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ORIGINAL ARTICLE

Evaluation of rice promoters conferring pollen-specific expression in a heterologous system, *Arabidopsis*

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Abstract Promoters can direct gene expression specifically to targeted tissues or cells. Effective with both crop species and model plant systems, these tools can help researchers overcome the practical obstacles associated with transgenic protocols. Here, we identified promoters that allow one to target the manipulation of gene expression during pollen development. Utilizing published transcriptomic databases for rice, we investigated the promoter activity of selected genes in *Arabidopsis*. From various microarray datasets, including those for anthers and pollen grains at different developmental stages, we selected nine candidate genes that showed high levels of expression in the late stages of rice pollen development. We named these *Oryza sativa* late pollen-specific genes. Their promoter regions contained various *cis*-acting elements that could be responsible for anther-/pollen-specific expression. Promoter::GUS–GFP reporters were constructed and introduced into *Arabidopsis* plants. Histochemical GUS staining revealed that six of the nine rice promoters conferred strong *GUS* expression that was restricted to the anthers in *Arabidopsis*. Further analysis showed that although the GUS signals were not detected at the unicellular stage, they strengthened in the bicellular or tricellular stages, peaking at the mature pollen stage. This paralleled their transcriptomic profiles in rice. Based on our results, we proposed that these six rice promoters, which are active in the late stages of pollen formation in the dicot *Arabidopsis*, can aid molecular breeders in generating new varieties of a monocot plant, rice.

Keywords *Arabidopsis* · GUS · Male gametophyte · Pollen-specific expression · Rice promoters

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Introduction

Billons of people rely on the double fertilization of flowering plants for products used in their daily life, including food and clothing. As demand for high-quality food increases with continued population growth, while the agricultural environment is altered through climate change, researchers will be challenged to develop new crop varieties. For example, the male gametophyte (pollen grain) from rice, one of the most important stable crop, presents an excellent target for molecular breeding experiments.

The most effective strategy for improving yields from fertile F1 hybrids is to create male sterile or restorer lines. This can be accomplished by either inhibiting endogenous hormone biosynthesis during pollen development or applying appropriate hormones to the reproductive tissue at the correct stage (Bae et al. 2010). Thus, identifying the best anther-/pollen-specific rice promoter is necessary so that one avoids undesirable effects on other sporophytic tissues that may alter plant growth or flowering time. Although such efforts that employ several constructs with rice tissue culture are feasible in the laboratory, field trials with transgenic plants are very labor-intensive, can usually be conducted only once in each growing season, and are constrained by safety regulations. Therefore, it would be advantageous to obtain rice promoters whose specificity for anther/pollen is equivalent to that found with the model plant *Arabidopsis*.

Pollen grains form within the anther during several distinct stages that involve three rounds of cell division (Borg et al. 2009). A pollen mother cell undergoes meiosis, giving rise to a tetrad of four haploid microspores enclosed in a callose wall. Each microspore is released upon the dissolution of the callose wall and undergoes pollen mitosis I (PMI). This highly asymmetric division results in a larger vegetative cell and a smaller generative cell with different fates. While the vegetative cell exits the cell cycle and differentiates for its biological role to deliver sperm cells in the vicinity of the female gametophyte via the pollen tube, the generative cell proceeds to pollen mitosis II (PMII), in which two sperm cells are produced. The majority of species, such as lily and tomato, sheds the bicellular pollen, with PMII taking place in the pollen tube. In contrast, *Arabidopsis* and rice shed tricellular pollen at anthesis, although their promoters did not necessarily work in bicellular plants (Ge et al. 2011).

Male gametophyte development succeeds because of the highly orchestrated expression of several genes. Numerous research groups have already identified some of the promoters that control pollen-specific expression (Honys and Twell 2004; Engel et al. 2005; Honys et al. 2006; Verelst et al. 2007; Gupta et al. 2007; Zhang et al. 2010; Khurana et al. 2013b). Both early and late pollen genes have been studied in various crops (Zimmermann et al. 2004; Twell et al. 2006; Borges et al. 2008; Zhang et al. 2010; Huang et al. 2011). Most of the late pollen genes exhibit similar patterns of expression, in which transcripts are generally accumulated beginning at PMI and then increase in abundance as the pollen grains mature (Twell et al. 1989; Xu et al. 1999).

Although several such promoters have been analyzed in crop plants, only *Osg6B*, *OsIPP3*, *OsbHLH*, *OsIPK*, *OsFbox*, *OsIPP3*, *OsIPA*, *OsSCP1*, *OsSCP2*, and *OsSCP3* have been reported in rice (Yokoi et al. 1997; Park et al. 2006; Swapna et al. 2011; Khurana et al. 2013a, b). Among these, the *OsIPK*, *OsIPA*, *OsbHLH*, *OsFbox* and *OsIPP3* promoters have conferred anther-/pollen-specific expression in the heterologous dicot system of *Arabidopsis* (Swapna et al. 2011; Khurana et al. 2013a, b). The most effective way to identify these promoters is to obtain genome-wide expression profiles during microsporogenesis, utilizing transcriptome analyses based on DNA microarray or RNA-seq technologies. The two most popular and reliable commercial array platforms in rice are Affymetrix and Agilent 44K. To date, 2,081 Affymetrix and 1,706 Agilent 44K array data are available from The Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov/geo/) (Jung et al. 2011; Barrett et al. 2013). Three genome-wide perspectives have recently been published on the use of transcriptomes from developing anthers and pollen in rice: (1) transcriptome analysis of pre-meiotic (PMA), meiotic (MA), anthers at single-celled (SCP), and tri-nucleate pollen (TPA) stages (Deveshwar et al. 2011); (2) 33 laser micro-dissection (LM) microarray data and 11 microarray data covering the four stages of anther development (Aya et al. 2011); and (3) transcriptomes for developing and germinated pollen, and spern cell (Wei et al. 2010; Russell et al. 2012). Meta-expression analyses of these data are useful for identifying pollen-preferred or pollen-specific genes. Furthermore, the Rice Oligonucleotide Array Database (www.ricearray.org/) provides comprehensive expression profiles for rice genes that function in anatomy or development. These sources offer an approach for filtering out genes expressed in tissues/organs other than those being targeted (Cao et al. 2012).

Here, we used pollen-specific promoters identified in rice to evaluate their activities in *Arabidopsis*, a heterologous model plant. Also, for the evaluation of pollen-specific GUS expression, we performed rice transformation. Based on meta-expression analysis using microarray datasets available in rice, we selected nine genes showing high expression during the later stages of pollen grain formation. We then characterized their promoter activities using promoter::GFP–GUS reporters in *Arabidopsis* and rice.

Materials and methods

Selection of candidate genes showing pollen-specific expression in rice

We collected publicly available microarray datasets for rice anthers and tricellular, mature, and germinating pollen from the GEO database (Supplemental Tables 1 and 2). These data were normalized via Affy software, as described by Cao et al. (2012). Average intensities for

Table 1 Nine candidate genes exhibiting microspore- or pollen-specific expression based on microarray data

Vector	ID of RFGED	NCBI accession no.	Gene description	Promoter size and location on vector
OsLPS1	LOC_Os08g44660.1	*Os08g0560700*	Similar to Polcalcin Phl p 7 (Calcium-binding pollen allergen Phl p7)	263 −411 to −148
OsLPS2	LOC_Os04g54600.1	*Os04g0638800*	DUF617, plant family protein with unknown function	2,283 −2,405 to −122
OsLPS3	LOC_Os11g45730.1 LOC_Os11g45730.2	*Os11g0683800*	Pectinesterase family protein	1,520 −1,815 to −295
OsLPS4	LOC_Os02g50770.1	*Os02g0741200*	Plant peroxidase family protein	963 −1,038 to −75
OsLPS5	LOC_Os04g49650.1 LOC_Os04g49650.2	*Os04g0585900*	DUF581 family protein with unknown function	1,792 −1,792 to 0
OsLPS6	LOC_Os01g69020.1 LOC_Os01g69020.2	*Os01g0919200*	Cell division protein in FtsZ family	1,779 −1,868 to −89
OsLPS7	LOC_Os05g46530.1	*Os05g0543000*	Protein containing plant invertase/pectin methylesterase inhibitor domain	1,784 −1,928 to −144
OsLPS8	LOC_Os07g14340.1	*Os07g0247000*	Protein containing plant invertase/pectin methylesterase inhibitor domain	1,634 −1,711 to −77
OsLPS9	LOC_Os04g25190.1	*Os04g0317800*	Protein for pollen allergen in Lol p2 family	1,498 −1,570 to −72

reproductive tissues, including the anthers and grains at the trinucleate and mature stages, were compared with those for other vegetative tissues and organs. Those that showed distinct patterns of expression were chosen for further analysis.

Isolation of promoter region and construction of promoter::GFP–GUS reporters

Primers to amplify the promoter region for our nine candidate genes were designed via the oligonucleotide properties calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html; Supplemental Table 3). Sizes for the selected promoter regions varied from 263 to 2,283 bp (Table 1).

We used the GATEWAY cloning system to generate promoter::GUS–GFP reporters according to the manufacturer's instructions (Invitrogen, http://www.invitrogen.com). First, promoter fragments were amplified by two-step PCR, using genomic DNA extracted from 3-week-old japonica rice seedlings ('Donjinbyeo') by the cetyl trimethyl ammonium bromide (CTAB) method (Ronald and Chen 1999). For the first step, gene-specific primers with 12 bp of attB1 and attB2 sites were added, and PCR was conducted at 94 °C/5 min; then 10 cycles of 94 °C/30 s, 58 °C/30 s, and 72 °C/2 min; followed by a final extension of 72 °C/5 min. In the second step, 10 μL from the first PCR reaction was added to a 40-μL PCR reaction mixture containing the attB1 and attB2 adapter primers. Conditions included 20 cycles of 94 °C/30 s, 55 °C/30 s, and 72 °C/2 min; then a final extension of 72 °C/5 min. BP recombination reactions were conducted between the amplified promoter fragments and the pDONR201 vector, using BP clonase mix II (Invitrogen). The resulting entry clones were verified by sequencing and subjected to LR recombination reactions with the pKGWFS7 plant destination vector (Karimi et al. 2005), using LR clonase mix II (Invitrogen). The final plant expression vectors were transferred into *Agrobacterium tumefaciens* (GV3101).

Agrobacterium-mediated transformation of *Arabidopsis* and rice

Plants of *Arabidopsis* ('Columbia', or 'Col-0') were grown at 20–22 °C in a controlled-environment room under a 16-h light regime. Wild-type (WT) plants were transformed by the floral-dip method (Clough and Bent 1998). Transgenic plants were selected on Murashige and Skoog agar media supplemented with 50 mg L^{-1} kanamycin and 200 mg L^{-1} cefotaxime.

Mature seeds of the japonica rice cultivar Dongjinbyeo were used for Agrobacterium-mediated transformation (Hiei et al. 1997; Toki et al. 2006). We extracted genomic DNAs from the leaf tissues of T_1 transgenic plants with a homogenization procedure that included TissueLyser (Qiagen, http://www.qiagen.com). PCR was performed in a 20 μL mixture containing 1 μL of genomic DNA, 1 μL of each primer (20 pmol), 0.4 μL of dNTPs (1 mM), 2 μL of

10 X PCR buffer, and 0.1 μL of e-Taq polymerase (5 units μL^{-1}; Solgent, http://www.solgent.com). Conditions included 95 °C/2 min; then 30 cycles of 94 °C/1 min, 55 °C/1 min, and 72 °C/2 min; followed by a final extension of 72 °C/5 min.

Histochemical staining for GUS and microscopic evaluation

Histochemical GUS staining was performed with transgenic plants as described by Oh et al. (2010). Seedlings or whole inflorescences were submerged in GUS buffer [0.1 M sodium phosphate (pH 7.0), 1 mM EDTA (pH 8.0), 0.1 % Triton X-100, and 0.5 mM potassium ferricyanide] that contained 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-D-glucuronic acid). They were then vacuum-infiltrated for 10 min and incubated overnight at 37 °C. Afterward, the tissues were cleared in 70 % ethanol and viewed under a stereomicroscope (Stemi 2000C, Zeiss). Stained seedlings and inflorescences were imaged using a ProgRes C3 camera (Jenoptik, Germany). To examine the developing microspores and pollen grains at different stages, we fixed the GUS-stained inflorescences in a 3:1 (v:v) solution of ethanol and acetic acid prior to staining with DAPI (4′, 6-diamidino-2-phenylindole). Anthers in DAPI solution on the microscope slides were then opened with dissecting needles and gently squashed under a cover slip. Images were captured from either the ProgResC3 camera to show GUS signals under a bright field or else the ProgRes MFcool camera for investigating DAPI epifluorescence under a Nikon ECLIPSE 80i microscope (Nikon).

Results

Candidate genes showing pollen-specific expression

To identify pollen-specific genes in rice, we collected 65 Affymetrix arrays from five datasets. From these, we selected six slides for anthers containing tricellular and mature pollen grains, and 11 with tricellular and mature pollen grains. As controls, we used other tissue/organ types from Affymetrix meta-anatomical expression data that we had recently produced (Cao et al. 2012). In all, 261 genes showed 500-fold change in expression when compared with the other tissues/organs. Our heat map of the patterns displayed by nine randomly selected candidate genes confirmed that expression was preferential in grains at the tri-nucleate, mature, and germinating stages (Fig. 1).

Sequence analysis of nine promoter regions

To evaluate the promoter activity for these pollen-specific genes, we used genomic DNA from ‘Dongjinbyeo’ rice as template for isolating the upstream regulatory region (URR), which comprised 263–2.3 kb, excluding the 5′-

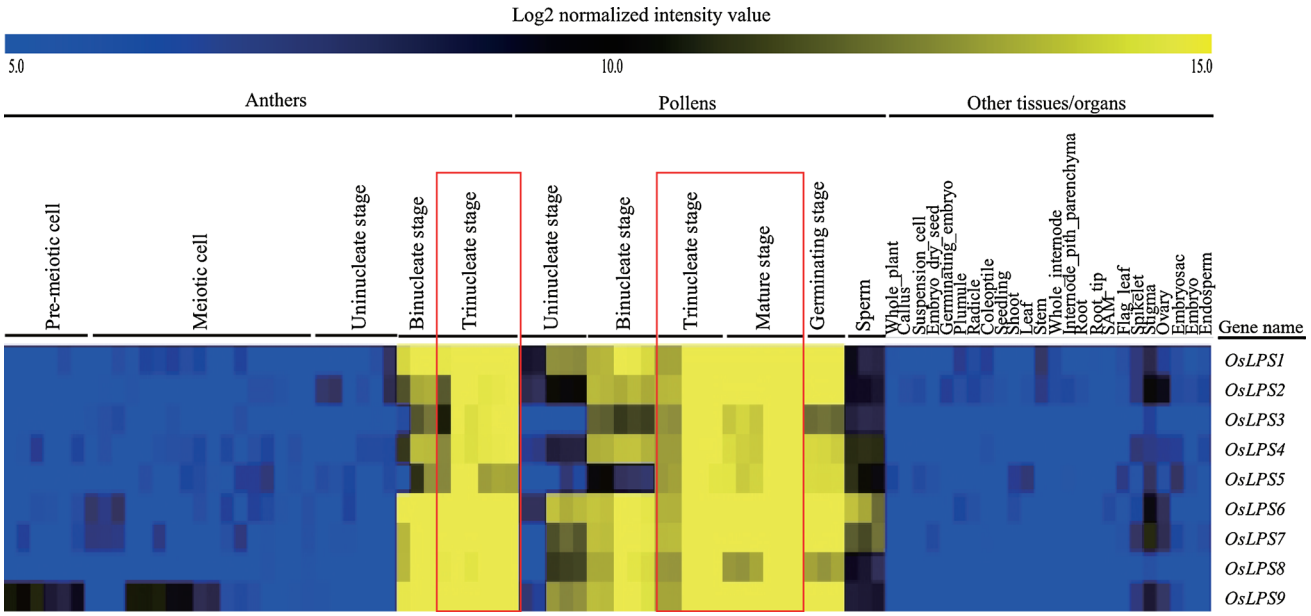

Fig. 1 Heat map analysis of expression patterns for nine genes, based on meta-anatomical expression. *Blue color* indicates low expression; *yellow* high expression. Values beneath *blue–yellow bar* indicate normalized average $\log_2$ intensity. Samples presented in heat map are detailed in Supplemental Tables 1 and 2 (color figure online)

Table 2 *Cis*-acting elements identified by the (PLACE DATABASE signal scan)

Promoter	Site	Sequence	Position from ATG/translation start codon
OsLPS1	MYCCONSENSUSAT	CANNTG	_176
	DOFCOREZM	AAAG	_405
	GTGANTG10	GTGA	_376
	ARR1AT	NGATT (N = A/C/T/G)	_281, _342
	POLLEN1LELAT52	AGAAA	_
	SITEIIATCYTC	TGGGCY (Y = C/T)	_325, _330, _367
	IBOXCORE	GATAA	_
OsLPS2	MYCCONSENSUSAT	CANNTG	_236, _281, _587, _954, _1417, _2053
	DOFCOREZM	AAAG	_148, _352, _478, _488, _547, _946, _1051, _1291, _1489, _1999, _2012, _2145
	GTGANTG10	GTGA	_481, _728, _1524, _1819, _1874, _2388, _2393
	ARR1AT	NGATT (N = A/C/T/G)	_485, _616, _776, _818, _859, _1088, _1129, _1158, _1207, _1267, _1584, _1720, _1928, _2087, _2167
	POLLEN1LELAT52	AGAAA	_989, _1030, _1144, _1518, _2015, _2037
	SITEIIATCYTC	TGGGCY (Y = C/T)	_364, _416, _440
	IBOXCORE	GATAA	_610, _1535
OsLPS3	MYCCONSENSUSAT	CANNTG	_1019, _1427, _1504
	DOFCOREZM	AAAG	_473, _555, _639, _807, _928, _1089, _1143, _1155, _1224, _1365
	GTGANTG10	GTGA	_983, _1065, _1261, _1450, _1724
	ARR1AT	NGATT (N = A/C/T/G)	_470, _765, __856, _1097, _1173, _1519, _1561, _1570
	POLLEN1LELAT52	AGAAA	_716, _757, _799, _842, 932
	SITEIIATCYTC	TGGGCY (Y = C/T)	_1775
	IBOXCORE	GATAA	_532, _803, _1228, _1539
OsLPS4	MYCCONSENSUSAT	CANNTG	_372, _431
	DOFCOREZM	AAAG	_112, _223, _394, _542, _607
	GTGANTG10	GTGA	_391, _ 513, _549, _974
	ARR1AT	NGATT (N = A/C/T/G)	_469, _492, _591, _604, _777, _896, _990
	POLLEN1LELAT52	AGAAA	_81, _193, _620, _1032
	SITEIIATCYTC	TGGGCY (Y = C/T)	_
	IBOXCORE	GATAA	_
OsLPS5	MYCCONSENSUSAT	CANNTG	_601, _982, _1004, _1079, _1349, _1394, _1663, _1671, _1706, _1749
	DOFCOREZM	AAAG	_392, _882, _888, _1199, _1264
	GTGANTG10	GTGA	_978, _1145, _1151, _1230, _1243, _1679
	ARR1AT	NGATT (N = A/C/T/G)	_353, _380, _485, _673, _807, _867, _1226, _1738
	POLLEN1LELAT52	AGAAA	_742, _875, _885, _1105
	SITEIIATCYTC	TGGGCY (Y = C/T)	_1294
	IBOXCORE	GATAA	_
OsLPS6	MYCCONSENSUSAT	CANNTG	_244, _356, _1281, _1507, _1541
	DOFCOREZM	AAAG	_114, _161, _277, _296, _335, _435, _591, _712, _840, _855, _905, _1596, _1675
	GTGANTG10	GTGA	_763, _943, _982, _1444, _1539, _1590
	ARR1AT	NGATT (N = A/C/T/G)	_314, _690, _894, _1207, _1233, _1273, _1744, _1836
	POLLEN1LELAT52	AGAAA	_297, _327, _592, _778, _841, _1610, _1677
	SITEIIATCYTC	TGGGCY (Y = C/T)	_
	IBOXCORE	GATAA	_

Table 2 continued

Promoter	Site	Sequence	Position from ATG/translation start codon
OsLPS7	MYCCONSENSUSAT	CANNTG	_607, _951, _1004, _1562, _1712, _1743
	DOFCOREZM	AAAG	_214, _337, _356, _794, _863, _1250, _1391
	GTGANTG10	GTGA	_1121, _1493
	ARR1AT	NGATT (N = A/C/T/G)	_543, _653, _802, _860, _1010, _1036, _1143, _1383, _1544
	POLLEN1LELAT52	AGAAA	_211, _334, _446, _864, _1581, _1594, _1819
	SITEIIATCYTC	TGGGCY (Y = C/T)	_
	IBOXCORE	GATAA	_1246, _1790
OsLPS8	MYCCONSENSUSAT	CANNTG	_133, _232, _284, _569, _685, _924, _933, _952, _1436, _1548, _1704
	DOFCOREZM	AAAG	_165, _273, _602, _767, _1096, _1254, _1371
	GTGANTG10	GTGA	_119, _326, _377, _377, _475, _976, _1441, _1616
	ARR1AT	NGATT (N = A/C/T/G)	_889, _986, _1155, _1411, _1421, _1497, _1563, _1628
	POLLEN1LELAT52	AGAAA	_242, _592, _1530
	SITEIIATCYTC	TGGGCY (Y = C/T)	_
	IBOXCORE	GATAA	_1192, _1613
OsLPS9	MYCCONSENSUSAT	CANNTG	_193, _334, _492, _598, _918, _1099, _1175, _1406
	DOFCOREZM	AAAG	_295, _397, _928,
	GTGANTG10	GTGA	_326, _453, _1336, _1550, _1554
	ARR1AT	NGATT (N = A/C/T/G)	_288, _324, _522, _615, _715, _734, _1363
	POLLEN1LELAT52	AGAAA	_1355
	SITEIIATCYTC	TGGGCY (Y = C/T)	_
	IBOXCORE	GATAA	_

untranslated region (UTR). Amplified promoter fragments were used for constructing reporter gene fusions. The resulting binary vectors were designated as *OsLPS1* through *9* (rice late pollen-specific gene 1–9) (Table 1). Sequences of the promoter fragments were compared with the 'Nipponbare' rice genomic sequence, using the BioEdit sequence alignment editor. Because 'Nipponbare' differs from 'Dongjinbyeo' rice, we found variations while verifying the promoter sequence of *OsLPS3*. Similar results have been described for the URRs of anther-/pollen-specific genes *OsIPK*, *OsbHLH*, and *OsFbox* (Gupta et al. 2007; Khurana et al. 2013b). By comparison, no differences between the two cultivars were found for the other eight promoters.

The *cis*-acting elements possess specific sequence information that is recognized by corresponding trans-acting factors. Various *cis*-acting elements in the promoter region are involved in anther-/pollen-specific expression (Tebbutt and Lonsdale 1995; Rogers et al. 2001; Swapna et al. 2011). We used the promoter analysis program PLACE DATABASE signal scan (Higo et al. 1999) to detect putative *cis*-acting elements. Regions examined here included 263 URR + 148 UTR (411 bp) for *OsLPS1*, 2283 URR + 122 UTR (2,405 bp) for *OsLPS2*, 1520 URR + 295 UTR (1,815 bp) for *OsLPS3*, 963 URR + 75 UTR (1,038 bp) for *OsLPS4*, 1792 URR (1,792 bp)) for *OsLPS5*, 1779 URR + 89 UTR (1,868 bp) for *OsLPS6*, 1784 URR + 144 UTR (1,928 bp) for *OsLPS7*, 1634 URR + 77 UTR (1,711 bp) for *OsLPS8*, and 1498 URR + 72 UTR (1,570 bp) for *OsLPS9*. Those elements present in all promoter regions are listed in Table 2.

Each promoter had several MYC recognition sites (CANNTG) that occur in the promoters of the dehydration gene *rd22* and many other genes in *Arabidopsis* (Abe et al. 2003). All promoter regions had the AAAG motif, which is the recognition sequence of Dof proteins, a family of transcription factors associated with diverse promoters of plant-specific genes (Yanagisawa and Schmidt 1999). They also contained the GTGA motif, which is similar to tobacco *g10* late pollen regulatory elements. The tobacco gene *g10*, which is homologous to pectate lyases and the tomato gene *lat56*, is preferentially and maximally expressed in mature pollen (Rogers et al. 2001). We also detected the DNA-binding motif for *Arabidopsis* ARR1 and ARR2 proteins, which are response regulators that work as transcriptional activators in plant cells (Sakai et al. 2000). All promoters, except for *OsLPS1*, had numerous AGAAA elements that are required for transcriptional activation of pollen-specific expression in tomato (Bate and Twell 1998). Their presence implied that all of the promoters examined here were likely to confer preferential expression during rice pollen development. Furthermore, the TGGGCY motif, which contains TCP domain-binding elements that are implicated in anther- and meristem-

Fig. 2 GUS expression driven by nine promoters (*OsLPS1* through *OsLPS9*) in whole inflorescences

specific gene expression (Welchen and Gonzalez 2005), was detected in four promoters, as was the GATAA motif, which is a conserved element in many light-regulated promoters in dicots and monocots (Martínez-Hernández et al. 2002). These analytical results suggested that our nine selected promoters direct mature pollen-specific expression in a stage-specific manner, operating as late pollen genes in a heterologous system.

Analysis of promoter::GFP–GUS reporters in *Arabidopsis*

To assess the activity of our selected promoters, we generated promoter::GFP–GUS constructs and introduced them into WT *Arabidopsis* plants. PCR analysis was conducted to verify that the transformants contained each T-DNA insertion, using a forward primer specific to each promoter sequence and a reverse primer for the GFP sequence (data not shown). For each construct, we obtained 20 T_1 transgenic plants and evaluated GUS activity in young buds and open flowers. Except for *OsLPS5*, all inflorescences accumulated GUS in the flowers and at the late stage of bud formation but showed no expression in small buds or at the early stage of pollen development. For the *OsLPS5* promoter, expression was very weak, making it difficult to conclude that it could be used to drive either pollen-specific or preferential gene expression in *Arabidopsis*. All tested plants showed the same pattern of expression in their respective constructs. Therefore, we used the other eight promoters to examine the anther-specific expression of genes in *Arabidopsis* (Fig. 2).

Promoter activity was also assessed in vegetative tissues from T_2 seedlings for each construct (Fig. 3). GUS activity was not detected in seedlings harboring six promoters—*OsLPS1* through *4*, *OsLPS6*, and *OsLPS9*—whereas plants harboring *OsLPS7* and *OsLPS8* promoters showed significant GUS signals in the roots and developing leaves, respectively. Because transcript levels for the first six promoters were much lower than in the open flowers and late-stage developing buds, they could be classified as pollen-preferred. Therefore, we concluded that six of the eight promoters could induce pollen-specific expression in *Arabidopsis*.

We produced transgenic *Arabidopsis* plants to monitor the expression patterns of all nine putative pollen-specific promoters fused with the GUS reporter gene. Twenty transgenic lines per construct were used for histochemical assays (Fig. 4). During pollen formation, GUS signals were detected in all promoters except for *OsLPS5*. At the unicellular stage (UC), no signals were detected for any of the

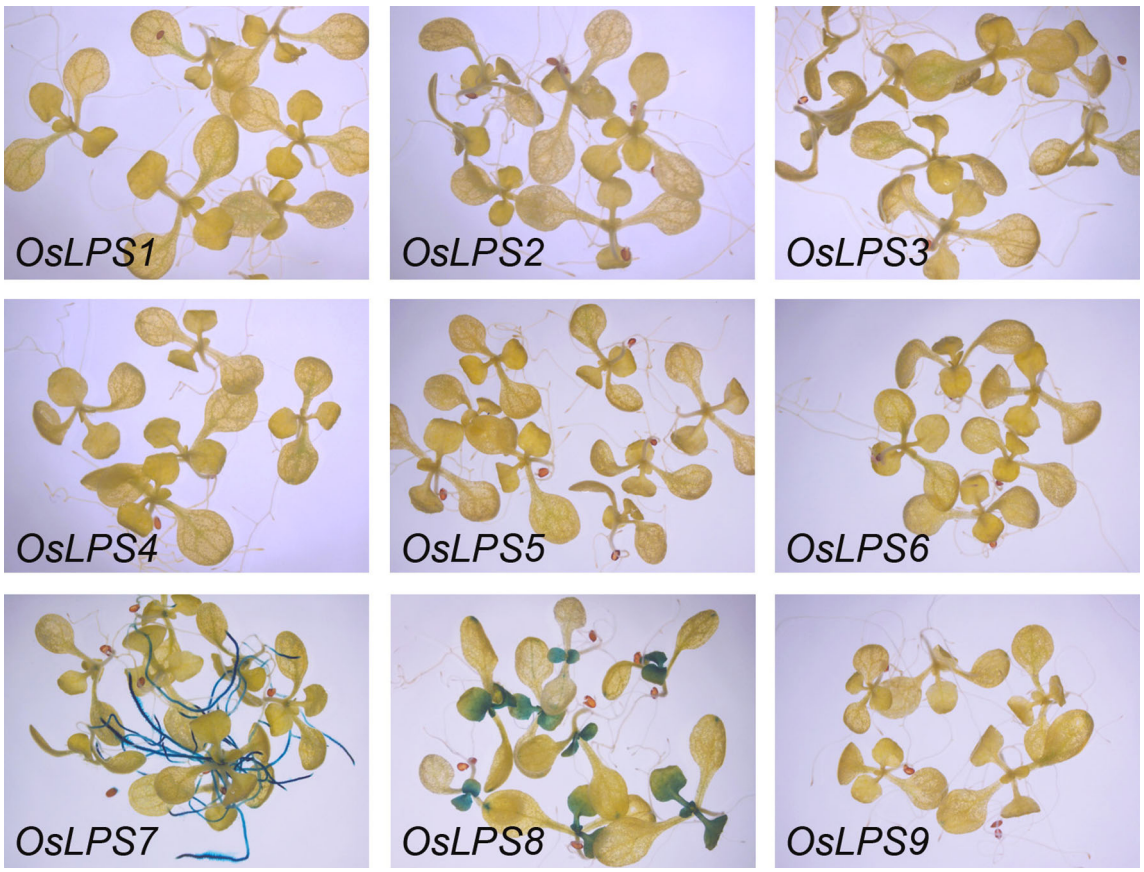

Fig. 3 GUS expression driven by nine promoters (*OsLPS1* through *OsLPS9*) in seedlings

promoters. GUS activity generally intensified over time, peaking at the mature pollen (MP) stage. This indicated that most of the promoters drove late pollen-specific or preferential expression, as supported by our microarray analysis.

Similar tissue-specific or preferential patterns of expression were observed in *Arabidopsis* plants harboring six vectors that expressed *GUS* under the control of the *OsLPS1*, *OsLPS3*, *OsLPS4*, *OsLPS6*, *OsLPS8*, and *OsLPS9* promoters. No GUS expression occurred in the early stage of microspore development (UC) for any of the promoters. During the bicellular stage (BC), GUS activity was either absent (*OsLPS3*, *OsLPS6*, and *OsLPS8*) or detected only in trace amounts (*OsLPS1*, *OsLPS4*, and *OsLPS9*). Activity was found at the tricellular (TC) stage after PMII, peaking at the mature pollen (MP) stage (Fig. 4). The T_1 transgenic plants carrying promoters for *OsLPS2* and *OsLPS7* showed similar patterns of GUS expression during male gametophyte development, being undetectable at the UC stage but beginning to appear at the BC stage. Activity gradually increased at the TC stage and peaked at the MP stage. In all, eight late pollen genes showed the highest expression at the MP stage. By contrast, *Arabidopsis* plants transformed by the *OsLPS5* vector did not demonstrate strong *GUS* expression in the microspore and pollen development stages, thereby suggesting that its promoter is likely rice or monocot pollen-specific.

We also evaluated the activity of promoters to examine the pollen-specific expression in rice. Two late pollen-specific promoters (*OsLPS1* and *OsLPS5*) exhibiting distinct expression patterns in *Arabidopsis* (Fig. 4) were analyzed in transgenic rice plants through the monitoring of the GUS gene expression pattern on developing microspore/pollen. Eight T_0 transgenic plants generated with respective constructs showed rice pollen-specific expression (Fig. 5). Rice transgenic plants carrying *OsLPS1* construct exhibited similar patterns with *Arabidopsis* during male gametophyte development in rice. On the contrary, transgenic plants with *OsLPS5* displayed the unique pattern showing strong expression at TC stage after PMII (Fig. 5). Hence, our rice data revealed that *OsLPS5* is rice pollen-specific.

Discussion

Using genome-wide expression analyses and microarray datasets, we have identified nine candidate genes that are

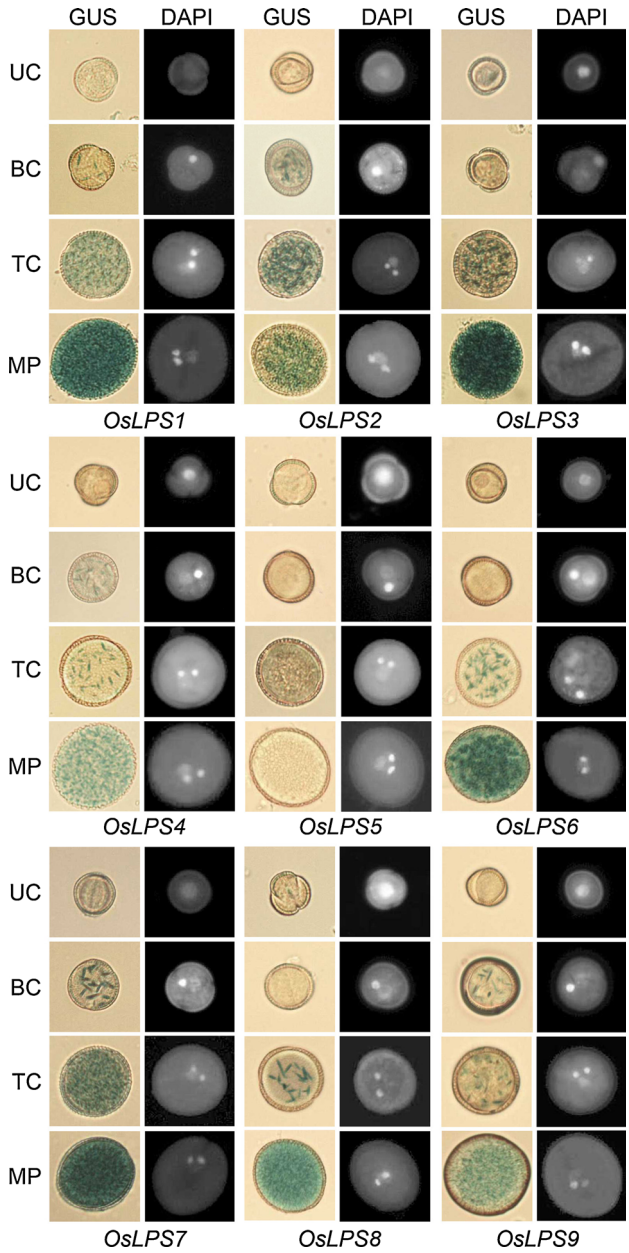

Fig. 4 GUS expression driven by nine promoters (*OsLPS1* through *OsLPS9*) during pollen development in *Arabidopsis*. GUS- and DAPI-stained images of unicellular microspores (UC), bicellular pollen (BC), immature tricellular pollen (TC), and mature pollen (MP) are shown

pollen-specific in rice. This approach has been proven efficient for identifying genes and/or promoters that confer spatio-temporal expression. For example, Thilmony et al. (2009) used anatomical meta-expression profiles from Genevestigator (Zimmermann et al. 2008; https://www.genevestigator.com/gv/) to determine that the promoter of *Leaf Panicle 2* in rice has expression that is specific to green tissues. Similarly, Jung et al. (2008) identified light-responsive genes in rice by evaluating the data from independent NSF45K and BGI60K arrays to obtain expression profiles for light- and dark-grown seedlings. In *Arabidopsis*, promoters have been found that direct pollen-specific expression at the microspore stage based on transcriptomic datasets that include pollen grains sampled at different developmental stages (Honys et al. 2006; da Costa-Nunes 2013).

We used upstream regions beyond the 5′-UTR to construct promoter::GUS reporters. The presence of *cis*-acting elements further supported our belief that their expression is either pollen-specific or preferential. The *Arabidopsis* ABORTED MICROSPOROGENESIS (AMS), belonging to a MYC superfamily of basic helix-loop-helix transcription factors, is required for tapetum cell development and microspore formation (Sorensen et al. 2003; Xu et al. 2010). It has the capacity to bind in vitro to a CANNTG motif. We noted that all nine of our selected promoters had one to 11 MYC recognition sites for that motif, which also commonly occurs in the upstream regions of genes expressed in the sperm cells of rice (Sharma et al. 2011). Therefore, these findings suggest that MYC-type transcription factors are involved in the expression of our tested genes during pollen development. In our nine promoter regions, we also detected two other *cis*-acting elements, AAAG and GTGA, that are considered anther- or pollen-specific (Bate and Twell 1998; Yanagisawa and Schmidt 1999; Rogers et al. 2001). Another element (AGAAA) common to late pollen-specific genes was found in all selected promoter regions except for *OsLPS1*. For that gene, this motif was situated within its 5′-UTR region. We also located three copies of the TGGGCY motif in *YY2* tapetum-specific genes, where it functions in anther- and meristem-specific gene expression for nuclear proteins PCF1 and PCF2 (Kuriakose et al. 2009). However, it remains unclear whether this motif is active in pollen-specific expression because it has been evaluated only through mutagenesis experiments and gain-of-function analysis (Gupta et al. 2007).

We performed a Blast search of genes that were homologous at the protein level for all nine rice late pollen-specific sequences tested here. Of these, five genes (*OsLPS1*, *OsLPS2*, *OsLPS3*, *OsLPS4*, and *OsLPS5*) had significant levels for their e-values, i.e., 4e−33, 7e−60, 2e−74, 5e−121, and 1e−13, respectively. By contrast, four other genes (*OsLPS6* through *OsLPS9*) were not detected at significant levels in the *Arabidopsis* genome, even though their protein information is publicly available for that species and others. We determined that OsLPS1 (*Os08g0560700*) shares approximately 64–66 % identity with amino acids encoding two calmodulin-like proteins: CML28 (*At3g03430*) and CML29 (*At5g17480*). The expression profile for the former is most significantly changed during pollen germination and tube growth (Wang et al. 2008). Moreover, OsLPS2 (*Os04g0638800*) is 42 %

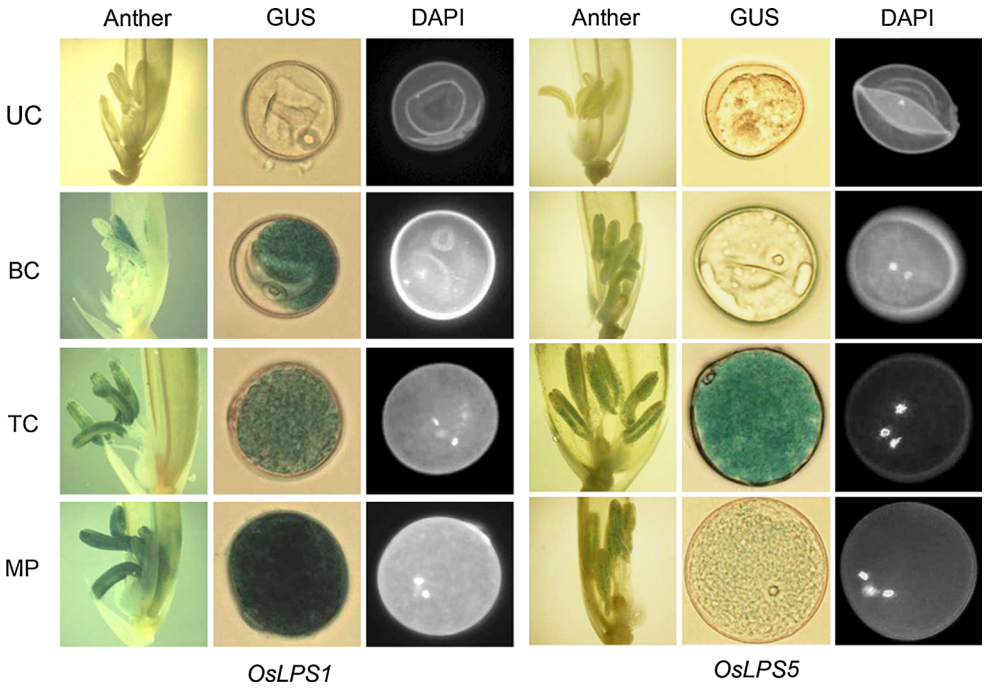

Fig. 5 GUS expression driven by 2 promoters (*OsLPS1* and *OsLPS5*) during pollen development in rice. Anther-, GUS- and DAPI-stained images of unicellular microspores (UC), bicellular pollen (BC), immature tricellular pollen (TC), and mature pollen (MP) are shown

identical to a DUF617 plant family protein with unknown function. The *Arabidopsis* gene family profiler (http://arabidopsisgfp.ueb.cas.cz/) has revealed that it is encoded by *At4G39610* and shows preferential expression in mature pollen grains (Dupl'áková et al. 2007).

OsLPS3 (*Os11g0683800*) encodes a pectinesterase family protein with roles in pollen wall development or the production of pollen-released proteins in *Arabidopsis* and canola (Noir et al. 2005; Sheoran et al. 2009). It shares approximately 42 % identity with *Arabidopsis* pectin methylesterases that are encoded by *At5g07410* and *At1g69940*. These are classified as pollen-specific genes because their expression is highly restricted to pollen grains and tubes (Bosch et al. 2005; Tian et al. 2006). OsLPS4 protein is 56 % identical to a peroxidase family protein encoded by *At5g47000*, for which expression is greatest in the late pollen stages based on NASCArrays (http://arabidopsisgfp.ueb.cas.cz/). OsLPS5 protein shows 63 % identity to DUF581 protein with unknown function encoded by *At5G47060*, which is expressed at a very low level in pollen grains (http://arabidopsisgfp.ueb.cas.cz/). The OsLPS6 protein encodes a Filamenting temperature-sensitive mutant Z (FtsZ) family protein involved in cell division. In *Lilium longiflorum*, *ftsZ* has roles in male gametogenesis and double fertilization (Mori and Tanaka 2000); transcripts are preferentially accumulated in the generative cells of mature pollen (Tang et al. 2009).

Both *OsLPS7* and *OsLPS8* encode for plant invertase/pectin methylesterase inhibitor domain proteins. The pectin methylesterase (PME) inhibitor has important roles in determining pollen development, tube growth (Bosch et al. 2005; Tian et al. 2006; Zhang et al. 2010), and the stability of cell walls at the tips of those pollen tubes (Rockel et al. 2008). A PME inhibitor gene from *Brassica oleracea* is preferentially expressed in the mature pollen grains and pollen tubes of *Arabidopsis* (Zhang et al. 2010). These findings suggest that such inhibitors have a conserved function among species.

Finally, *OsLPS9* encodes Lol p 2-family proteins, which are well-characterized allergens in ryegrass pollen. These proteins are highly homologous to Zea m 3, the major Group-1 allergen from maize pollen, displaying the cell wall-loosening activity of beta expansins (Li et al. 2003). Also, OsLPS9 protein is 40 % homologous to the major Group-1 allergen from rice (Ory s 1) (Xu et al. 1995). Analysis of their regulatory regions has demonstrated that genes in this major group from *Arabidopsis* and tobacco show characteristic late pollen expression (Gupta et al. 2007; Swapna et al. 2011). In the rice genome, putative pollen allergens have been identified and their expression characterized using public database (Jiang et al. 2005) and the Affymetrix 57K rice GeneChip microarray (Russell et al. 2008). All of these results implicate the conserved functioning of major Group-1 allergen genes in the development of mature pollen.

Although more rice pollen-specific genes are being identified, few rice promoters have been shown to be equally specific for pollen in transgenic *Arabidopsis* plants (Swapna et al. 2011). However, the promoter of rice anther-specific *OsIPP3* (*Os05g543000*), identical to *OsLPS7* in our study, exhibits broad activity in anthers and roots from *Arabidopsis* (Khurana et al. 2013a). This is

consistent with the findings reported here. Our transcriptomic data revealed that GUS activity in the *Arabidopsis* inflorescences and pollen grains was driven to varying degrees by the nine *OsLPS* promoters and followed spatiotemporal patterns. For example, at all stages, the *OsLPS5* promoter led to only limited GUS signals in whole inflorescences and no signals in the grains. The other eight promoters followed similar patterns for *GUS* expression, starting at low levels in the young buds, but gradually increasing to a peak in the mature pollen. In particular, the earliest GUS activity was detected at the BC stage from the *OsLPS1*, *OsLPS2*, *OsLPS4*, *OsLPS7*, and *OsLPS9* promoters, and at the TC stage for *OsLPS3*, *OsLPS6*, and *OsLPS8*. Moreover, histochemical analysis using promoter::GFP–GUS reporters indicated that six of these were late pollen-specific in our *Arabidopsis* model plants.

The outcome of this study provides a new set of pollen-specific promoters that can be used with both rice and *Arabidopsis*. This strategy will allow us to design a more efficient means for applying biotechnological approaches to rice breeding programs. For example, when combined with the expression of cytotoxic genes or the downregulation of genes essential for pollen development, constructs containing these promoters can be used to induce male sterility, a highly desirable agronomic trait (Bae et al. 2010). Therefore, future investigations should utilize a large number of transgenic *Arabidopsis* plants harboring the same constructs. This will greatly improve our ability to evaluate the *in planta* impacts of a wide variety of constructs, which is otherwise a very demanding process because of constraints related to space, time, and safety regulations that arise when working with transgenic rice.

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