Plant Reprod (2016) 29:291–300
DOI 10.1007/s00497-016-0293-7

ORIGINAL ARTICLE

Application of rice microspore-preferred promoters to manipulate early pollen development in Arabidopsis: a heterologous system

Tien Dung Nguyen[1] · Sunok Moon[2] · Moe Moe Oo[1] · Rupesh Tayade[1] · Moon-Soo Soh[3] · Jong Tae Song[1] · Sung Aeong Oh[1] · Ki Hong Jung[2] · Soon Ki Park[1]

Received: 12 July 2016 / Accepted: 23 October 2016 / Published online: 31 October 2016

Key message **Rice microspore-promoters.**

Abstract Based on microarray data analyzed for developing anthers and pollen grains, we identified nine *rice microspore-preferred* (*RMP*) genes, designated *RMP1* through *RMP9*. To extend their biotechnological applicability, we then investigated the activity of *RMP* promoters originating from monocotyledonous rice in a heterologous system of dicotyledonous *Arabidopsis*. Expression of *GUS* was significantly induced in transgenic plants from the microspore to the mature pollen stages and was driven by the *RMP1*, *RMP3*, *RMP4*, *RMP5*, and *RMP9* promoters. We found it interesting that, whereas *RMP2* and *RMP6* directed *GUS* expression in microspore at the early unicellular and bicellular stages, *RMP7* and *RMP8* seemed to be expressed at the late tricellular and mature pollen stages. Moreover, *GUS* was expressed in seven promoters, *RMP3* through *RMP9,* during the seedling stage, in immature leaves, cotyledons, and roots. To confirm microspore-specific expression, we used complementation analysis with an *Arabidopsis* male-specific gametophytic mutant, *sidecar pollen-2* (*scp-2*), to verify the activity of three promoters. That mutant shows defects in microspore development prior to pollen mitosis I. These results provide strong evidence that the SIDECAR POLLEN gene, driven by *RMP* promoters, successfully complements the *scp-2* mutation, and they strongly suggest that these promoters can potentially be applied for manipulating the expression of target genes at the microspore stage in various species.

Keywords *Arabidopsis* · Heterologous GUS expression · Microspore · Promoter · Rice

Communicated by David Twell.

Electronic supplementary material The online version of this article (doi:10.1007/s00497-016-0293-7) contains supplementary material, which is available to authorized users.

✉ Ki Hong Jung
khjung2010@khu.ac.kr

✉ Soon Ki Park
psk@knu.ac.kr

[1] School of Applied Biosciences, Kyungpook National University, Daegu 41566, Korea

[2] Graduate School of Biotechnology and Crop Biotech Institute, Kyung Hee University, Yongin 17104, Korea

[3] Department of Molecular Biology, Sejong University, Seoul 143-747, Korea

Introduction

In flowering plants, microgametogenesis is defined as the early stages of pollen development, during which unicellular microspores are released from tetrads to produce two daughter cells via pollen mitosis I (PMI) at the bicellular stage (Borg et al. 2009). Microspores obtain nutrients from closely linked tissues the innermost layer of the anther wall, i.e., the tapetum, and undergo further division to generate twin sperm cells after pollen mitosis II (PMII).

Several mutants defective at the microspore stages have been isolated and functionally characterized for genes, such as *SIDECAR POLLEN* (*SCP*), *GEMINI POLLEN 1* and *3*, that are required for microspore polarity at PMI (Chen and McCormick 1996; Park et al. 1998; Twell et al. 2002; Oh et al. 2010, 2016). Also, many genes involved in pollen development and male gametophyte-specific promoters have been identified. Genes such as *MSP-1*, *-2*, and *-3* are specifically active in either the microspores or developing pollen (Honys et al. 2006). In addition, transcriptome

analyses based on various microarray experiments have provided a collection of 13,977 genes, including 11,565 that exhibit male-gametophytic expression in unicellular microspores from *Arabidopsis* (reviewed by Rutley and Twell 2015).

Although numerous genes have been identified and characterized in rice (*Oryza sativa*), most are related to the tapetum/anther and late pollen development, such as *TDR*, *Osg6B*, *RA8*, *RTS*, *UDT1*, *CSA*, *OsLTP6*, *OsSCP1–3*, *RIP1*, *OSIPA*, *OSIPK*, *OsLPS1–9*, *OsLSP10*, and *OsLPS11* (Yokoi et al. 1997; Jeon et al. 1999; Jung et al. 2005; Han et al. 2006; Li et al. 2006; Luo et al. 2006; Park et al. 2006; Gupta et al. 2007; Zhang et al. 2010; Swapna et al. 2011; Khurana et al. 2013b; Liu et al. 2013; Oo et al. 2014; Nguyen et al. 2015). However, only a few genes associated with early pollen/microspore development have been described for rice. Transcriptome data are now publicly available for developing pollen and anthers in rice (Suwabe et al. 2008; Fujita et al. 2010; Wei et al. 2010; Aya et al. 2011). These data should be valuable sources for identifying genome-wide candidate genes that are significantly expressed during microspore development. Furthermore, manipulation of rice genes through promoters driving gene expression at this specific stage may give novel insight about various reproductive processes in plants, and might also be useful in biotechnological approaches.

We recently selected eight promoters based on a meta-analysis of expression data from developing anthers and pollen (Nguyen et al. 2016). The activity of these promoters was characterized in rice, a homologous species, and confirmed the early pollen-stage-specific expression, designated as *rice microspore-preferred* (*RMP*), by evaluating *GUS* expression from the uni-nucleated stage to mature pollen (Nguyen et al. 2016). Here, we extend the applicability of these *RMP* promoters by analyzing their expression patterns in dicotyledonous *Arabidopsis* plants. We previously reported a similar approach using late pollen-preferred (*OsLPS*) promoters (Oo et al. 2014). In this study, we tested stage-specific expression patterns of nine *RMP* promoters (*RMP1* through *RMP9*) by using promoter-GUS reporter gene vectors in a heterologous system, *Arabidopsis*. To confirm their functionality, we utilized the *RMP1*, *RMP2*, and *RMP5* promoters to complement *sidecar pollen-2* (*scp-2*) homozygous mutants in *Arabidopsis*, which exhibit defects in their early pollen development.

Materials and methods

Meta-expression analysis

Based on the Affymetrix microarray datasets generated from developing anthers and pollen grains, we selected nine *RMP* genes (*RMP1* through *RMP9*) out of 410 candidates because they are highly expressed in early stages of pollen development, including the microspores (Nguyen et al. 2016). We also compared the expression patterns of these genes by using laser-captured microdissection (LCM) microarray data (Suwabe et al. 2008) (Fig. 1, Table S1). Orthologs of *RMP*s in *Arabidopsis* were searched for using the online programs Rice Gene Annotation tool (Ouyang et al. 2007; http://rice.plantbiology.msu.edu/index.shtml) and PANTHER classification system (Mi et al. 2013; http://pantherdb.org).

Comparative analysis of *cis*-acting elements (CREs) between promoters of *RMP* and *OsLPS* groups

To identify consensus CREs in the promoter sequences of *RMP* genes, we analyzed three upstream sequences of 1,343 bp for the *RMP1* (*LOC_Os01g34920*, from −18 to −1360), 754 bp for the *RMP2* (*LOC_Os04g47400*, from −97 to −850), and 1,962 bp for the *RMP5* (*LOC_Os12g44080*, from −19 to −1980) (Table S1) that had been analyzed through *GUS* assays. In addition, promoter sequences of 9 genes of rice late pollen-specific gene (*OsLPS*) group, which have previously been reported (Oo et al. 2014), were used as a reference group to identify unique CREs conserved in promoters of these *RMP* genes. Gene group analysis was performed to identify the co-occurrence of transcription factor binding site or *cis*-acting elements in a group of gene promoter regions. We looked for up to four CREs in only *RMPs* by using gene group analysis tool from the Plant Promoter Analysis Navigator (PlantPAN, http://plantpan2.itps.ncku.edu.tw/).

Construction of promoter::GUS gene vectors

The promoter regions of *RMP* genes are located in the 5′ region upstream from the start codon (ATG). These were amplified by two-step Gateway PCR from rice genomic DNA (japonica "Nipponbare"), using specific primers (Table S2) as described by Nguyen et al. (2015). After verification by sequencing, promoter sequences were fused to the GUS reporter gene in the binary Gateway vector pKGWFS7 (http://www.psb.ugent.be/) and were transformed into *Agrobacterium tumefaciens* strain GV3101.

Transformation and selection of transgenic plants

Wild-type *Arabidopsis* plants (ecotype Columbia, or Col-0) were grown in a controlled environment chamber (22 °C, 16-h photoperiod). They were transformed with *Agrobacterium* strain GV3101, harboring *RMP-GUS* vectors, by the standard floral-dip method (Clough and Bent 1998). Transgenic T_1 plants were selected on a solid Murashige

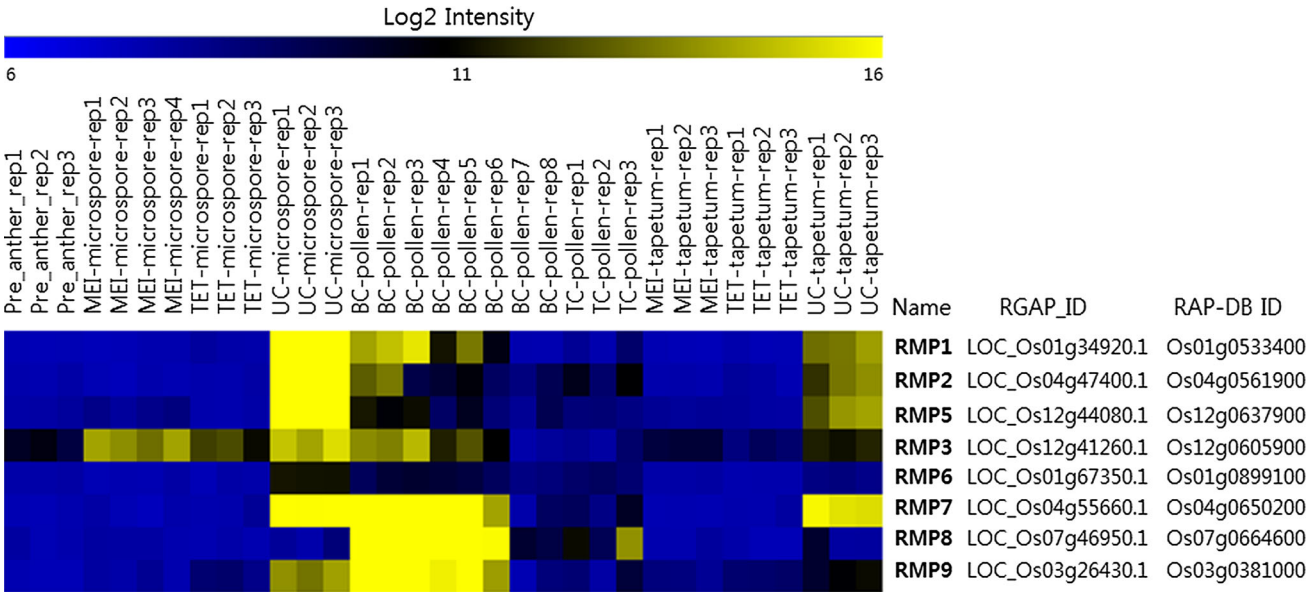


Fig. 1 Heatmap analysis of *RMP* genes using laser-captured microdissection (LCM) microarray data for developing anthers, starting from pre-meiosis to mature pollen stages. All LCM data, except *RMP4*, are presented. Most candidate genes were highly expressed in unicellular (UC) or bicellular (BC) pollen. *Blue boxes* indicate low level of expression; *yellow boxes*, high level

and Skoog (MS) medium [4.4 g L^{-1} MS salts and vitamins (Duchefa Biochemie, RV Haarlem, The Netherlands), and 6 g L^{-1} phyto agar (pH 5.8)] that also contained 200 mg L^{-1} cefotaxime (Duchefa Biochemie) and 50 mg L^{-1} kanamycin (Duchefa Biochemie).

Histochemical GUS assay

Expression of *GUS* was examined in both reproductive and vegetative organs of *Arabidopsis* as described by Nguyen et al. (2015). Samples 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 $K_3Fe(CN)_6$] that was supplemented with 1 mM 5 bromo-4-chloro-indoxyl β-D-glucuronic acid (X-GlcA; Duchefa Biochemie). After vacuum infiltration for 10 min, the samples were incubated at 37 °C for 48 h. Stained tissues were subsequently cleared in 70% ethanol and viewed under a stereomicroscope (Zeiss Stemi 2000-C; Carl-Zeiss, Oberkochen, Germany). Images were captured using a ProgC3 camera (Jenoptik, Jena, Germany) under 0.65× magnification.

DAPI staining

Samples were stained with DAPI (4′,6-diamidino-2-phenylindole) as described by Nguyen et al. (2015). Anthers of various sizes were dissected to release microspores/pollen at four stages—unicellular (UC), bicellular (BC), tricellular (TC), and mature pollen (MP)—into the DAPI staining solution (GUS buffer containing 0.4 µg mL^{-1} DAPI) as described by Park et al. (1998). Images of the pollen were viewed by light and UV epi-illumination, using a Nikon ECLIPSE 80i microscope (Nikon, Melville, NY, USA), and were captured with a Prog ResMFcool camera (Jenoptik) at 40× magnification.

Genetic complementation analysis

To examine functionally whether the *RMP* promoters can direct gene expression in *Arabidopsis* microspores, we performed a complementation analysis using *Arabidopsis scp-2* homozygous mutants, which are developmentally defective at the microspore stage (Oh et al. 2010). For genetic complementation, vectors were constructed by introducing each promoter into a pBluescript (pBS) vector, using *AscI* and *NotI* restriction enzymes. The full-length SCP coding region and a double HA fragment from the proSCP-SCP:dHA construct (Oh et al. 2010) were ligated into pBS-proRMP between the *NotI* and *PacI* sites to create proRMP-SCP:dHA. After verification by enzyme digestion and sequencing, the proRMP-SCP:dHA fragments generated from double digestion by *AscI* and *PacI* were ligated into binary vector pER8, which contains a hygromycin resistance gene as a selection marker (Fig. S1a). To generate complementing lines, we transformed *scp-2* homozygotes with *Agrobacterium* harboring the proRMP-SCP::dHA construct. Transgenic T_1 seedlings were selected on an MS medium containing 20 mg L^{-1} hygromycin (Bio Basic Inc., Markham, ON, Canada) and transferred to soil. The generated plants were examined for the T-DNA insertion and genotypes of the *scp-2* mutant allele (Fig. S2) via PCR-based analysis using the specific primers listed in

Table S2. The pollen phenotypes of those transgenic *scp-2* homozygotes were observed with the Nikon ECLIPSE 80i microscope. In addition, T-DNA genotypes of individual T_2 complementing lines were evaluated based on frequencies of hygromycin-resistant (hyg^R) plants. Briefly, T_2 seeds were grown on an MS medium containing 20 mg L^{-1} hygromycin. Resistant seedlings were scored 10 d after germination.

Results

Heatmap analysis of selected *RMP* expression using LCM microarray data

Using genome-wide microarray data of developing rice anthers and pollen samples, we recently identified 410 *RMP* genes showing rice microspore-preferred expression patterns (Nguyen et al. 2016). From these, we selected eight genes and used promoter-GUS reporter gene vectors to test expression in the homologous system, rice (Nguyen et al. 2016). For the current study, we added one gene, *RMP9* (*LOC_Os03g26430.1*), which encodes a putative aldose 1-epimerase-like protein and exhibited UC and BC microspore-preferred expression (Fig. 1; Table S1). Furthermore, we investigated accurate expression patterns via LCM microarray data during microspore/pollen development, from pre-meiosis to the mature pollen stage (Suwabe et al. 2008). As we had predicted, all of the *RMP* genes except *RMP4* (which had no probe in the Agilent 44 K array) were most highly expressed in the early stages of pollen development, including the microspores (Fig. 1), However, their expression in the tapetum was much lower at corresponding stages, which strongly suggested that these genes have microspore-preferred patterns. Of these, *RMP1*, *RMP2*, *RMP3, RMP7,* and *RMP9* were expressed preferentially at both stages of UC microspore and BC pollen. Interestingly, *RMP5* was UC-preferred, while *RMP8* was BC-preferred expression. But *RMP6* showed weak expression at UC compared to other stages. We also determined that eight of those nine genes have putative orthologs in *Arabidopsis* (Table S1). Of these, *At1g31740*, *At2g76160*, and *At3g47800* demonstrated high amino acid identity with *RMP1* (51%), *RMP2* (65%), and *RMP9* (53%), respectively. No ortholog for *RMP6* was identified in *Arabidopsis* (Table S1).

Evaluation of the activity of *RMP* promoters in heterologous plants

To examine the activity of these promoters in *Arabidopsis*, a heterologous dicot, we conducted *Agrobacterium*-mediated transformation with *Arabidopsis*. In situ *GUS* expression driven by *RMP* promoters was examined in both reproductive and vegetative tissues from 50 independent transgenic plants per construct. Distinct expression patterns were observed, including those for the *RMP2* and *RMP6* promoters, which directed *GUS* expression in young buds but not in mature flowers (Fig. 2a). To determine the earliest appearance of GUS signals, we examined expression patterns in microspores/pollen grains that were released from dissected anthers at four stages: UC, BC, TC, and MP, and found signals only at the UC and BC stages for *RMP2* and *RMP6*. In contrast, *RMP7* and *RMP8* exhibited *GUS* expression in mature flowers at the late pollen stages, TC and MP. The other five promoters showed expression at all stages, from UC through MP (Fig. 2b). We also investigated the activities of these promoters in vegetative tissues from 10-day-old T_2 seedlings (20 lines per construct). Although GUS signals were not found in *RMP1* and *RMP2* seedlings, slight expression was detected in the cotyledon regions of *RMP7* and *RMP8* and the apical regions of *RMP3* seedlings (Fig. 2c). Unexpectedly, we observed a high level of GUS signals in seedlings driven by the other promoters. For example, signals were detected in emerging leaves of *RMP4* seedlings, but those signals were much higher in the leaves and shoots of transgenics driven by the *RMP5* promoter. Expression under the control of the *RMP6* and *RMP9* promoters was similar among seedlings for each promoter and was strongest in root tissues (Fig. 2c).

RMP promoters are effective in genetic complementation analysis for functional verification through a heterologous system

To verify the activity of promoters at the microspore stage, we selected three candidate promoters—*RMP1*, *RMP2*, and *RMP5*—for genetic complementation of gametophytic mutant *scp-2*. All three showed UC/BC microspore-preferred expression. Whereas ~86% of pollen from non-transformed *scp*-2 homozygotes had the expected phenotype of aberrant grains, frequencies were reduced for the transgenic *scp-2* homozygotes (Fig. S3). Specifically, 45 transformed T_1 plants containing the *RMP1* promoter showed frequencies of aberrant pollen ranging from 36.9 to 73.8%, including 12 lines with strongly reduced frequencies of less than 50%. Moreover, frequencies for 29 T_1 transgenics of *RMP2* ranged from 35.3 to 95.9%, which was less than the 80.2 to 89.6% calculated for their corresponding non-transformed plants (Fig. S3). Finally, the 20 T_1 transgenic lines of *RMP5* showed frequencies of 44.2 to 92.3%.

For analyzing the T_2 generation, we selected two T_1 plants that showed strong reductions in the occurrence of mutant pollen in each complementing line. In all, 20 hyg^R

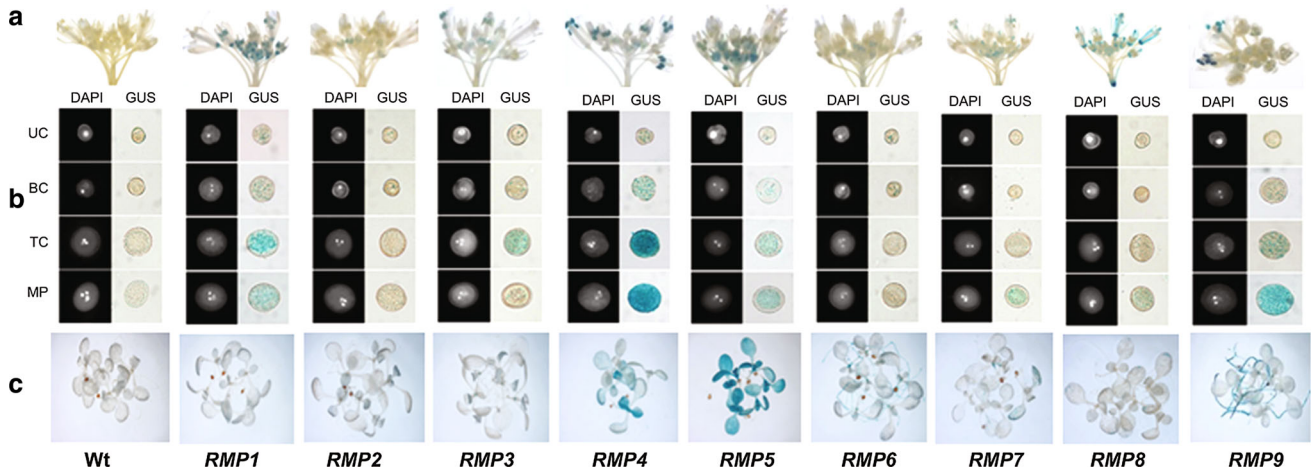


Fig. 2 GUS expression driven by *RMP* promoters during pollen development stages and at seedling stage in *Arabidopsis*: **a** in inflorescences. **b** DAPI- and GUS-stained images of unicellular microspores (UC), bicellular (BC), tricellular (TC), and mature (MP) pollen grains. **c** *GUS* expression at seedling stage. Images of inflorescences and seedlings were taken under ×0.65 magnification. Pollen grains were taken under ×40 magnification lens

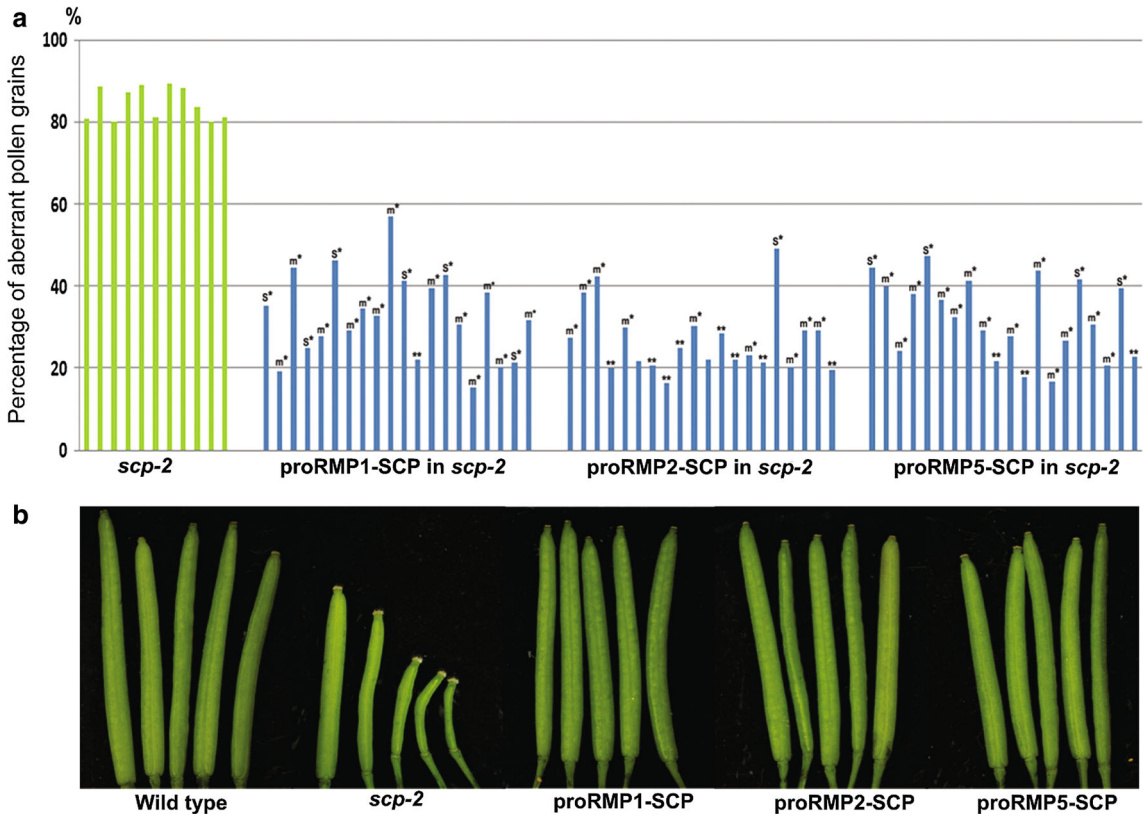


Fig. 3 **a** Percentages of aberrant pollen grains from non-transformed *scp*-2 homozygotes (*scp-2*) and transformed *scp*-2 homozygotes harboring proRMP-SCP:dHA were calculated at mature pollen stage in T_2 generation. **Homozygous plants; *heterozygous plants carrying either single (s*) or multiple (m*) T-DNA copies. **b** Silique production in complementing lines (proRMP-SCP) compared with *scp*-2 hm mutant background (*scp-2*) and wild-type plants

T_2 progenies for each promoter construct were evaluated by calculated the frequency of the *scp* phenotype. Most T_2 plants showed much stronger reductions in aberrant pollen grains (average of 27.0–33.0%; Fig. 3a). Specifically, frequencies of *scp* pollen ranged from 15.4 to 56.9% for the *RMP1* promoter, 19.5 to 49.1% for *RMP2*, and 16.9 to 47.5% for *RMP5*. This strongly contrasted with the 80.2 to 89.6% aberrant pollen produced by non-transgenic *scp-2*

back-crossed plants. Frequencies of hyg^R were also analyzed in T_3 seedlings to determine the genotype or the copy number of T-DNA. Those frequencies ranged from 76.3 to 100.0%, suggesting that at least one was a homozygote among the 20 T_2 plants harboring the *RMP1* promoter. The others were likely to be heterozygous for T-DNA, with at least one copy (Fig. 3a, Table S3). Eight of the 20 T_2 plants containing the *RMP2* promoter seemed to be homozygous for T-DNA, exhibiting a lower frequency of *scp-2* pollen. Moreover, among 12 plants presenting hygromycin segregation, only one showed an obvious 3:1 ratio, indicating a single copy of T-DNA as a heterozygote. The other 11 displayed higher frequencies of resistant seedlings, demonstrating that they carried more than one T-DNA copy (Fig. 3a, Table S4). For the *RMP5* promoter, three homozygous plants were observed on hygromycin plates while the other 17 plants showed various frequencies, suggesting that all were heterozygous for T-DNA. Among these 17 plants, five carried a single copy of T-DNA (based on frequencies), with approximately 75% having a 3:1 segregation ratio. The remaining plants were expected to contain more than one copy in their genomes because they had higher hyg^R frequencies (Fig. 3a, Table S5). As predicted, most homozygous and heterozygous T_2 plants that had high frequencies of hyg^R T_3 seedlings and were thought to have multiple copies of T-DNA, showed strong reductions in the occurrence of *scp-2* mutant pollen when compared with heterozygotes carrying single copies (Fig. 3a).

The high frequencies of aberrant pollen in *scp-2* homozygous plants led to reduced fertility and, ultimately, shorter siliques. By comparison, most of the T_2 complementing lines exhibited strong decreases in mutant pollen frequencies and highly restored fertility, and they produced siliques with lengths relatively similar to those of normal wild-type plants (Fig. 3b, Fig. S1b). These results provided solid evidence that expression levels of *SCP* driven by RMP promoters are biologically relevant, enough to complement the *scp-2* mutation.

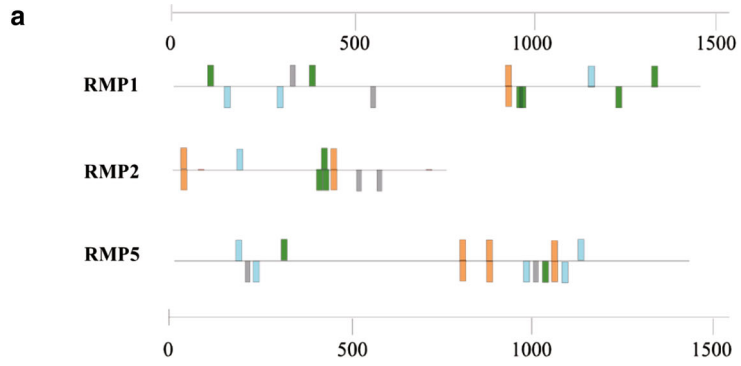
Analysis of *cis*-acting regulatory elements for the promoters of three *RMP* genes confirmed by the *GUS* reporter system

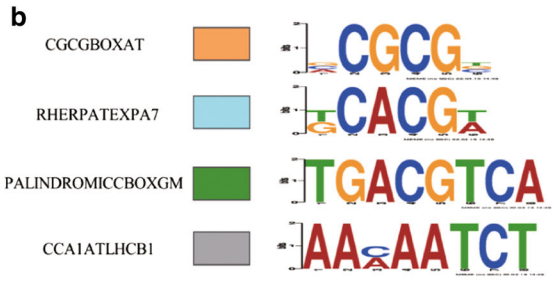
To identify the CREs associated with microspore-preferred expression, we examined three promoters of *RMP1*, *RMP2,* and *RMP5* used for complementation analysis and nine late pollen-specific genes (*OsLPS)* which have previously been reported (Oo et al. 2014) as a control. The promoter sequences were analyzed using the PlantPAN 2.0 (Chang et al. 2008; Chow et al. 2016). As a result, we found four CREs identified only in the promoters of three *RMP* genes but not in the *OsLPS* genes, including CGCGBOXAT, RHERPATEXPA7, PALINDROMICCBOXGM, and CCA1ATLHCB1. The functions and sequences of these CREs are summarized in Fig. 4 and Table S6. Of these, CGCGBOXAT is related to Ca^{2+}/calmodulin (Yang and Poovaiah 2002); RHERPATEXPA7, root hair distribution patterns (Kim et al. 2006); PALINDROMICCBOXGM, soybean apical hypocotyl (Cheong et al. 1998); and CCA1ATLHCB1, regulation by phytochrome (Wang et al. 1997). These CREs are not related to previously identified motifs involved in pollen expression, suggesting the potential as novel CREs for early pollen development. Thus, we propose that they will be useful in future efforts to manipulate useful agronomic traits in rice microspore.

Discussion

We have identified nine *RMP* genes from rice and performed several experiments with a *GUS* reporter system to examine the patterns of tissue/developmental stage-specific expression when their promoters are used with *Arabidopsis*. All nine promoters exhibited *GUS* expression in anther tissues (Fig. 2a). For *RMP2* and *RMP6*, *GUS* expression was first observed at the UC stage but then was arrested at the BC stage. In contrast, expression in the *RMP7* and *RMP8* transgenic lines was detected in the pollen only at the TC and MP stages (Fig. 2b). For the *RMP1*, *RMP3*, *RMP4*, *RMP5*, and *RMP9* promoters, *GUS* was expressed during pollen development throughout all stages, from UC through TC and MP (Fig. 2b). Although expression was not detected in other tissue types from transgenic plants driven by the *RMP1* and *RMP2* promoters, it was found in immature leaves, cotyledons, and young roots of seedlings with the *RMP3* through *RMP9* promoters. These findings confirmed the conclusions predicted from microarray data, indicating that *RMP* genes are preferentially expressed at the microspore stages.

We have previously taken a transgenic approach to characterize the eight *RMP* promoters in rice and have verified that eight (*RMP1* through *RMP8*) drive *GUS* expression in reproductive and vegetative tissues (Nguyen et al. 2016). All eight promoters exhibit similar patterns in the anthers, with GUS activity peaking from the UC through the MP stages of pollen development. In extending this research to a heterologous plant system, we have now confirmed that *RMP* promoters also confer distinct patterns of *GUS* activity in *Arabidopsis* (Fig. 2a, b). This strengthens earlier conclusion on the existence of evolutionarily conserved and divergent transcriptional machinery for pollen developmental program (Khurana et al. 2013a; Oo et al. 2014; Nguyen et al. 2015). Further detailed analysis to identify *cis*-acting elements, we found that *RMP1*, *RMP2*, and *RMP5* contained four conserved





Name	Locus_ID	Name of CRE	Positions	Occurrence frequency
RMP1	LOC_Os01g34920	CGCGBOXAT	979, 979(-)	2
		RHERPATEXPA7	1, 89, 342, 1186	3
		PALINDROMICCBOXGM	131, 386, 947, 1167, 1200	5
		CCA1ATLHCB1	335, 579	2
RMP2	LOC_Os04g47400	CGCGBOXAT	13, 13(-), 369, 369(-), 371, 371(-), 373, 373(-)	8
		RHERPATEXPA7	275	1
		PALINDROMICCBOXGM	435, 443, 443(-)	3
		CCA1ATLHCB1	532, 596	2
RMP5	LOC_Os12g44080	CGCGBOXAT	762, 762(-), 859, 859(-), 1091, 1091(-)	6
		RHERPATEXPA7	26, 034, 491, 510, 611, 100	5
		PALINDROMICCBOXGM	3, 021, 078	2
		CCA1ATLHCB1	220, 873	2

Fig. 4 Conserved CREs analysis in the promoter sequences of three *RMP* genes. **a** Diagram of distribution of four conserved CREs in the promoter sequences of *RMP1*, *RMP2*, and *RMP5*. *Numeric values* indicate the upstream position from ATG, start codon sequence. **b** LOGOs of consensus sequences in four CREs and summary information of these CREs collected from PlantPAN 2.0. *Numeric values* in position column indicate the position in the plus strand promoter, and "(−)" symbols indicate the position in the minus strand promoter

CREs in the promoter sequences which were not be identified in the late pollen (*OsLPS*) promoters. Although these conserved CREs have not been reported for pollen expression, they might play an important role for early pollen expression of *RMP* genes.

We also demonstrated here that *RMP1*, *RMP2*, and *RMP5* can be applied to this heterologous system, based on our findings from complementation analysis using *Arabidopsis* male-gametophytic mutant *scp-2* homozygotes, which display developmental defects at the microspore stage (Oh et al. 2010). Our current results indicated that the occurrence of mutant pollen is critically diminished in most lines harboring proRMP-SCP:dHA in the T_2 generation, when compared with frequencies calculated for non-transgenic *scp-2* homozygous mutants (Fig. 3a). This provides evidence that the *RMP* promoters can effectively direct the expression of *SCP* to recover the *scp-2* mutation at the precise time before the BC stage begins.

A protein member of the glycoside hydrolase (GH) 35 family is encoded by *RMP1*. Its deduced biological functions in plants are mainly the degradation of structural pectins and xyloglucans in cell walls, and family member genes such as *OsBgals* are expressed in rice anthers (Tanthanuch et al. 2008). Two other genes—*At1g31740* and *At5g20710*—in the GH35 family encode *β*-galactosidases and share high identities with *RMP1*. Both function in the early stages of microspore and pollen development in *Arabidopsis* (Hrubá et al. 2005; Ahn et al. 2007) (Table S1). *RMP2* encodes peptidase S9A in the prolyl oligopeptidase (POP) family. That group of serine peptidases has been implicated in the degradation of biologically important peptide hormones and neuropeptides (Rosenblum and Kozarich 2003). These POP members are involved in regulating the development of reproductive organs in rice, and they also confer tolerance to abiotic stresses in *Escherichia coli* (Yoshida and Kuboyama 2001; Tan et al. 2013). In this study, we found an *Arabidopsis* gene, *At1g76160*, that encodes SKU5 (monocopper oxidase-like protein) similar five protein and shares 65% amino acid identity with *RMP2*. Although no SKU5 functions have been reported in pollen, the eFP database (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) has indicated that *At1g76160* is expressed in flower tissues from stages 9 to 15. Another gene, *At5g09570*, encodes a Cox19-like coiled-coil-helix-coiled-coil-helix domain (CHCHD)-containing protein family similar to *RMP5* (Table S1). However, the functions of this protein have previously been described primarily in humans (Banci et al. 2012; Darshi et al. 2012), but not in plants. Our results are the first to

show that CHCHD protein is also accumulated in pollen grains.

Promoters that control expression at a specific stage during microspore/pollen development are ideal tools for the biotechnological manipulation of targeted genes involved in pollen functions. For example, these promoters can be potentially applied for modifying the hormonal biosynthesis pathway that generates male sterile plants or for improving other agronomic traits associated with fertility (Bae et al. 2010). Thus far, several microspore-specific promoters have been identified in plants, including *TNM19*, *Bp4*, *BECLIN 1*, and *TA29* (Albani et al. 1991; Kriete et al. 1996; Oldenhof et al. 1996; Singh et al. 2010). When these promoters are combined with the cytotoxic *barnase,* TNM19- and Bp4-*barnase* plants clearly show lethal pollen phenotypes, and pollen from these plants does not germinate, causing sterility in species such as *Nicotiana tabacum* (Custers et al. 1997). However, the source of such promoters is quite restrictive for the development of elite GM rice. In our previously report, more than 410 *RMP* genes, which are explored by analyzing meta-expression data, showed that most of the genes (285 genes) have no GO terms and only two genes have been functionally characterized. In contrast, we found 23 out of 263 tapetum-preferred genes have been functionally investigated, and 52% had GO terms (Nguyen et al. 2016). These suggest that the functions of microspore genes are still in vague and need to be addressed. *RMP* genes could be new targets to understand male-gametophytic development.

Recently, several research groups have focused on male gametophyte development under stressful conditions including genomics and proteomics, suggesting that pollen is new target for development of plants adapting to environmental changes (reviewed by Grover et al. 2016). Microspores in plants are very sensitive to abiotic stresses, such as drought, heat, and cold, which are becoming serious challenges in agricultural regions. The introduction of stress during key reproductive stages can reduce or completely eliminate crop yields due to pollen sterility (Solomon et al. 2007; Zinn et al. 2010). The metabolic alterations involved in male reproductive development may be associated with abiotic stresses such as abscisic acid, gibberellic acid, and excess sugar (Sharma 2014; Burke and Chen 2015). Among the nine *RMP* promoters described in this paper, three promoters of *RMP1*, *RMP2*, and *RMP5* which are active and preferential at the microspore stage in both monocotyledonous and dicotyledonous plant species will be applicable to manipulate tissue-specific transgenic expression in the pollen-preferential way.

Author contribution statement TDN and SM performed the experiments and generated the data. SAO, KHJ, and SKP designed the experiments. TDN, SM, SAO, KHJ, and SKP wrote the manuscript.

Acknowledgements This work was carried out with the support of the "Cooperative Research Program for Agriculture Science and Technology Development (Project Nos. PJ01182602 to KHJ; PJ01194201 to SKP)," Rural Development Administration, Republic of Korea.

References

Ahn YO, Zheng M, Bevan DR, Esen A, Shiu SH, Benson J, Peng HP, Miller JT, Cheng LI, Poulton JE, Shih MC (2007) Functional genomic analysis of *Arabidopsis thaliana* glycoside hydrolase family 35. Phytochemistry 68:1510–1520

Albani D, Altosaar I, Arnison PG, Fabijanski SF (1991) A gene showing sequence similarity to pectin esterase is specifically expressed in developing pollen of *Brassica napus*. Sequences in its 5′ flanking region are conserved in other pollen-specific promoters. Plant Mol Biol 16:501–513

Aya K, Suzuki G, Suwabe K, Hobo T, Takahashi H, Shiono K, Yano K, Tsutsumi N, Nakazono M, Nagamura Y, Matsuoka M, Watanabe M (2011) Comprehensive network analysis of anther-expressed genes in rice by the combination of 33 laser microdissection and 143 spatiotemporal microarrays. PLoS ONE 6:e26162

Bae HK, Kang HG, Kim GJ, Eu HJ, Oh SA, Song JT, Chung IK, Eun MY, Park SK (2010) Transgenic rice plants carrying RNA interference constructs of *AOS* (*allene oxide synthase*) genes show severe male sterility. Plant Breed 129:647–651

Banci L, Bertini I, Coffi-Baffoni S, Jaiswal D, Peruzzini R, Winkelman J (2012) Structural characterization of *CHCHD5* and *CHCHD7*: two atypical twin CX9C proteins. J Struct Biol 180:190–200

Borg M, Brownfield L, Twell D (2009) Male gametophyte development: a molecular perspective. J Exp Bot 60:1465–1478

Burke JJ, Chen J (2015) Enhancement of reproductive heat tolerance in plant. PLoS ONE 10:e0122933

Chang WC et al (2008) PlantPAN: plant promoter analysis navigator, for identifying combinatorial *cis*-regulatory elements with distance constraint in plant gene groups. BMC Genom 9:561

Chen YC, McCormick S (1996) *sidecar pollen*, an *Arabidopsis thaliana* male gametophytic mutant with aberrant cell divisions during pollen development. Development 122:3243–3253

Cheong YH, Yoo CM, Park JM, Ryu GR, Goekjian VH, Nagao RT, Key JL, Cho MJ, Hong JC (1998) STF1 is a novel TGACG-binding factor with a zinc-finger motif and a bZIP domain which heterodimerizes with GBF proteins. Plant J 15:199–209

Chow CN, Zheng HQ, Wu NY, Chien CH, Huang HD, Lee TY, Chiang-Hsieh YF, Hou PF, Yang TY, Chang WC (2016) PlantPAN 2.0: an update of plant promoter analysis navigator for reconstructing transcriptional regulatory networks in plants. Nucleic Acids Res 44(D1):D1154–D1160

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Custers JBM, Oldenhof MT, Schrauwen JAM, Cordewender JHG, Wullems GJ, van Lookeren Campagne MM (1997) Analysis of microspore-specific promoters in transgenic tobacco. Plant Mol Biol 35:689–699

Darshi M, Trinh K, Murphy A, Taylor S (2012) Targeting and import mechanism of coiled-coil helix coiled-coil helix domain-containing protein 3 (ChChd3) into the mitochondrial intermembrane space. J Biol Chem 287:39480–39491

Fujita M, Horiuchi Y, Ueda Y, Mizuta Y, Kubo T, Yano K, Yamaki S, Tsuda K, Nagata T, Niihama M, Kato H, Kikuchi S, Hamada K, Mochizuki T, Ishimizu T, Iwai H, Tsutsumi N, Kurata N

(2010) Rice expression atlas in reproductive development. Plant Cell Physiol 51:2060–2081

Grover A, Twell D, Schleiff E (2016) Pollen as a target of environmental changes. Plant Reprod 29:1–2

Gupta V, Khurana R, Tyagi AK (2007) Promoters of two anther-specific genes confer organ-specific gene expression in a stage-specific manner in transgenic systems. Plant Cell Rep 26:1919–1931

Han MJ, Jung KH, Yi G, Lee DY, An G (2006) *Rice immature pollen 1* (*RIP1*) is a regulator of late pollen development. Plant Cell Physiol 47:1457–1472

Honys D, Oh SA, Renak D, Donders M, Solcova B, Johnson JA, Boudova R, Twell D (2006) Identification of microspore-active promoters that allow targeted manipulation of gene expression at early stages of microgametogenesis in *Arabidopsis*. BMC Plant Biol 6:31

Hrubá P, Honys D, Twell D, Capková V, Tupý J (2005) Expression of beta-galactosidase and beta-xylosidase genes during microspore and pollen development. Planta 220:931–940

Jeon JS, Chung YY, Lee S, Yi GH, Oh BG, An G (1999) Isolation and characterization of an anther-specific gene, *RA8*, from rice (*Oryza sativa* L.). Plant Mol Biol 39:35–44

Jung KH, Han MJ, Lee YS, Kim YW, Hwang I, Kim MJ, Kim YK, Nahm BH, An G (2005) *Rice undeveloped tapetum* 1 is a major regulator of early tapetum development. Plant Cell 17:2705–2722

Khurana R, Kapoor S, Tyagi AK (2013a) Spatial and temporal activity of upstream regulatory regions of rice anther-specific genes in transgenic rice and *Arabidopsis*. Transgenic Res 22:31–46

Khurana R, Kathuria H, Mukhopadhyay A, Kapoor S, Tyagi AK (2013b) 286 bp upstream regulatory region of a rice anther specific gene, *OSIPP3*, confers pollen-specific expression in *Arabidopsis*. Biotechnol Lett 35:455–462

Kim DW, Lee SH, Choi SB, Won SK, Heo YK, Cho M, Park YI, Cho HT (2006) Functional conservation of a root hair cell-specific *cis*-element in angiosperms with different root hair distribution patterns. Plant Cell 18:2958–2970

Kriete G, Niehaus K, Perlick AM, Pühler A, Broer I (1996) Male sterility in transgenic tobacco plants induced by tapetum-specific deacetylation of the externally applied non-toxic compound *N*-acetyl-L-phosphinothricin. Plant J 9:809–818

Li N, Zhang DS, Liu HS, Yin CS, Li XX, Liang WQ, Yuan Z, Xu B, Chu HW, Wang J, Wen TQ, Huang H, Luo D, Ma H, Zhan DB (2006) The rice *tapetum degeneration retardation* gene is required for tapetum degradation and anther development. Plant Cell 18:2999–3014

Liu X, Shangguan Y, Zhu J, Lu Y, Han B (2013) The rice *OsLTP6* gene promoter directs anther-specific expression by a combination of positive and negative regulatory elements. Planta 238:845–857

Luo H, Lee JY, Hu Q, Nelson-Vasilchik K, Eitas TK, Lickwar C, Kausch AP, Chandlee JM, Hodges TK (2006) *RTS*, a rice anther-specific gene is required for male fertility and its promoter sequence directs tissue-specific gene expression in different plant species. Plant Mol Biol 62:397–408

Mi H, Muruganujan A, Casagrande JT, Thomas PD (2013) Large-scale gene function analysis with the PANTHER classification system. Nat Protoc 8:1551–1556

Nguyen TD, Oo MM, Moon S, Bae HK, Oh SA, Soh MS, Song JT, Kim JH, Jung KH, Park SK (2015) Expression analysis of two rice pollen-specific promoters using homologous and heterologous systems. Plant Biotechnol Rep 9:297–306

Nguyen TD, Moon S, Nguyen VNT, Gho Y, Soh MS, Song JT, An G, Oh SA, Park SK, Jung KH (2016) Genome-wide identification and analysis of rice genes preferentially expressed in pollen at an early developmental stage. Plant Mol Biol 92:71–88. doi:10.1007/s11103-016-0496-1

Oh SA, Park KS, Twell D, Park SK (2010) The *SIDECAR POLLEN* gene encodes a microspore-specific LOB/AS2 domain protein required for the correct timing and orientation of asymmetric cell division. Plant J 64:839–850

Oh SA, Jeon J, Park HJ, Grini PE, Twell D, Park SK (2016) Analysis of *gemini pollen 3* mutant suggests a broad function of AUGMIN in microtubule organization during sexual reproduction in *Arabidopsis*. Plant J 87:188–201

Oldenhof MT, Groot PF, Visser JH, Schrauwen JA, Wullems GJ (1996) Isolation and characterization of a microspore-specific gene from tobacco. Plant Mol Biol 31:213–225

Oo MM, Bae HK, Nguyen TD, Moon M, Oh SA, Kim JH, Soh MS, Song JT, Jung KH, Park SK (2014) Evaluation of rice promoters conferring pollen-specific expression in a heterologous system, *Arabidopsis*. Plant Reprod 27:47–58

Ouyang S, Zhu W, Hamilton J, Lin H, Campbell M, Childs K, Thibaud-Nissen F, Malek RL, Lee Y, Zheng L, Orvis J, Haas B, Wortman J, Buell CR (2007) The TIGR rice genome annotation resource: improvements and new features. Nucleic Acids Res 35:D883–D887

Park SK, Howden R, Twell D (1998) The *Arabidopsis thaliana* gametophytic mutation *gemini pollen1* disrupts microspore polarity, division asymmetry and pollen cell fate. Development 125:3789–3799

Park JI, Hakozaki H, Endo H, Takada Y, Ito H, Uchida M, Okabe T, Watanabe M (2006) Molecular characterization of mature pollen-specific genes encoding novel small cysteine-rich proteins in rice (*Oryza sativa* L.). Plant Cell Rep 25:466–474

Rosenblum JS, Kozarich JW (2003) Prolyl peptidases: a serine protease subfamily with high potential for drug discovery. Curr Opin Chem Biol 7:496–504

Rutley N, Twell D (2015) A decade of pollen transcriptomics. Plant Reprod 28:73–89

Sharma KD (2014) Pollen development under cold stress: a molecular perspective. Austin J Genet Genom Res 1:4

Singh SP, Pandey T, Srivastava R, Verma PC, Singh PK, Tuli R, Sawant SV (2010) *BECLIN1* from *Arabidopsis thaliana* under the generic control of regulated expression systems, a strategy for developing male sterile plants. Plant Biotechnol 8:1005–1022

Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB (2007) Contribution of working group I to the fourth assessment report of the intergovernmental panel on climate change. Cambridge University Press, Cambridge, p 996

Suwabe K, Suzuki G, Takahashi H, Shiono K, Endo M, Yano K, Fujita M, Masuko H, Saito H, Fujioka T, Kaneko F, Kazama T, Mizuta Y, Kawagishi-Kobayashi M, Tsutsumi N, Kurata N, Nakazono M, Watanabe M (2008) Separated transcriptomes of male gametophyte and tapetum in rice: validity of a laser microdissection (LM) microarray. Plant Cell Physiol 49:1407–1416

Swapna L, Khurana R, Vijaya-Kumar S, Tyagi AK, Rao KV (2011) Pollen-specific expression of *Oryza sativa* indica pollen allergen gene (*OSIPA*) promoter in rice and *Arabidopsis* transgenic systems. Mol Biotechnol 48:49–59

Tan CM, Chen RJ, Zhang JH, Gao XL, Li LH, Wang PR, Deng XJ, Xu ZJ (2013) *OsPOP5*, a prolyl oligopeptidase family gene from rice confers abiotic stress tolerance in *Escherichia coli*. Int J Mol Sci 14:20204–20219

Tanthanuch W, Chantarangsee M, Maneesan J, Ketudat-Cairns J (2008) Genomic and expression analysis of glycosyl hydrolase family 35 genes from rice (*Oryza sativa* L.). BMC Plant Biol 8:84

Twell D, Park SK, Hawkins TJ, Schubert D, Schmidt R, Smertenko A, Hussey PJ (2002) MOR1/GEM1 has an essential role in the

plant-specific cytokinetic phragmoplast. Nat Cell Biol 4:711–714

Wang ZY, Kenigsbuch D, Sun L, Harel E, Ong MS, Tobin EM (1997) A Myb-related transcription factor is involved in the phytochrome regulation of an Arabidopsis *Lhcb* gene. Plant Cell 9:491–507

Wei LQ, Xu WY, Deng ZY, Su Z, Xue Y, Wang T (2010) Genome-scale analysis and comparison of gene expression profiles in developing and germinated pollen in *Oryza sativa*. BMC Genom 11:338

Yang T, Poovaiah BW (2002) A calmodulin-binding/CGCG box DNA-binding protein family involved in multiple signaling pathways in plants. Biol Chem 277:45049–45058

Yokoi S, Tsuchiya T, Toriyama K, Hinata K (1997) Tapetum-specific expression of the *Osg6B* promoter-β-glucuronidase gene in transgenic rice. Plant Cell Rep 16:363–367

Yoshida KT, Kuboyama T (2001) A subtilisin-like serine protease specifically expressed in reproductive organs in rice. Sex Plant Reprod 13:193–199

Zhang H, Liang W, Yang X, Luo X, Jiang N, Ma H, Zhang D (2010) *Carbon starved anther* encodes a MYB domain protein that regulates sugar partitioning required for rice pollen development. Plant Cell 22:672–689

Zinn KE, Tunc-Ozdemir M, Harper JF (2010) Temperature stress and plant sexual reproduction: uncovering the weakest links. J Exp Bot 61:1959–1968