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An ABC transporter, OsABCG26, is required for anther cuticle and pollen exine formation and pollen-pistil interactions in rice

Zhenyi Chang[a,e,1], Zhufeng Chen[a,1], Wei Yan[c,1], Gang Xie[a], Jiawei Lu[a], Na Wang[a], Qiqing Lu[a], Nan Yao[d], Guangzhe Yang[b], Jixing Xia[b,**], Xiaoyan Tang[a,e,*]

[a] Shenzhen Institute of Molecular Crop Design, Shenzhen 518107, China
[b] State Key Laboratory of Conservation and Utilization of Subtropical Agro-bioresources, College of Life Science and Technology, Guangxi University, Nanning 530005, China
[c] School of Life Sciences, Capital Normal University, Beijing 100048, China
[d] School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, China
[e] Guangdong Key Lab of Biotechnology for Plant Development, College of Life Sciences, South China Normal University, Guangzhou 510631, China

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ABSTRACT

Wax, cutin and sporopollenin are essential components for the formation of the anther cuticle and the pollen exine, respectively. Their lipid precursors are synthesized by secretory tapetal cells and transported to the anther and microspore surface for deposition. However, the molecular mechanisms involved in the formation of the anther cuticle and pollen exine are poorly understood in rice. Here, we characterized a rice male sterile mutant *osabcg26*. Molecular cloning and sequence analysis revealed a point mutation in the gene encoding an ATP binding cassette transporter G26 (OsABCG26). *OsABCG26* was specifically expressed in the anther and pistil. Cytological analysis revealed defects in tapetal cells, lipidic Ubisch bodies, pollen exine, and anther cuticle in the *osabcg26* mutant. Expression of some key genes involved in lipid metabolism and transport, such as *UDT1*, *WDA1*, *CYP704B2*, *OsABCG15*, *OsC4* and *OsC6*, was significantly altered in *osabcg26* anther, possibly due to a disturbance in the homeostasis of anther lipid metabolism and transport. Additionally, wild-type pollen tubes showed a growth defect in *osabcg26* pistils, leading to low seed setting in *osabcg26* cross-pollinated with the wild-type pollen. These results indicated that *OsABCG26* plays an important role in anther cuticle and pollen exine formation and pollen-pistil interactions in rice.

1. Introduction

Male reproductive development in flowering plants is a complex biological process from the formation of the anther primordium, to the production of mature pollen within the anther and dehiscence [1]. Anther and pollen development is a prerequisite for male reproductive success. The anther consists of four distinct layers of cells, from exterior to interior the epidermis, the endothecium, the middle layer, and the tapetum [1]. At the center of each anther lobe, microspores are produced, differentiated, and developed into mature pollen grains. The anther and pollen are respectively covered by anther cuticle and pollen exine, which have a protective role against environmental and biological stresses during reproductive development [2].

The anther cuticle consists of cutin and wax [2–4]. Cutin is a mixture of lipophilic biopolymers comprised of hydroxylated and epoxy C16 and C18 fatty acids, while cuticular wax is a biopolymer containing a mixture of alcohols, ketones, aldehydes, alkanes, and long-chain fatty acids [2–5]. The pollen exine is composed of a rigid material called sporopollenin, which is made up of complex biopolymers derived mainly from phenolics and aliphatic derivatives [2,6,7]. The tapetum is considered to act as a supplier of sporopollenin and anther cuticle precursors [8,9]. By analyzing male sterile mutants defective in the production of the anther cuticle and pollen exine, a number of different proteins, such as enzymes for lipid synthesis and modification, lipid transfer proteins (LTPs), ABCG transporters, and transcription factors, have been identified [2,9,10]. They are required for the synthesis and deposition of sporopollenin and cuticle in *Arabidopsis* and rice. For

* Corresponding author at: Shenzhen Institute of Molecular Crop Design, Xinjianxing Industrial Park, Fengxin Street, Guangming New District, Shenzhen, 518107, China.
** Corresponding author.
E-mail addresses: xiajx@gxu.edu.cn (J. Xia), txy@frontier-ag.com (X. Tang).
[1] These authors contributed equally to this work.

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example, *MS2*, *ACOS5*, *PKSA*, *PKSB*, *TKPR1*, *CYP703A2*, and *CYP704B1* encode enzymes functioning in the synthesis of sporopollenin and cutin in *Arabidopsis* [2,9,10]. *AtABCG11*, *AtABCG12* and *AtABCG26* in *Arabidopsis* encode plasma membrane-localized ABC transporters acting in lipidic precursor export from the tapetum [11–16]. Recently, *OsABCG15*, the homolog of *AtABCG26*, was reported to transport lipidic precursors from tapetal cells to the anther cuticle and pollen wall in rice [17–20]. WDA1, CYP704B2, DPW and CYP703A3 were identified as enzymes involved in the synthesis of sporopollenin in rice [21–24]. OsC4 and OsC6 were considered to be LTPs related to lipid trafficking during rice anther and pollen exine formation [25]. Transcription factors such as UDT1, TDR and GAMYB were proposed to be major regulators in rice pollen development [26–28]. Despite these recent progresses, our knowledge of the mechanism underlying the lipid transport from the tapetum is still limited.

Very recently, *OsABCG26* was reported to cooperate with *OsABCG15* functioning in the formation of anther cuticle and pollen exine by the transport of lipidic precursors in rice [29]. Here, we reported the isolation and characterization of an allelic mutant of *OsABCG26* caused by a point mutation. Similar to what was reported, we found that *OsABCG26* was involved in the formation of anther cuticle and pollen exine by regulating lipid transport from the tapetum. More interestingly, we found that wild-type pollen germinated on the mutant stigma normally, but most of the pollen tubes ceased growth in the mutant pistil and failed to reach the micropyle, suggesting that *OsABCG26* plays an important role in pollen-pistil interaction by affecting pollen tube growth in the pistil.

2. Materials and methods

2.1. Plant materials and growth conditions

Mutants *osabcg26* and *osabcg15* were derived from the *indica* cv. Huanghuazhan (HHZ) by ethyl methanesulfonate (EMS) mutagenesis [30]. HHZ was used as the wild-type control. All the plants (*Oryza sativa*) were grown in the paddy field of Shenzhen, China, under natural conditions with regular care.

2.2. Characterization of the mutant phenotype

To analyze pollen fertility, pollen grains at mature stage were stained with 1% I_2-KI solution and photographed using Nikon AZ100 microscope.

Spikelets at different developmental stages were collected and embedded as described previously with some modifications [27]. Briefly, spikelets were pre-fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M PBS (pH 7.0) at 4 °C for 24 h, and post-fixed with 1% osmium tetroxide in 0.1 M PBS (pH 7.0) for 1–2 h. Subsequently, samples were dehydrated with ethanol and embedded in SPI-PON 812 resin (SPI-CHEM). Semi-thin sections (1 μm) were obtained on an ultramicrotome (Leica EM-UC6) using a diamond knife (DiATOME) and stained with 0.5% toluidine blue. Ultra-thin sections (100 nm) were double stained with uranyl acetate and lead citrate solution, and examined with a transmission electron microscope (JEOL, JEM1400) at an accelerating voltage of 120 kV.

For scanning electron microscopy, anthers were fixed in FAA solution (38% formaldehyde 5 mL, acetic acid 5 mL, 50% alcohol 90 mL). Following ethanol dehydration, samples were processed for critical point drying and gold coated [18]. Finally, samples were observed under scanning electron microscope (Hitachi S-3400N) with an acceleration voltage of 10 kV.

2.3. Mutant gene cloning and HRM analysis

The *osabcg26* mutant was backcrossed with wild-type HHZ, and the resulted F1 was further selfed to generate the F2 population. Thirty male sterile plants in F2 population were randomly selected for DNA extraction, and equal amount of DNA was pooled and sequenced to 43x of genome coverage using the Illumina Hiseq 2000 platform. The data were subjected to computational analysis for identification of the mutant gene as described [30]. Co-segregation of the candidate mutation with the male sterile phenotype in F2 population was analyzed using high resolution melting (HRM) analysis [31]. Primer set OsABCG26-P1 used for HRM analysis is listed in Table S1.

2.4. Plasmid construction and rice transformation

The 8.0 kb HHZ genomic DNA fragment for *OsABCG26*, including a 2.0 kb upstream region, 5.3 kb coding region, and 0.7 kb downstream region, was PCR-amplified with primer set OsABCG26-P2. The PCR products were digested with *Kpn*I and *BamH*I, and cloned into the binary vector pCAMBIA1300 to generate construct pCAMBIA1300-*OsABCG26* for mutant complementation. The 2.0 kb upstream region of *OsABCG26* was PCR-amplified with primer set OsABCG26-P3, and cloned into binary vector pHPG between *Kpn*I and *Sal*I, to yield $OsABCG26_{pro}$:*GUS* for promoter analysis. Both constructs were sequence-confirmed before transformation. Constructs were introduced into *Agrobacterium tumerfaciens* AGL10 strain and transformed into rice calli. pCAMBIA1300-*OsABCG26* was introduced into offspring of *osabcg26* heterozygote plants. $OsABCG26_{pro}$:*GUS* was introduced into *japonica* cv. Zhonghua11. The positive transgenic lines were determined by PCR with primer set HPTII. To identify the background genotype of pCAMBIA1300-*OsABCG26* transgenic plants, specific genomic fragment covering the mutant site of *osabcg26* was amplified using primer set OsABCG26-P4, and the product was diluted 1000 times and further subjected to HRM analysis with primers OsABCG26-P1. Primers used are listed in Table S1.

2.5. qRT-PCR assay

Rice tissues were collected at the reproductive stage. The stages of anthers were classified as previously described [1]. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and then reverse-transcribed with PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China), according to the manufacturer protocols. qRT-PCR was performed with an Applied Biosystems 7500 Real-Time PCR System using SYBR Premix Ex Taq™ II (Takara, Dalian, China), following the manufacturer instructions. Each experiment was biologically repeated three times, each with three replicates. *OsACTIN1* was used as the normalized reference. The relative expression levels were measured using the $2^{-\Delta\Delta Ct}$ analysis method. Primer sequences used are listed in Table S1.

2.6. GUS staining

Histochemical GUS assay was performed as described [28], except for the addition of 0.1% (v/v) Triton X-100 to the staining solution. After staining, samples were cleared with 70% (v/v) ethanol and photographed using Nikon AZ100 microscope.

2.7. Histological analyses of ovule

Wild-type and *osabcg26* spikelets at different developmental stages were fixed with FAA solution, dehydrated with an ethanol series followed by a xylene series, and then embedded in Paraplast Plus (Sigma, St. Louis, MO), as described [32]. Samples were