Planta
DOI 10.1007/s00425-013-1934-9

ORIGINAL ARTICLE

The rice *OsLTP6* gene promoter directs anther-specific expression by a combination of positive and negative regulatory elements

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Received: 10 May 2013 / Accepted: 19 July 2013

Abstract Characterization of tissue-specific plant gene promoters will benefit genetic improvement in crops. Here, we isolated a novel rice anther-specific plant lipid transfer protein (*OsLTP6*) gene through high through-put expressional profiling. The promoter of *OsLTP6* was introduced to the upstream of the *uidA* gene, which encodes β-glucuronidase (GUS), and transformed into rice plants for functional analysis. Histochemical and fluorometric GUS assay showed that GUS was specifically expressed in the anthers and pollens in *OsLTP6* promoter::*uidA* transgenic plants. Transverse section of the rice anther further indicated that the *OsLTP6* promoter directed the reporter gene specifically expressed in anther tapetum. To identify regulatory elements within *OsLTP6* promoter region, four progressive deletions of the *OsLTP6* promoter were constructed. The results indicated that the *OsLTP6* promoter achieved anther-specific expression through a combination of positive and negative regulatory elements. A 26-bp motif upstream of TATA box was a key transcriptional activator for *OsLTP6* gene. CAAT box and GTGA box were the putative motifs to increase the transcription level to full expression. Two negative regulatory elements were also found in two distinct regions within this promoter. They repressed the expression in leaf and stem, respectively. These results revealed the regulating complexity of anther-specific expression.

Keywords Anther-specific · Lipid transfer protein · *Oryza* · *OsLTP6* promoter

Abbreviations

4-MU	4-Methylumbelliferone
CaMV	Cauliflower mosaic virus
GUS	β-Glucuronidase
MMC	Microspore mother cell
MUG	4-Methylumbelliferyl β-D-glucuronide
nsLTP	Non-specific lipid transfer protein
OsLTP6	*Oryza sativa* lipid transfer protein 6
TA29 promoter	Tobacco anther-specific gene 29 promoter
TSS	Transcriptional start site
X-gluc	5-Bromo-4-chloro-3-indoyl-β-D-glucuronide solution
St	Anther developmental stage

Introduction

Plant gene promoter is one of key components for crop transgenic study. Over the decades, several well-characterized constitutive expression promoters have been made available for transgenic studies in crops. The cauliflower mosaic virus (CaMV) 35S gene promoter (Odell et al. 1985) and the maize polyubiquitin1 (*Ubi1*) promoter (Christensen et al. 1992) have been widely used for high

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Published online: 02 August 2013

expression of transgenes in crops. Several the rice gene promoters, such as actin1 (*Act1*) promoter (McElroy et al. 1990, 1991), actin2 (*Act2*) promoter (He et al. 2009), ubiquitin1 (*OsUbi1*) promoter (Bhattacharyya et al. 2012), cytochrome c1 (*OsCc1*) promoter (Jang et al. 2002), and L-ascorbate peroxidase (*APX*) and cytosolic 6-phosphogluconate dehydrogenase (*PGD1*) promoters (Park et al. 2010), can drive the constitutively high expression of transgenes in rice. In transgenic *Bt* crops, insecticidal genes from *Bacillus thuringiensis* are expressed constitutively (Shelton et al. 2002; High et al. 2004; Chen et al. 2005). Tissue-specific promoters are isolated to meet the demand for precise control of transgene expression in certain tissues/cells. The endosperm-specific glutelin promoter is isolated and utilized in the production of golden rice (Ye et al. 2000; Beyer et al. 2002; Paine et al. 2005). The promoter of rice β-glucanase 9 (*Gns9*) directs the selectable marker gene expressed in calli, but not in leaves, roots or seeds, thus preventing the accumulation of selectable marker gene product in transgenic rice seeds (Huang et al. 2001). The promoter of *AGPL1* gene, the large subunit of ADP-glucose pyrophosphorylase, drives fruit-specific gene expression in transgenic tomato plants (Yin et al. 2009).

Microspores/pollen grains are formed in the anthers. The formation and release of pollen requires the coordinated expression of a complex array of genes. Only a small fraction of these genes is thought to be anther-specific. Anther-specific expression promoter can be used in generating male-sterile plants (Mariani et al. 1990; Paul et al. 1992; Luo et al. 2006; Roque et al. 2007; Konagaya et al. 2008). Mariani et al. (1990, 1992) reported the strategy for the production of male sterile plants using anther-specific promoter and a cytotoxic gene. The cytotoxic gene which encodes a ribonuclease is selectively expressed in the anthers under the control of tobacco anther-specific gene29 promoter (*TA29* promoter). The promoter of *Pisum sativum ENDOTHECIUM1* (*PsEND1*), a pea anther-specific promoter, when fused to the cytotoxic *barnase* gene, can induce specific ablation of the cell layers where *PsEND1* is expressed and consequently produce male sterile plants in Solanaceae and Brassicaceae species (Gomez et al. 2004; Roque et al. 2007). The promoter of *PsEND1* is also utilized to target *barnase* to the anthers of tomato plants to produce seedless and good quality fruits (Medina et al. 2013). Another biotechnological alternative for generating male sterile plants is metabolically engineered male sterility under the control of the anther-specific promoter (Hirsche et al. 2009; Engelke et al. 2010, 2011). Anther-specific cell wall-bound invertase (*Nin88*) is an essential enzyme to supply the developing microspores with carbohydrates during anther development. Pollen development is blocked by repressing the activity of invertase under the control of the anther-specific *Nin88* promoter, thus generating male sterile plants in tobacco, *Arabidopsis* and rapeseed.

Male-sterile plants have no viable pollen grain. They greatly facilitate the production of hybrids for eliminating the need of manual or mechanical removal of anthers from flowers. Moreover, male-sterile plants prevent transgene flow from genetically modified crops to food crops and wild relatives (Daniell 2002). This helps to cope with the public awareness and fears about the environmental impact of genetically modified plants.

Nowadays, with the demand for the application of multigene stacking, it is necessary to develop a range of promoters to fine modulate gene expression as this is highly advantageous in reducing homology-based transcriptional gene silencing (Halpin 2005). So, one of the major limitations in generating designed monocot transgenic plants is the shortage of an enough array of efficient tissue-specific promoters.

Till now, only a few rice anther-specific promoters have been characterized. The promoter of *Osg6B* gene directs tapetum-specific expression in rice (Yokoi et al. 1997). The promoter of rice anther-specific cDNA clone8 (*RA8*) is active in the tapetum, endothecium, and connective tissues of rice anthers (Jeon et al. 1999). The rice tapetum-specific gene (*RTS*) promoter directs gene predominantly expressed in the anther's tapetum during meiosis and disappears before anthesis (Luo et al. 2006). The promoters of three rice small cysteine-rich proteins, *OsSCP1*, *OsSCP2*, and *OsSCP3*, are active in the mature pollen grains and pollen tubes in transgenic tobacco (Park et al. 2006). The promoters of *Oryza sativa indica* pollen allergen/expansion gene (*OSIPA*) and *Oryza sativa indica* calcium-dependent protein kinase gene (*OSIPK*) are active in different pollen developmental stages in transgenic tobacco and *Arabidopsis* (Gupta et al. 2007). However, the anther-specific regulating mechanism of these promoters has not been elucidated yet.

In this study, a novel anther-specific plant lipid transfer protein (*OsLTP6*) gene was identified through cDNA expressional profiling. Functional analysis of *OsLTP6* promoter was carried out by stable transformation of rice. The *OsLTP6* promoter directed GUS reporter gene specifically expressed in the anthers from microspore mother cell (MMC) developmental stage (anther developmental stage 6, st6) to mature pollen stage (st14) (Zhang and Wilson 2009). Moreover, we elucidated the anther-specific regulatory mechanism for *OsLTP6* promoter. *OsLTP6* promoter achieved anther-specific expression through a combination of positive and negative regulatory elements.

Materials and methods

Promoter selection according to cDNA abundance and real-time RT-PCR confirmation of gene expression patterns

Four full-length cDNA libraries were constructed from *Oryza sativa* ssp. *indica* Guangluai 4, including germinated seeds, 14-day seedling shoots, 14-day seedling roots and young panicles (Liu et al. 2007). Guangluai 4 was obtained from National Center for Gene Research, Chinese Academy of Sciences (Shanghai, China). In each library, 20,000 clones were sequenced randomly from the 5′ ends. Then, cDNA abundance was summarized in each library. The top twenty most abundant cDNAs were selected for further investigation. If these sequences showed no occurrence or appeared only one or two copies in the other three libraries, they were considered as candidate cDNAs for tissue-specific promoter isolation. Next, the expression patterns of these cDNAs in different rice tissues were further confirmed by real-time RT-PCR according to the manufacturer's procedures. Real-time RT-PCR primers for *OsLTP6* gene amplification were forward primer: 5′-cttggtgaagttggctgaca-3′; and reverse primer: 5′-caacatgctgaaggttgacg-3′. Rice *actin1* gene was used as endogenous reference for RT- PCR. The real-time RT-PCR primers for rice *actin1* gene amplification were forward primer: 5′-ttgctgacaggatgagcaag-3′; and reverse primer: 5′-cacatctgctggaatgtgct-3′.

Amplification of the promoter region of *OsLTP6* gene and vector construction

BAC clone, AC091665 (OSJNBb0016M10, Clemson University Genomics Institute), was used as PCR template for the amplification of *OsLTP6* promoter region. *Pfu* DNA polymerase was used for amplification. The primer sequences for promoter amplification were forward primer 5′-cgggatccacctcagccaaaaccgaaga-3′ and reverse primer 5'-agtacggtctccatggcagccaggctagaagacttg-3'. The underlined sequence denoted *Bam*HI and *Nco*I sites that were added to facilitate directional cloning of the amplified fragment. Because there was an *Nco*I site inside the promoter region, *Nco*I could not be used to digest the amplified fragment. So, a *Bsa*I site (in box) was added in the primer sequence. The PCR product was digested with *Bam*HI and *Bsa*I to give the *Bam*HI and *Nco*I ends. The digested promoter fragment was ligated to the binary vector pCAMBIA1305.2 to generate the *OsLTP6* promoter::*uidA* construct. The *OsLTP6* promoter sequence in the vector was further confirmed by DNA sequencing.

The forward primer sequences for four progressive deletions were M1, 5′- cgggatccgtaccgccgaaggaccttat-3′. M2, 5′-cgggatcccatatcaaacagtcacggatgg-3′. M3, 5′-cgggatcc ctgctgcacaggctaaatca-3′. M4, 5′-cgggatcctagccatgaaccattca cct-3′. The reverse primer sequence was all the same.

Rice transformation and regeneration of *OsLTP6* promoter::*uidA* transgenic plants

OsLTP6 promoter::*uidA* was transformed into a *japonica* rice variety Nipponbare through *Agrobacterium tumefaciens* EHA105. The procedures for transformation, regeneration, and transfer of plantlets to soil were according to Hiei's (Hiei et al. 1994) methods. The positive transgenic T0 plants were selected as resistant to hygromycin B (40 mg/l) on culture medium. The resistant transgenic plants were further confirmed their *GUS* gene presence by PCR before transferring to soil. The T1 generation of over five independent lines for each of the promoters was used for the subsequent detailed analysis.

Histochemical localization of GUS expression in *OsLTP6* promoter::*uidA* transgenic rice

The tissues from transgenic rice were washed briefly with 50 mM (pH 7.0) phosphate buffer, then immersed in 1 mM GUS substrate (5-bromo-4-chloro-3-indoyl-β-D-glucuronide solution, X-gluc) in 50 mM phosphate buffer (pH 7.0), 10 % methanol, and 0.1 % Triton X-100 solution. Vacuum was applied for three times to let the tissues immerse fully into the GUS staining solution (Triton X-100 was omitted in the vacuum procedure.), then changed with fresh GUS staining solution, and the tissues were incubated at 37 °C overnight (Jefferson 1987). Chlorophyll was removed from the samples with 70 % ethanol. The GUS expression patterns were recorded by Nikon SMZ1000 steroscopes.

To prepare the transverse section of the anthers, the spikelets after overnight GUS staining were washed briefly with 50 mM (pH 7.0) phosphate buffer and then immersed in FAA solution (70 % ethanol: formaldehyde: acetic acid = 18: 1: 1, by vol.). Vacuum was applied for three times to let the tissues immerse fully into the FAA solution and then treated for resin section. The transverse-section was 8 μm in thickness. GUS expression patterns in the anther transverse-section were recorded by Olympus AX70 microscopes.

Fluorometric quantitative assay of GUS activity in *OsLTP6* promoter::*uidA* transgenic rice

Fluorometric quantitative assay of GUS activity was performed using 4-methylumbelliferyl β-D-glucuronide (MUG) as a substrate, as described by Jefferson (Jefferson 1987). Protein concentration was assessed by Bradford (Bradford 1976) method, using bovine serum albumin

(BSA) as a standard. The fluorescence of 4-methylumbelliferone (4-MU) was measured with Modulus 9300-002 (Turner biosystems). GUS activity was normalized to the protein concentration and calculated as nmol of 4-MU per milligram of soluble protein per minute.

Results

Identification of a novel anther-specific expressed gene in rice and real-time RT-PCR confirmation for gene expression patterns

The candidate panicle-specific full-length cDNA, *osigcfa001g12* (Accession Number CT833313) (Liu et al. 2007), was isolated through redundancy analysis of the rice full-length cDNA libraries. This cDNA sequence was found highly abundant in the panicle library, but did not appear in the other three libraries.

The expression patterns of *osigcfa001g12* were further confirmed by real-time RT-PCR (Fig. 1). Rice *actin1* gene was used as an endogenous reference gene. The relative expression of *osigcfa001g12* in germinated seed, seedling root, seedling shoot, and panicles were 0, 0.005, 0.0014, and 1413.408, respectively. *Osigcfa001g12* was highly expressed in panicles than in the other tissues. This result of gene expression was in good accordance to that from the cDNA library studies. Because we did not utilize PCR amplification during cDNA library construction, the cDNA abundance in libraries mainly reflected the redundancy of mRNAs. We, therefore, identified a novel panicle-specific cDNA *osigcfa001g12* through expressional profiling. This gene has also shown its specific expression pattern in inflorescence via public microarrays (Wang et al. 2010).

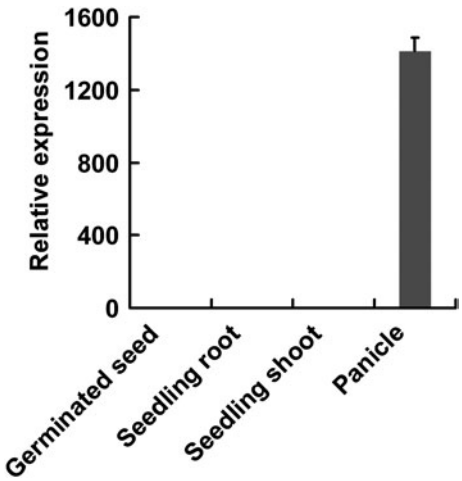

Fig. 1 Quantitative RT-PCR analysis of *OsLTP6*. Each data point is the average of three independent experiments. *Y axis*, relative expression values between transcript levels of *OsLTP6* and *Actin1*. *Vertical bars* SD

Sequence analysis of *osigcfa001g12* gene and its promoter region

Osigcfa001g12 was located on chromosome 10 and encoded a protein of 109 amino acids (LOC_Os10g05720.2). This protein belonged to the plant non-specific lipid transfer protein family (nsLTPs). The function of this gene has not been characterized yet. We designated this gene as *OsLTP6*. Figure 2a shows the amino acid sequence of *OsLTP6* with eight cysteine motifs in boxes.

OsLTP6 gene consisted of two exons and one intron (Fig. 2b). TSS was designated as +1. A putative TATA box (5′-TATAAAT-3′) was located at −35 to −29 relative to the transcriptional start site. A putative CAAT-box (5′-CCAATGCA-3′) was located at −325 to −318 relative to the transcription initiation site (Higo et al. 1999; Lescot et al. 2002).

Besides the consensus sequences common to eukaryotic promoters, an E-box sequence (5′-CATTTG-3′) was found located at −193 to −188 relative to TSS of *OsLTP6*. E-box was found presented in the promoter region of *OsC6* which was a lipid transfer protein that mainly expressed in anther tapetal cells (Zhang et al. 2010). E-box was also found to be present in the promoter region of the other four rice anther specific expressed genes, *RA8*, *Tapetum Degeneration Retardation* (*TDR*) (Li et al. 2006), *Undeveloped Tapetum1* (*UDT1*) (Jung et al. 2005), and *Defective Pollen Wall* (*DPW*) (Shi et al. 2011). Furthermore, the GTGA motif was present in two copies at position −387 to −384 and −409 to −406 relative to TSS of *OsLTP6* (Rogers et al. 2001). GTGA motif was an anther-specific *cis*-regulatory element. Another *cis*-element, CAAACAC sequence, was located at −166 to −160 relative to TSS of *OsLTP6*. CAAACAC sequence was a *cis*-acting element found in the promoter region of a LTP gene, *napA*. Deletion of CAAACAC sequence resulted in strong reduction in the promoter activity (Stalberg et al. 1996). These were the putative *cis*-regulatory motifs found in *OsLTP6* gene promoter region through sequence analysis.

The *OsLTP6* promoter directs anther-specific GUS expression

Functional analysis of *OsLTP6* gene promoter was carried out by stable transformation of rice. A 552-bp 5′ flanking region from −470 to +82 was amplified and introduced upstream of the *uidA* gene, which encodes β-glucuronidase (GUS). The 552-bp region contained all the analyzed *cis*-elements, TATA box, CAAT box, GTGA motifs,

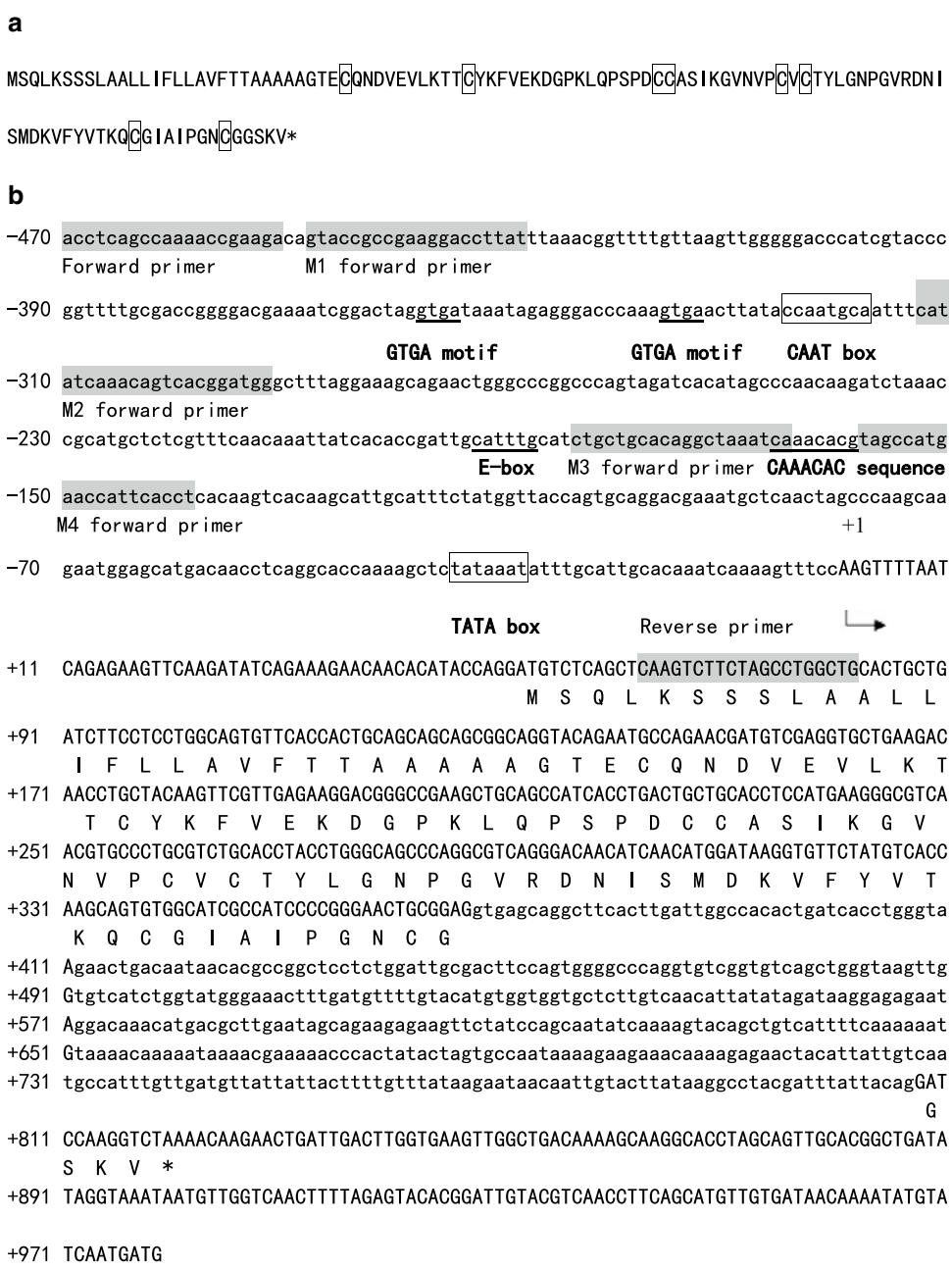

Fig. 2 The protein and genomic sequences of the *OsLTP6* gene. **a** The protein sequence of *OsLTP6* gene. The eight conserved cysteines are shown in *boxes*. The *asterisk* indicates the translation stop codon. **b** The genomic sequence of *OsLTP6* gene. Nucleotides are numbered on the *left side*, with the position of the transcriptional start site (TSS) designated as +1. Two exons of *OsLTP6* gene are indicated with *uppercase letters*. The TATA box and CAAT box are shown in *boxes*. The E-box, CAAACAC sequence, and GTGA motifs are *underlined*. The primer sequences for promoter amplification are blocked in *gray color*. The *arrow* indicates the position of TSS

E-box, and CAAACAC sequence. Ten amino acids (aa) of *OsLTP6* was fused to GUS protein at the *N*-terminal. GUS expression pattern was determined by the promoter sequences and would not be interfered with the *N*-terminal 10 aa fusion.

GUS activity was analyzed in *OsLTP6* promoter::*uidA* transgenic T1 plants. *OsLTP6* promoter directed anther-specific expression (Fig. 3). To identify the exact spatial and temporal regulation patterns, GUS activity in panicles was characterized at different developmental stages. At the microspore mother cells stage (st6), GUS staining was prominent and specifically restricted to the anthers as indicated by the arrows (Fig. 3a). Then, MMCs underwent meiosis. During meiosis stage (st7–9), GUS activity was also prominent in the anthers. No GUS activity was found in the palea, the lemma, and the glume (Fig. 3b). Figure 3c showed the spikelet at the same developmental stage as Fig. 3b with the palea and the lemma open to reveal the internal stained anthers. After meiosis, the haploid microspores were generated and they subsequently matured to become pollens. Before anthesis (st12), GUS activity was restricted in the anthers, while no GUS activity was found in the stigma, the ovary, and the filament of the stigmen (Fig. 3d). After anthesis (st14),

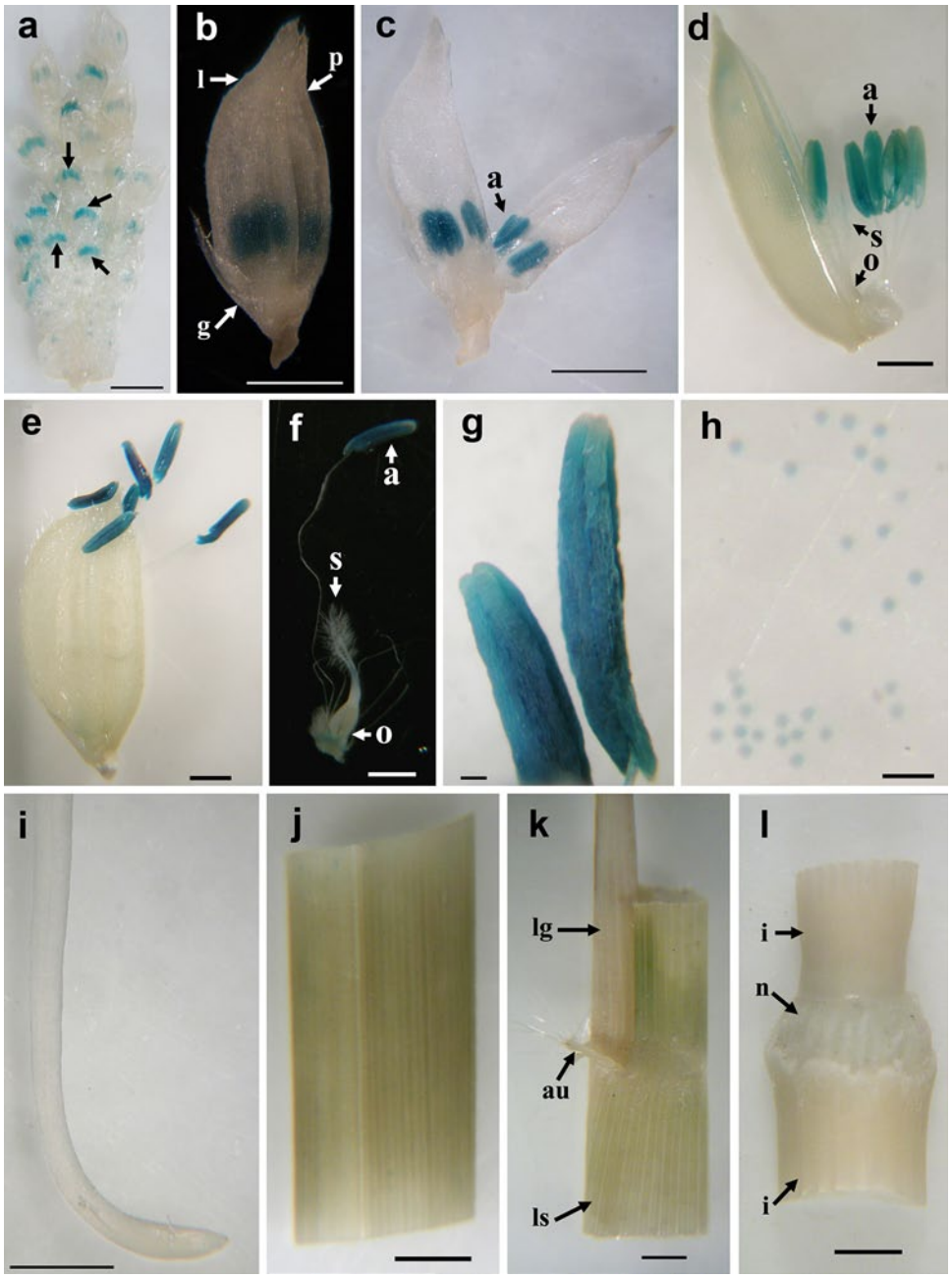

Fig. 3 Histochemical assay of GUS activity in *OsLTP6* promoter::*gus* transgenic rice. **a** A young panicle at early anther development. The *arrows* indicate the anthers with GUS staining. **b** A spikelet at meiosis stage. *P* palea, *l* lemma, *g* glume. **c** A spikelet at meiosis stage with the palea and lemma *open* to show the internal stained anthers. *A* anther. **d** A spikelet before anthesis. *S* stigma, *o* ovary. **e** A spikelet after anthesis. **f** The stamen and pistil. **g** The rice anthers at a bigger magnification. **h** The rice pollens. **i** A segment of rice root. **j** A segment of rice leaf blade. **k** Rice leaf sheath, auricles, and ligule. *Ls* leaf sheath, *au* auricles, *lg* ligule. **l** A segment of rice stem. *I* internode, *n* node. *Bar* 1 mm (**a**–**f**, **i**–**l**), 100 μm (**g**, **h**)

the anthers stretched outside the spikelet. The anthers were highly stained, while the palea, the lemma, and the glume were unstained (Fig. 3e). Figure 3f shows the inner pistil and stamen. GUS activity was remarkable in the anthers, while no GUS activity was found in the stigma, the main body of the ovary, and the filament of the stigmen. The ovary was faintly stained at the top site and the bottom site. Figure 3g showed the highly stained anthers at a bigger magnification. The mature pollens inside the anthers were GUS positive (Fig. 3h). Results of GUS staining in vegetative tissues were presented in Fig. 3i–l. No GUS expression was observed in root (Fig. 3i), leaf blade (Fig. 3j), leaf sheath, auricles, ligule (Fig. 3k) and the internode of the stem (Fig. 3l), while the node of the stem was faintly stained (Fig. 3l). In summary, the promoter of *OsLTP6* directed GUS reporter gene specifically expressed in anthers during anther development, from microspore mother cell developmental stage (st6) to mature pollen stage (st14).

Quantitative analysis of *OsLTP6* promoter activity

Fluorometric analysis was carried out to determine the GUS expression levels in plant tissues transformed with

OsLTP6 promoter::*uidA*. Five independent T1 transgenic lines were tested for quantification. Leaf blades, leaf sheaths, young stems, and young panicles were collected from transgenic plants growing in paddy field.

The results were shown in Fig. 4. GUS activity in the panicles was higher whereas no GUS activity was detected in leaf blades, leaf sheaths, and stems. The fluorometric quantification results indicated that *OsLTP6* promoter directed reporter gene specifically expressed in rice panicles.

In stem tissues, GUS activity was not detected in any lines of the transgenic plants. This result indicated that although GUS activity was faintly visible in stems by histochemical assay, its expression in stems was very low. Both histochemical staining and fluorometric assay indicated that *OsLTP6* promoter directed anther-specific expression.

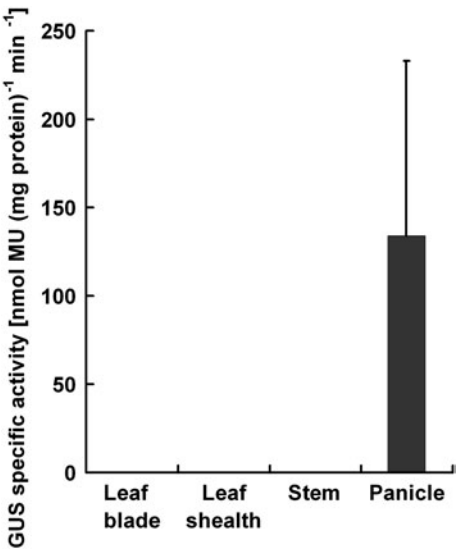

Fig. 4 Fluorometric GUS analysis in leaf blade, leaf sheath, stem, and panicles of transformed *OsLTP6* promoter::*gus* plants. Each value represents the average of five independent *transgenic lines*. The *error bar* indicates the SD. *MU* 4-methylumbelliferone

Transverse section of rice anther indicating GUS activity in anther tapetum

The rice anther consists of four layers of cells enclosing a central locule in which the microspores mature. The four layers are the epidermis, the endothecium, the middle layer, and the tapetum, from the surface to the interior. Specifically, the tapetum is the most important layer to provide nutrients and enzymes for pollen development. It effects the successful development of viable pollen grains within the anther (Huang et al. 2009; Zhang and Wilson 2009).

To determine the precise spatial regulatory patterns of *OsLTP6* promoter within the anthers, the anthers at two representative development stage, the microspore mother cell stage (st6) and the vacuolated pollen stage (st10), were transverse sectioned after GUS staining (Zhang and Wilson 2009). Figure 5a shows the transverse section of a spikelet. The palea and the lemma were located on the left side and bottom right, respectively, showing no GUS activity. The anthers were in the center of the picture. Each anther contained four lobes that were linked together with connective tissues. Figure 5b shows one lobe of the anther at a bigger magnification. GUS was predominantly expressed in the inner layer of the anther wall, the tapetum, as indicated by the arrow. The microspore mother cells were in the central locule. After meiosis, the haploid microspores were generated and they subsequently vacuolated to become round in shape (Fig. 5c; st10). GUS was specifically expressed in the inner layer of the anther wall, the tapetum (Fig. 5c). The transverse section of a lemma showed no GUS expression (Fig. 5d). In summary, transverse section of the rice anther indicated that *OsLTP6* promoter drove GUS specifically expressed in the anther tapetum.

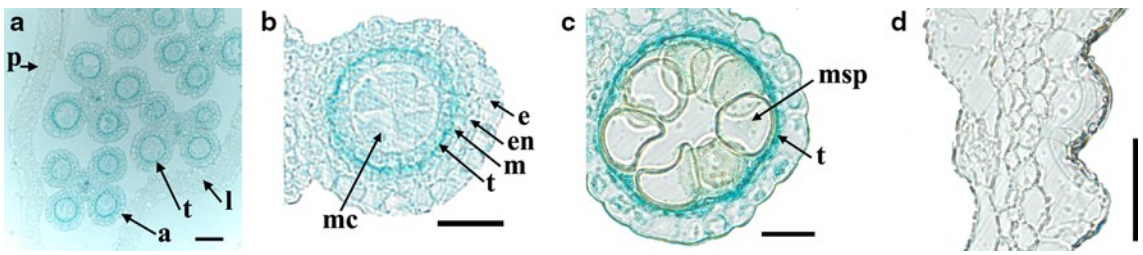

Fig. 5 Transverse section of the rice anther after GUS staining. *Blue color* indicates GUS expression at this location. **a** Transverse section of a spikelet. *P* palea, *l* lemma, *a* anther, *t* tapetum. **b** Transverse section of an anther locule at meiosis stage (st6). *E* epidermis, *en* endothecium, *m* middle layer, *mc* meiotic cell. **c** Transverse section of an anther locule at vacuolated stage (st10). *Msp* microspore. **d** Transverse section of a lemma. All the transverse sections are 8 μm in thickness. *Bar* 50 μm (**a**), 20 μm (**b**–**d**)

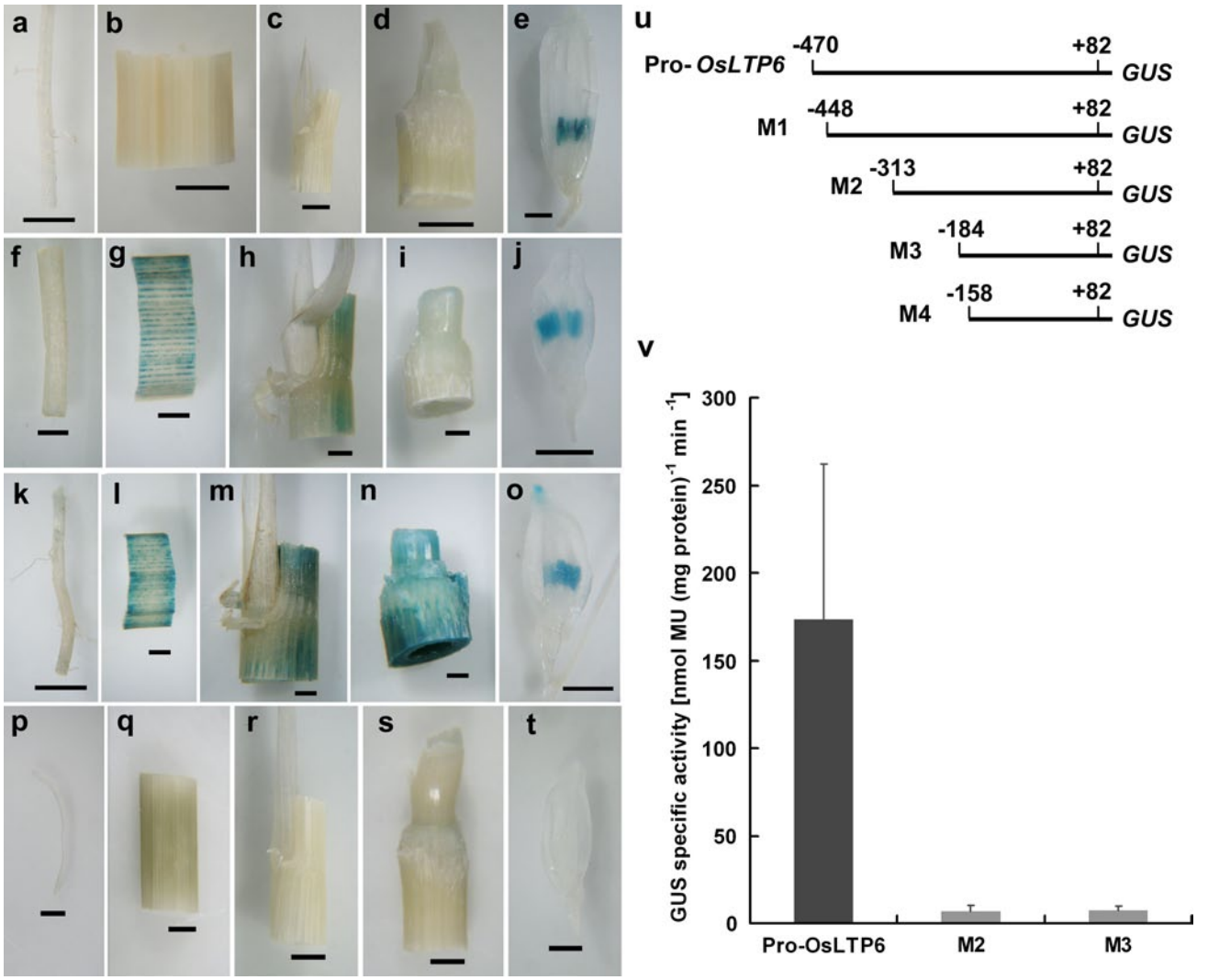

Fig. 6 Deletion analysis of *OsLTP6* promoter. **a–t** Histochemical assay of GUS activity in *OsLTP6* truncted promoter::*gus* transgenic rice. **a–e** GUS staining patterns of promoter M1 in root (**a**); leaf blade (**b**); leaf sheath, auricles, and ligule (**c**); stem (**d**) and spikelet (**e**). **f–j** GUS staining patterns of promoter M2 in root (**f**); leaf blade (**g**); leaf sheath, auricles and ligule (**h**); stem (**i**) and spikelet (**j**). **k–o** GUS staining patterns of promoter M3 in root (**k**); leaf blade (**l**); leaf sheath, auricles, and ligule (**m**); stem (**n**) and spikelet (**o**). **p–t** GUS staining patterns of promoter M4 in root (**p**); leaf blade (**q**); leaf sheath, auricles, and ligule (**r**); stem (**s**) and spikelet (**t**). *Bar* 1 mm. **u** Schematic representation of the native (Pro-*OsLTP6*) and truncated (M1, M2, M3 and M4) promoters. Each was fused with the *GUS* reporter gene and transformed into rice plants to study the *cis*-regulatory elements within *OsLTP6* promoter. The numbers referred to positions relative to the transcriptional start site (TSS). **v** Fluorometric GUS analysis of *OsLTP*6 promoter, promoter M2, and promoter M3 in rice panicles of transformed rice. Each value represents the average of five independent *transgenic lines*. The *error bar* indicates the SD. *MU* 4-methylumbelliferone

Deletion analysis indicates *OsLTP6* promoter achieved anther-specific expression through a combination of positive and negative regulatory elements

To identify regulatory elements within *OsLTP6* promoter region, four progressive deletions were applied at the promoter 5′ region. These truncated promoters, M1, M2, M3, and M4, were introduced upstream of the *uidA* gene and transformed into rice plants, respectively.

Promoter M1 sequence from −448 to +82 from the TSS had a deletion of 22-bp at the promoter 5′ region. Promoter M1 contained all the analyzed motifs,including CAAT-box, GTGA motif, E-box, CAAACAC motif, and TATA box. GUS staining results of promoter M1 were shown in Fig. 6a–e. GUS activity was specifically restricted in the anthers (Fig. 6e). No GUS activity was observed in root (Fig. 6a), leaf blade (Fig. 6b), leaf sheath, auricles, ligule (Fig. 6c), and stem (Fig. 6d). Promoter M1 showed the

same GUS expression pattern as the original promoter. This observation implied that M1 sequence and the sequence longer than M1 were sufficient to drive anther-specific gene expression.

Promoter M2 sequence from −313 to +82 from the TSS had deletions of both CAAT-box and GTGA motif. Promoter M2 exhibited a different GUS staining pattern compared with the original promoter (Fig. 6f–j). No GUS activity was detected in root (Fig. 6f), stem (Fig. 6i), and palea, lemma, glume of the spikelet (Fig. 6j), while GUS expression was observed in leaf blade (Fig. 6g), leaf sheath (Fig. 6h), and the anthers of the spikelet (Fig. 6j). Promoter M2 directed GUS expression not only in the anthers, but also in leaf blade and leaf sheath. This observation indicated that the motifs controlling leaf expression were presented in the 135-bp region from −448 to −314. This sequence suppressed GUS expression in leaf and deletion of this sequence released the suppression.

Promoter M3 sequence from −184 to +82 from the TSS made a progressive deletion of E-box motif. The GUS staining results of promoter M3 were shown in Fig. 6k–o. In vegetative tissues, GUS activity was observed in leaf blade (Fig. 6l), leaf sheath (Fig. 6m), and stem (Fig. 6n), while no GUS activity was observed in root (Fig. 6k). In rice spikelet, GUS activity was observed not only in the anthers of the spikelet, but also the top site of palea and lemma (Fig. 6o). Promoter M3 initiated GUS reporter gene expression in stem. This result indicated that the motifs responsible for stem repression were presented in the 129-bp region from −313 to −185 and deletion of these motifs released stem suppression.

Promoter M4 sequence from −158 to +82 from the TSS made a progressive deletion of CAAACAC motif. No GUS activity was observed in root (Fig. 6p), leaf blade (Fig. 6q), leaf sheath, auricles, ligule (Fig. 6r), the internode of stem (Fig. 6s), and the anthers (Fig. 6t). GUS activity was only faintly detectable in the node of the stem (Fig. 6s). Although there was only a 26-bp variation between M4 and M3, the GUS activities varied significantly. GUS activity was almost eliminated in promoter M4. This observation indicated that the 26-bp motif from −184 to −159 was essential for the downstream gene activation.

The truncated promoters were further analyzed for the quantitative expression of β-glucuronidase in panicles. For the reason that promoter M1 gave the same GUS expression pattern as the original promoter and promoter M4 showed no GUS activity in panicles, we compared the intensity of GUS expression among promoter M2, M3, and the original promoter (Fig. 6v). Both promoter M2 and M3 showed lower GUS activity than the original promoter, and the promoter intensity between M2 and M3 did not show obvious difference. The intensity of the original promoter was more than 20 times higher than that of promoter M2 and M3. This observation indicated that the motifs determining the intensity of *OsLTP6* expression were located on the 157-bp region from −470 to −314 from TSS. The CAAT-box and the GTGA motif were the putative motifs for increasing GUS expression intensity. In summary, promoter deletion analysis indicated that *OsLTP6* promoter achieved anther-specific expression by a combination of positive and negative regulatory elements.

Discussion

Based on deletion analysis, a regulatory model for *OsLTP6* promoter was developed (Fig. 7a). The TATA box (−35 to −29 from TSS) was the basal transcriptional regulatory element for the initiation of transcription. However, it only gave a low level of transcription as shown in the promoter M4. A 26-bp transcriptional regulatory sequence (−159 to −184) upstream of TATA box was a key element for transcriptional activation. It initiated the expression of GUS reporter gene in anthers as well as in leaves and stems as shown in the promoter M3. It was the putative binding site for transcriptional activator of *OsLTP6* gene. Activator binding to this motif greatly promoted the downstream gene transcription. In addition, CAAT box (−325 to −318) and GTGA motifs (−358 to −355 and −336 to −333) were the other two putative positive regulatory elements leading to full expression activity for *OsLTP6* gene.

The 135-bp sequence from −314 to −448 from TSS was responsible for leaf repression. This sequence was the putative binding sites for transcriptional regulators. Blast analysis showed that the 135-bp sequence had a good deal of homologous sequence in the rice genome. This sequence was not specific to *OsLTP6* gene. The homologous study indicated that the model for leaf repression in *OsLTP6* promoter region might also be shared by other genes in rice genome. Especially the 27-bp sequence from −353 to −327 from TSS had the highest match. The 27-bp sequence comprised a group of motifs, including DOFCOREZM (S000265), GTGANTG10 (S000378), CACTFTPPCA1 (S000449), INRNTPSADB (S000395), and TBOXATGAPB (S000383) (Higo et al. 1999; Lescot et al. 2002). Upstream the 27-bp sequence was the GTGA motif and downstream the sequence was the CAAT box. This region was the putative binding sites for a batch of transcriptional regulators. The regulators might interact with each other and perform their function in a complex to regulate the rate of gene transcription. The *cis*-regulatory region for stem repression was located from −313 to −185 from TSS. This sequence suppressed gene expression in stem and deletion of this sequence released the suppression.

Furthermore, the 26-bp sequence (−159 to −184) was compared with the upstream sequence (2 kilobase from the

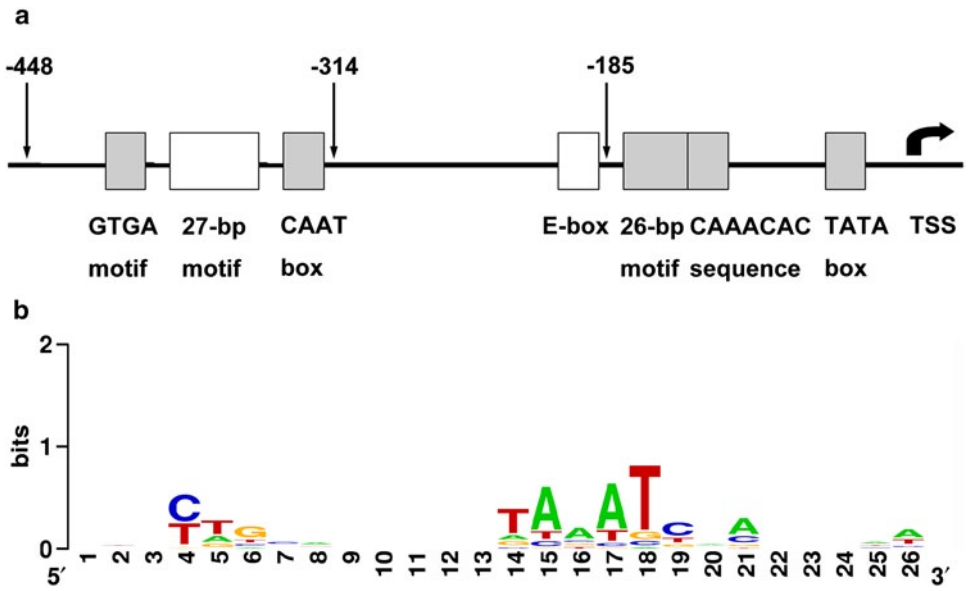

Fig. 7 Schematic representation of *cis*-elements in *OsLTP6* promoter region. **a** Organization of *cis*-elements in the 5′ flanking region of the *OsLTP6* gene. The *numbers* refer to positions relative to the transcriptional start site (TSS). TSS is indicated by an *arrow*. *Gray boxes* indicate positive elements. The region from −448 to −314 suppresses gene expression in leaf. The region from −313 to −185 suppresses gene expression in stem. **b** 26-bp sequence alignment generated by Weblogo 3. Conserved nucleotides were shown in *color*. *X-axis* showed position in the 26-bp sequence from 5′ to 3′ end. *Y-axis* showed stacks of nucleotides at each position and the overall height of each stack indicated the sequence conservation at that position (measured in bits)

transcriptional start site) of the identified rice anther-specific expressed genes, such as *RA8* (Jeon et al. 1999), *RTS* (Luo et al. 2006), *GaMYB* (Kaneko et al. 2004; Aya et al. 2009), *UDT1* (Jung et al. 2005), *TDR* (Li et al. 2006), *OsC6* (Zhang et al. 2010), *DPW* (Shi et al. 2011), *Persistent Tapetal Cell1* (*PTC1*) (Li et al. 2011), and *Eternal Tapetum1* (*EAT1*) (Niu et al. 2013). Blast analysis identified 26 homologous pairs (seven base pairs or longer than seven base pairs). The 26 homologous sequences and the 26-bp sequence were aligned using Weblogo 3 (http://weblogo.berkeley.edu/logo.cgi) (Crooks et al. 2004); (Fig. 7b). The aligned results indicated a conserved TAAATC region within 26-bp sequence. Especially the nucleotides at position 14 (T), 15 (A), 17 (A), and 18 (T) were more conserved. We designated this sequence as TAAATC motif. The TAAATC motif was the putative binding site for transcriptional activator to initiate gene transcription in rice anther.

In summary, *OsLTP6* promoter achieved anther-specific expression through a combination of positive and negative regulatory elements. The 26-bp motif upstream of TATA box was a key transcriptional activator for *OsLTP6* gene. The 26-bp motif together with TATA box promoted the recruitment of RNA polymerase and increased the rate of gene transcription. CAAT box and GTGA motif increased the transcription level to full expression. Two negative regulatory elements repressed the expression in leaf and stem, respectively, thus achieving anther-specific expression.

OsLTP6 belonged to the plant non-specific lipid transfer protein family. The proteins of this family were characterized by an eight cysteine motif (8 CM) backbone (Jose-Estanyol et al. 2004). The eight cysteine residues form four disulfide bonds and stabilize a hydrophobic cavity, which allow the binding of different lipids and hydrophobic compounds. Type I nsLTPs are characterized by a long tunnel-like cavity (Gincel et al. 1994; Lerche and Poulsen 1998; Cheng et al. 2004) while type II nsLTPs have two adjacent hydrophobic cavities (Samuel et al. 2002; Hoh et al. 2005). Genome-wide analysis of nsLTP gene families in *Oryza sativa* identified 52 rice nsLTP genes and they are clustered in nine different clades (Boutrot et al. 2008). *OsLTP6* belongs to the Type VI.

nsLTPs facilitate the transfer of fatty acids, phospholipids, and steroids between membranes. They play diverse roles in various biological processes, such as cutin biosynthesis (Kim et al. 2008; Lee et al. 2009), defense response (Lee et al. 2009; Sarowar et al. 2009), plant signaling (Blein et al. 2002; Maldonado et al. 2002), and seed maturation (Thoma et al. 1994). Most nsLTPs have been identified on the basis of sequence homology; only a limited number of rice nsLTPs has been characterized.

OsLTP1 promoter directs GUS expression in both vegetative and reproductive organs and is inducible by pathogen attack (Guiderdoni et al. 2002). The proposed role of OsLTP1 is strengthening of structural barriers and organ

protection against mechanical disruption and pathogen attack. Rice *LTP2* mRNA is accumulated only in mature seeds under normal conditions. After treatment with abscisic acid (ABA), mannitol or NaCl, *OsLTP2* mRNA can also be detected in vegetative tissues (Garcia-Garrido et al. 1998). *OsLTP5* transcript is expressed prominently in stems and flowers and is induced by cutin monomer (Kim et al. 2008). *OsC6*, a Type VII nsLTP, is required for postmeiotic anther development. *OsC6* expression is mainly detectable in tapetal cells and weakly in microspores during anther development (Zhang et al. 2010). Different nsLTP has different expression pattern and function. *OsLTP6* promoter directs gene expression, especially in anther tapetum and pollen from microspore mother cell stage (st6) to mature pollen stage (st14). The anther cuticle and pollen exine are composed of cutin matrixes with waxes embedded in and deposited on the surface of the matrix. They are the main protective barriers for microspores against various environmental and biological stresses (Jung et al. 2006; Li et al. 2010). We infer that the function of *OsLTP6* is the transfer and binding of lipid for the formation of the anther cuticle and pollen exine to protect inner microspores.

The promoter of *OsLTP6* gene is shorter in length as comparing with other anther-specific promoters such as 2.5 kb *RA8* gene promoter (Jeon et al. 1999), 1.2 kb *RTS* promoter (Luo et al. 2006), and 2,073 bp *OsC6* promoter (Yokoi et al. 1997; Zhang et al. 2010), thus facilitating its application in vector construction. The *OsLTP6* promoter, 530 bp (from −448 to +82 from TSS) in length, is sufficient to drive anther-specific gene expression. Shorter promoter sequence reduces plasmid size and improves cloning efficiency. The potential usage of *OsLTP6* promoter is to engineer male sterility for hybrid production as well as for gene confinement for genetically modified plants in field trials and agricultural production.

In conclusion, we identified a novel rice anther-specific plant lipid transfer protein (*OsLTP6*) gene. The *OsLTP6* promoter directed GUS reporter gene specifically expressed in anther tapetum and pollen. Moreover, the anther-specific regulatory mechanism for *OsLTP6* promoter was elucidated. *OsLTP6* promoter achieved anther-specific expression through a combination of positive and negative regulatory elements. It revealed the regulating complexity of anther-specific expression. At least for the case of *OsLTP6* promoter, anther-specific expression was not achieved by a single *cis*-element. The *OsLTP6* promoter study also suggested that *OsLTP6* played a crucial role in rice anther development. However, it needs further investigated whether the *OsLTP6* involves in lipid binding activity or lipidic component synthesis during anther development.

Acknowledgments The authors are grateful to Yiwen Yan and Tingting Lu for their technical help in this research. This work was supported by the grants from the National Natural Science Foundation of China (Grant No. 30771159, 30871319).

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