. 0.99, respectively) were achieved for primers pRT1 and pRT2, the abundance of *OsLpa1.2* can be calculated by deduction of *OsLpa1.1* from that measured by using pRT2.

The *OsLpa1.3* transcript could be barely detected in leaf and root tissues at all growth stages (Fig. 3a); its abundance was also the lowest in developing seeds, with only about 1/4 that of *OsLpa1.2* at its maximum level in seeds of 14 DAF (Fig. 3b).

Both *OsLpa1.1* and *OsLpa1.2* were abundant compared with *OsLpa1.3*, their amount being spatially and developmentally regulated (Figs. 3a and 3b). During the vegetative growth stage, the abundances of *OsLpa1.1* and *OsLpa1.2* changed inversely, i.e., *OsLpa1.2* decreased in both leaf and root tissues from germinating seeds (3 DAG), to seedlings (14 DAG), to plants at tillering stage (45 DAG), while *OsLpa1.1* increased continuously during the same period (Fig. 3a).

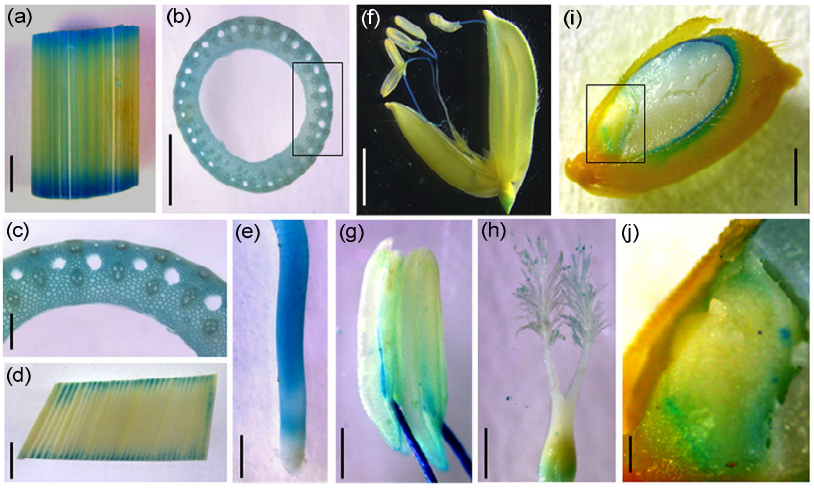

Fig. 2 Histochemical assay of GUS activity in transgenic rice plants expressing the *OsLpa1* promoter-GUS fusion gene
(a) Stem; (b) Cross-cut of stem; (c) Enlargement of a section of (b) (framed); (d) Leaf segment; (e) Root; (f) A floret; (g) Anther with filaments; (h) Unfertilized ovary with stigmas; (i) Longitudinal cut of a seed; (j) Enlargement of the embryo of (i) (framed). Bars in (a), (b), (d), (e), (f), and (i) equal to 2.0 mm, and those in (c), (g), (h), and (j) 0.5 mm (Note: for interpretation of the references to color in the text and legend, the reader is referred to the web version of this article)

Consequently, *OsLpa1.2* was 5.7- and 5.2-fold more abundant than *OsLpa1.1* in coleoptiles and roots of germinating seeds, respectively, but the amount of *OsLpa1.1* became 2.8- and 5.8-fold greater than that of *OsLpa1.2* in leaf and root tissues, respectively, at tillering stage. In the flag leaves and roots of plants at flowering stage, the abundances of all three transcripts decreased significantly compared with tillering plants (Fig. 3a). The abundances of both *OsLpa1.1* and *OsLpa1.2* were also low in unfertilized florets and young seeds but increased significantly in developing seeds starting from 14 DAF. For example, *OsLpa1.1* was 11.9- and 27.8-fold more abundant in seeds of 14 and 28 DAF than in those of 7 DAF, respectively. Compared with *OsLpa1.1*, *OsLpa1.2* was less abundant in developing seeds at all stages, although it was also highly abundant (Fig. 3b).

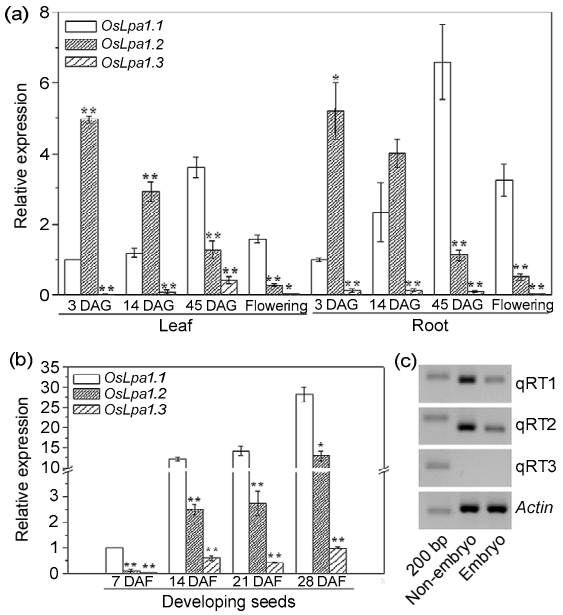

Fig. 3 Abundance analyses of three *OsLpa1* transcripts
(a, b) Relative abundances of three *OsLpa1* transcripts in various tissues of plants at different developmental stages, revealed by qRT-PCR analyses. (c) Semi-quantitative RT-PCRs of *OsLpa1* in the embryo and non-embryo parts of mature seeds using the three primer sets shown in (a). DAG: days after germination; DAF: days after flowering. Data are mean±standard error (SE) of three independent experiments. Relative abundance was calculated using the *Actin* gene as an internal control and compared with *OsLpa1.1* (set as 1) in leaf and root tissues of 3 DAG (a) and seeds of 7 DAF (b). * and ** mean significant differences compared with *OsLpa1.1* in each stage at $P<0.05$ and $P<0.01$, respectively

Because GUS assay showed that *OsLpa1* is expressed less in the embryo (Fig. 2j), we manually separated embryo from the whole seeds and subjected them to semi-RT-PCR analysis. The result was in accordance with the GUS staining: both *OsLpa1.1* and *OsLpa1.2* were more profoundly expressed in the non-embryo than in the embryo, and *OsLpa1.3* was barely detected in either (Fig. 3c).

3.3 OsLpa1 proteins anchored to the chloroplast

Through bioinformatic analysis (http://www.cbs.dtu.dk/services/TargetP), a chloroplast localizing signal peptide (45 amino acids) was detected in the protein encoded by *OsLpa1.1* at the N terminal, but not in those encoded by *OsLpa1.2* and *OsLpa1.3*. To reveal the localization of proteins encoded by the three *OsLpa1* transcripts, the coding sequences were fused with the *GFP* gene to generate three chimeric *OsLpa1::GFP* genes driven by the CaMV 35S promoter (Fig. 4a). They were transiently expressed in rice protoplasts for protein localization. Under the confocal microscopy, three chimeric proteins with green fluorescence can completely match the chloroplast with red autofluorescence (Fig. 4b), which indicated that they are all localized to the chloroplast.

4 Discussion

Knowledge of tissue-specific expression and protein subcellular localization provides important clues about the function of a gene. Our present study revealed such information about *OsLpa1* in rice, helping to illuminate its role in rice biology and potentially the role of the *OsLpa1* family in other plant species. Recent studies have shown that more than half of these genes in rice undergo AS, either induced by stress conditions or developmentally regulated (E *et al.*, 2013). However, detailed analyses of tissue-specific and developmental AS have so far been very limited (Wang *et al.*, 2014) until our present study which provides new insights into AS of *OsLpa1* in particular and gene AS in general in plants.

Our present study first demonstrated that the AS of *OsLpa1* is regulated both spatially and developmentally through a mechanism yet to be uncovered.

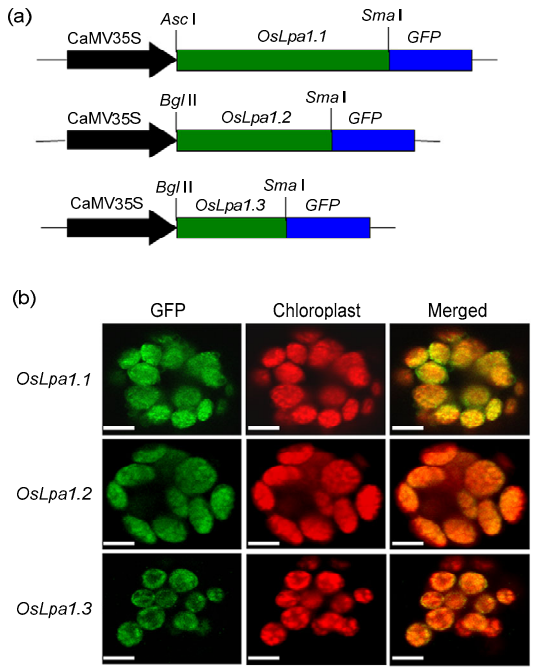

Fig. 4 Subcellular localization of OsLpa1.1, OsLpa1.2, and OsLpa1.3 in rice protoplasts by confocal microscopy
(a) The construction of *OsLpa1.1*, *OsLpa1.2*, and *OsLpa1.3* green fluorescence protein (*GFP*) fusion genes driven by the CaMV 35S promoter. (b) The green fluorescence from three proteins merged with red fluorescence from the chloroplast using confocal microscopy. Overlay of two images indicated that all three proteins are localized to the chloroplast. The bars equal to 1.5 μm (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

The significant and dynamic changes of the abundances of three *OsLpa1* transcripts in different tissues and at different stages suggested that they may play different roles in rice development. The greater abundance of *OsLpa1.2* compared with *OsLpa1.1* in seedling (Fig. 2a), for example, may suggest that the protein encoded by *OsLpa1.2* plays a more important role in seedling growth. In contrast, *OsLpa1.1* may play a more active role than *OsLpa1.2* in seed development.

OsLpa1 was found to be involved in PA metabolism through gene mapping and candidate gene analysis (Kim *et al.*, 2008a; Zhao *et al.*, 2008), with the biochemical function of the protein it encodes being largely unknown. Raboy (2007) discussed three possible roles for *OsLpa1*, i.e. it encodes a 2-phosphoglyceric acid (2-PGA) kinase producing 2,3-PGA2 (which is possibly a competitive inhibitor of the Ins polyphosphate-5-phosphate) or an Ins phosphate kinase (that catalyzes the phosphorylation of Ins monophosphates), or encodes an enzyme that has both of the above functions. However, none of the putative functions has been experimentally confirmed so far. Our present study showed that both *OsLpa1.1* and *OsLpa1.2* are abundant in developing seeds; it is intriguing to further test whether the two different transcripts encode the two enzymes.

The results of *OsLpa1* expression in different parts of the rice plant provide information about where the *OsLpa1* gene plays a role. In addition to rice seeds, expression of *OsLpa1* was observed in other organs (Fig. 2). In particular, high expression was observed in roots (except the root tip) (Fig. 2e) and in the filaments (Fig. 2g). It is yet to be revealed whether the high expression is related to biosynthesis of PA or other unknown molecules in these tissues. It is well-known that PA is accumulated in both aleurone layers and embryos (Raboy, 2009), and Sato *et al.* (2011) also showed that *LOC_Os02g57400* had the highest expression level in rice Nipponbare embryos (7–42 DAF). However, the promoter::GUS transgenic plant assay showed a high level of *OsLpa1* expression in the aleurone layers and to a lesser extent in the embryo (Figs. 2i and 2j). Therefore our results are not consistent with previous studies and further studies are needed to identify the cause of these differences.

In the present study, we also observed high expression of *OsLpa1* in the filament (Fig. 2g), suggesting that it may play an essential role, possibly in filament elongation at anthesis (Thole *et al.*, 2008; Zhao *et al.*, 2010). This would not be surprising given that two phosphatidylinositol compounds in the lipid-dependent PA synthesis pathway, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3), participate in the recruitment and activation of a wide variety of actin regulatory proteins at the plasma membrane, controlling cell shape, motility, and cytokinesis (di Paolo and de Camilli, 2006).

It is not yet known where PA is synthesized, although it is known that PA biosynthesis starts with the synthesis of Ins(3)P_1. Studies on *Arabidopsis* indicated that the first steps of PA biosynthesis are carried out in the cytosol and the final steps (InsP_3 to InsP_6) in the endoplasmic reticulum (Otegui *et al.*,

2002). Otegui *et al.* (2002) also noted that an increase in Mn-phyate in the embryo coincides with higher chlorophyll content. In the present study, we revealed that all proteins are anchored to chloroplast (Fig. 4), suggesting that PA biosynthesis may be related to molecules produced in the chloroplast, mediated or catalyzed by *OsLpa1*.

Sequence identity searches revealed that OsLpa1.1 has homology to the P-loop kinase domain of 2-PGK. The P-loop NTPase domain super-family is characterized by two conserved nucleotide phosphate-binding motifs, i.e. the Walker A motif (GXXXX GK[S/T], where X is any residue) and the Walker B motif (hhhhEG, where h is a hydrophobic residue). While OsLpa1.1 has both the Walker A and Walker B motifs, OsLpa1.2 and OsLpa1.3 have only the latter (Fig. 1a), suggesting OsLpa1.2 and OsLpa1.3 could be non-functional proteins. Therefore, *OsLpa1.2* and *OsLpa1.3* or their encoded proteins may function as competitors of *OsLpa1.1* in transcription as genes involved in stress response (Staiger and Brown, 2013) or in catalysis through formation of homo- and hetero-dimer proteins. In addition, because the 5' untranslated regions (5' UTRs) could be a part of a promoter (Guo *et al.*, 2010; Suhandono *et al.*, 2014), further studies are needed to reveal the spatiotemporal expression patterns of *OsLpa1.2* and *OsLpa1.3*.

5 Conclusions

The present study demonstrates that *OsLpa1* was strongly expressed in stem, leaf, and root tissues, and in filaments in the floral organ. In seeds, GUS staining was concentrated in the aleurone layers. Three *OsLpa1* transcripts (*OsLpa1.1*, *OsLpa1.2*, *OsLpa1.3*) are produced by AS; quantitative RT-PCR analysis revealed that *OsLpa1.2* is predominant in germinating seeds, but its abundance decreases quickly with plant development, whereas the abundance of *OsLpa1.1* rises and falls in both leaves and roots. In seeds the abundance of *OsLpa1* continuously increases with seed growth. Transient expression of chimeric genes with GFP in rice protoplasts demonstrated that all proteins encoded by the three *OsLpa1* transcripts are localized to the chloroplast.

Compliance with ethics guidelines

Hai-ping LU, Wei-qin PANG, Wen-xu LI, Yuan-yuan TAN, Qing WANG, Hai-jun ZHAO, and Qing-yao SHU declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Aravind, L., Wolf, Y.I., Koonin, E.V., 2000. The ATP-CONE: an evolutionarily mobile, ATP-binding regulatory domain. *J. Mol. Microbiol. Biotechnol.*, **2**(2):191-194.

di Paolo, G., de Camilli, P., 2006. Phosphoinositides in cell regulation and membrane dynamics. *Nature*, **443**(7112): 651-657. http://dx.doi.org/10.1038/nature05185

E, Z.G., Wang, L., Zhu, J.H., 2013. Splicing and alternative splicing in rice and humans. *BMB Rep.*, **46**(9):439-447. http://dx.doi.org/10.5483/BMBRep.2013.46.9.161

Filichkin, S.A., Priest, H.D., Givan, S.A., *et al*., 2010. Genome-wide mapping of alternative splicing in *Arabidopsis thaliana*. *Genome Res.*, **20**(1):45-58. http://dx.doi.org/10.1101/gr.093302.109

Guo, L.L., Yu, Y.H., Xia, X.L., *et al*., 2010. Identification and functional characterisation of the promoter of the calcium sensor gene *CBL1* from the xerophyte *Ammopiptanthus mongolicus*. *BMC Plant Biol.*, **10**(1):18. http://dx.doi.org/10.1186/1471-2229-10-18

Hiei, Y., Komari, T., 2008. *Agrobacterium*-mediated transformation of rice using immature embryos or calli induced from mature seed. *Nat. Protoc.*, **3**(5):824-834. http://dx.doi.org/10.1038/nprot.2008.46

Janetopoulos, C., Devreotes, P., 2006. Phosphoinositide signaling plays a key role in cytokinesis. *J. Cell Biol.*, **174**(4):485-490. http://dx.doi.org/10.1083/jcb.200603156

Kim, S.I., Andaya, C.B., Goyal, S.S., *et al*., 2008a. The rice *Oslpa1* gene encodes a novel protein involved in phytic acid metabolism. *Theor. Appl. Genet.*, **117**(5):769-779. http://dx.doi.org/10.1007/s00122-008-0818-z

Kim, S.I., Andaya, C.B., Newman, J.W., *et al*., 2008b. Isolation and characterization of a low phytic acid rice mutant reveal a mutation in the rice orthologue of maize MIK. *Theor. Appl. Genet.*, **117**(8):1291-1301. http://dx.doi.org/10.1007/s00122-008-0863-7

Lee, H.S., Lee, D.H., Cho, H.K., *et al*., 2015. $InsP_6$-sensitive variants of the Gle1 mRNA export factor rescue growth and fertility defects of the *ipk1* low-phytic-acid mutation in *Arabidopsis*. *Plant Cell*, **27**(2):417-431. http://dx.doi.org/10.1105/tpc.114.132134

Lu, H.P., Edwards, M., Wang, Q.Z., *et al.*, 2015. Expression of cytochrome P450 *CYP81A6* in rice: tissue specificity, protein subcellular localization, and response to herbicide application. *J. Zhejiang Univ.-Sci. B (Biomed. & Biotechnol.)*, **16**(2):113-122. http://dx.doi.org/10.1631/jzus.B1400168

Marquez, Y., Brown, J.W.S., Simpson, C., *et al*., 2012. Transcriptome survey reveals increased complexity of the alternative splicing landscape in *Arabidopsis*. *Genome Res.*, **22**(6):1184-1195. http://dx.doi.org/10.1101/gr.134106.111

Monserrate, J.P., York, J.D., 2010. Inositol phosphate synthesis and the nuclear processes they affect. *Curr. Opin. Cell Biol.*, **22**(3):365-373. http://dx.doi.org/10.1016/j.ceb.2010.03.006

Otegui, M.S., Capp, R., Staehelin, L.A., 2002. Developing seeds of *Arabidopsis* store different minerals in two types of vacuoles and in the endoplasmic reticulum. *Plant Cell*, **14**(6):1311-1327. http://dx.doi.org/10.1105/tpc.010486

Raboy, V., 2001. Seeds for a better future: 'low phytate' grains help to overcome malnutrition and reduce pollution. *Trend Plant Sci.*, **6**(10):458-462. http://dx.doi.org/10.1016/S1360-1385(01)02104-5

Raboy, V., 2003. *myo*-Inositol-1,2,3,4,5,6-hexa*kis*phosphate. *Phytochemistry*, **64**(6):1033-1043. http://dx.doi.org/10.1016/S0031-9422(03)00446-1

Raboy, V., 2007. Forward genetics studies of seed phytic acid. *Israel J. Plant Sci.*, **55**(2):171-181. http://dx.doi.org/10.1560/IJPS.55.2.171

Raboy, V., 2009. Approaches and challenges to engineering seed phytate and total phosphorus. *Plant Sci.*, **177**(4):281-296. http://dx.doi.org/10.1016/j.plantsci.2009.06.012

Reddy, A.S.N., Roger, M.F., Richardson, D.N., *et al*., 2012. Deciphering the plant splicing code: experimental and computational approaches for predicting alternative splicing and splicing regulatory elements. *Front. Plant Sci.*, **3**:18. http://dx.doi.org/10.3389/fpls.2012.00018

Reddy, A.S.N., Marquez, Y., Kalyna, M., *et al*., 2013. Complexity of the alternative splicing landscape in plants. *Plant Cell*, **25**(10):3657-3683. http://dx.doi.org/10.1105/tpc.113.117523

Rogers, M.F., Thomas, J., Reddy, A.S.N., *et al*., 2012. SpliceGrapher: detecting patterns of alternative splicing from RNA-Seq data in the context of gene models and EST data. *Genome Biol.*, **13**(1):R4. http://dx.doi.org/10.1186/gb-2012-13-1-r4

Sato, Y., Antonio, B., Namiki, N., *et al*., 2011. Field transcriptome revealed critical developmental and physiological transitions involved in the expression of growth potential in *japonica* rice. *BMC Plant Biol.*, **11**(1):10. http://dx.doi.org/10.1186/1471-2229-11-10

Sieburth, L.E., Meyerowitz, E.M., 1997. Molecular dissection of the *AGAMOUS* control region shows that *cis* elements for spatial regulation are located intragenically. *Plant Cell*, **9**(3):355-365. http://dx.doi.org/10.1105/tpc.9.3.355

Staiger, D., Brown, J.W.S., 2013. Alternative splicing at the intersection of biological timing, development and stress response. *Plant Cell*, **25**(10):3640-3656. http://dx.doi.org/10.1105/tpc.113.113803

Stevenson-Paulik, J., Bastidas, R.J., Chiou, S.T., *et al*., 2005. Generation of phytate-free seeds in *Arabidopsis* through disruption of inositol polyphosphate kinases. *PNAS*, **102**(35):12612-12617. http://dx.doi.org/10.1073/pnas.0504172102

Suhandono, S., Apriyanto, A., Ihsani, N., 2014. Isolation and characterization of three cassava elongation factor 1α (MeEF1A) promoters. *PLoS ONE*, **9**(1):e84692. http://dx.doi.org/10.1371/journal.pone.0084692

Thole, J.M., Nielsen, E., 2008. Phosphoinositides in plants: novel functions in membrane trafficking. *Curr. Opin. Plant Biol.*, **11**(6):620-631. http://dx.doi.org/10.1016/j.pbi.2008.10.010

Thole, J.M., Vermeer, J.E.M., Zhang, Y.L., *et al*., 2008. ROOT HAIR DEFECTIVE4 encodes a phosphatidylionositol-4-phosphate phosphatase required for proper root hair development in *Arabidopsis thaliana*. *Plant Cell*, **20**(2):381-395. http://dx.doi.org/10.1105/tpc.107.054304

Wang, L., Cao, C.L., Ma, Q.B., *et al*., 2014. RNA-seq analyses of multiple meristems of soybean: novel and alternative transcripts, evolutionary and functional implications. *BMC Plant Biol.*, **14**:169. http://dx.doi.org/10.1186/1471-2229-14-169

Xu, X.H., Zhao, H.J., Liu, Q.L., *et al*., 2009. Mutations of the multi-drug resistance-associated protein ABC transporter gene 5 result in reduction of phytic acid in rice seeds. *Theor. Appl. Genet.*, **119**(1):75-83. http://dx.doi.org/10.1007/s00122-009-1018-1

York, J.D., 2006. Regulation of nuclear processes by inositol polyphosphates. *Biochim. Biophys. Acta*, **1761**(5-6):552-559. http://dx.doi.org/10.1016/j.bbalip.2006.04.014

Zhang, G.J., Guo, G.W., Hu, X.D., *et al*., 2010. Deep RNA sequencing at single base-pair resolution reveals high complexity of the rice transcriptome. *Genome Res.*, **20**(5):646-654. http://dx.doi.org/10.1101/gr.100677.109

Zhang, Y., Su, J.B., Duan, S., *et al*., 2011. A high efficient rice green tissue protoplast system for transient gene expression and studying light/chloroplast-related process. *Plant Methods*, **7**(1):30. http://dx.doi.org/10.1186/1746-4811-7-30

Zhao, H.J., Liu, Q.L., Ren, X.L., *et al*., 2008. Gene identification and allele-specific marker development for two allelic low phytic acid mutations in rice (*Oryza sativa* L.). *Mol. Breeding*, **22**(4):603-612. http://dx.doi.org/10.1007/s11032-008-9202-6

Zhao, H.J., Cui, H.R., Xu, X.H., *et al*., 2013. Characterization of *OsMIK* in a rice mutant with reduced phytate content reveals an insertion of a rearranged retrotransposon. *Theor. Appl. Genet.*, **126**(12):3009-3020. http://dx.doi.org/10.1007/s00122-013-2189-3

Zhao, Y., Yan, A., Feijo, J., *et al*., 2010. Phosphoinositides regulate clathrin-dependent endocytosis at the tip of

pollen tubes in *Arabidopsis* and tobacco. *Plant Cell*, **22**(12):4031-4044. http://dx.doi.org/10.1105/tpc.110.076760

中文概要

题　目：水稻低植酸基因 *OsLpa1* 可变剪接和表达的时空特征及编码蛋白的亚细胞定位

目　的：揭示水稻低植酸基因 *OsLpa1* 的分子生物学特征，特别是深化对其可变剪切和表达的时空和组织特征，以及蛋白亚细胞定位的认识。

创新点：确定了 *OsLpa1* 存在的三种剪切方式，明确了三种转录本在不同组织和发育时期丰度的变化；揭示了 *OsLpa1* 表达的组织和时空差异，确定其在根、种子糊粉层细胞和花丝中高度表达；明确了三种转录本编码的蛋白均定位于亚叶绿体。

方　法：通过培育 *OsLpa1* 启动子与 β-葡萄糖醛酸糖苷酶（GUS）杂合基因的转基因植株，通过不同组织的 GUS 组织化学染色确定 *OsLpa1* 表达的组织特异性；通过设计特异性引物确定 *OsLpa1* 存在的转录方式，采用实时荧光定量聚合酶链式反应（PCR）分析三种转录本在不同组织和发育时期的丰度；采用 *OsLpa1* 三种转录本与绿色荧光蛋白（*GFP*）基因构建杂合基因并在水稻原生质体中的瞬时表达，在共聚焦显微镜下观察蛋白的亚细胞定位。

结　论：*OsLpa1* 在根、茎、叶和花丝有强烈的表达。它存在三种可变剪切方式，产生的三种转录本存在明显的时空和组织差异，但其编码的蛋白均定位于叶绿体。

关键词：*OsLpa1*；低植酸；表达模式；可变剪接；亚细胞定位