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Short communication

Arabidopsis mutant of AtABCG26, an ABC transporter gene, is defective in pollen maturation

Takashi Kuromori[a,*,1], Takuya Ito[b,1], Eriko Sugimoto[a], Kazuo Shinozaki[a]

[a] *Gene Discovery Research Group, RIKEN Plant Science Center, 1-7-22 Suehiro, Tsurumi, Yokohama 230-0045, Japan*
[b] *Chemical Biology Core Facility, RIKEN Advanced Science Institute, 2-1 Hirosawa, Wako 351-0198, Japan*

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ABSTRACT

In plants, pollen is the male gametophyte that is generated from microspores, which are haploid cells produced after meiosis of diploid pollen mother cells in floral anthers. In normal maturation, microspores interact with the tapetum, which consists of one layer of metabolically active cells enclosing the locule in anthers. The tapetum plays several important roles in the maturation of microspores. ATP-binding cassette (ABC) transporters are a highly conserved protein super-family that uses the energy released in ATP hydrolysis to transport substrates. The ABC transporter gene family is more diverse in plants than in animals. Previously, we reported that an *Arabidopsis* half-size type ABC transporter gene, *COF1*/*AtWBC11*/*AtABCG11*, is involved in lipid transport for the construction of cuticle layers and pollen coats in normal organ formation, as compared to *CER5*/*AtWBC12*/*AtABCG12*. However, physiological functions of most other ABCG members are unknown. Here, we identified another family gene, *AtABCG26*, which is required for pollen development in *Arabidopsis*. An *AtABCG26* mutant developed very few pollen grains, resulting in a male-sterile phenotype. By investigating microspore and pollen development in this mutant, we observed that there was a slight abnormality in tetrad morphology prior to the formation of haploid microspores. At a later stage, we could not detect exine deposition on the microspore surface. During pollen maturation, many grains in the mutant anthers got aborted, and surviving grains were found to be defective in mitosis. Transmission of the mutant allele through male gametophytes appeared to be normal in genetic transmission analysis, supporting the view that the pollen function was disturbed by sporophytic defects in the *AtABCG26* mutant. AtABCG26 can be expected to be involved in the transport of substrates such as sporopollenin monomers from tapetum to microspores, which both are plant-specific structures critical to pollen development.

Introduction

In plants, pollen, the male gametophyte that interacts with the female reproductive organ, is produced in anthers located on floral stamens (Bedinger, 1992; Goldberg et al., 1993; Borg et al., 2009). The development of functional pollen is critical for plant reproduction, and the control of pollen release is important for crop breeding. Pollen development is a post-meiotic process that produces mature pollen grains from microspores (Bedinger, 1992; Goldberg et al., 1993; Borg et al., 2009). In addition to activities within the microspore itself, sporophytic anther tissues play important roles in this process not only by providing physical support but also by supplying signals and necessary materials. In particular, the tapetum, which is the innermost somatic cell layer of the anther locule, plays an essential role in pollen development (Ito et al., 2007).

The pollen wall is composed of ultrastructurally distinct layers. The outer layers (exine) consist of two parts, the sculptured sexine on the outside and the inner nexine(s). The exine is principally made of sporopollenin, an aliphatic polymer of long-chain fatty acids, phenylpropanoids, and oxygenated aromatic rings, which is synthesized predominantly by the tapetum (Wilson and Zhang, 2009). Throughout the maturation of microspores, the tapetum cells transfer nutrients and necessary compounds for maturation to the locule fluid (Wilson and Zhang, 2009). However, the molecular mechanism of substrate transport from the tapetum to microspores is currently unknown.

ATP-binding cassette (ABC) transporter is a highly conserved family of ATP-binding proteins that use ATP-derived energy to transport molecules across cell membranes (Higgins, 1992). ABC transporters are present in organisms from bacteria to animals,

Abbreviations: ABC, transporter; ATP, binding cassette transporter; TB, toluidine blue O; T-DNA, transfer DNA; WBC, White–brown complex homolog.

* Corresponding author. Tel.: +81 45 503 9625; fax: +81 45 503 9586.
E-mail address: kuromori@psc.riken.jp (T. Kuromori).

[1] These authors contributed equally to this work.

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including humans, but are especially prevalent in plants. For example, *Arabidopsis*, rice and some legumes have more than 100 ABC transporter genes (Sánchez-Fernández et al., 2001a; Verrier et al., 2008). Overall, plants have two to three times the number of ABC transporter genes compared to other groups of organisms (Garcia et al., 2004; Sugiyama et al., 2006; Verrier et al., 2008), suggesting that some members of the ABC transporter family evolved to have important plant-specific functions in development and physiological regulation (Sánchez-Fernández et al., 2001b).

The largest subfamily in *Arabidopsis* comprises the half-size ABC transporter genes, represented by a cluster of 28 genes in the AtABCG subfamily, previously called the white–brown complex homolog (WBC) subfamily (Sánchez-Fernández et al., 2001a; Verrier et al., 2008). The functions of four subfamily members have been reported: *CER5*/*AtWBC12*/*AtABCG12* and *COF1*/*AtWBC11*/*AtABCG11* are localized in the plasma membrane of epidermal cells and are required for wax export and elaboration of the cuticle (Pighin et al., 2004; Bird et al., 2007; Panikashvili et al., 2007; Ukitsu et al., 2007). Activation of *AtWBC19*/*AtABCG19* improves antibiotic resistance because overexpression of AtWBC19 confers kanamycin resistance in transgenic plants (Mentewab and Stewart, 2005). *AtABCG25* is involved in ABA transport and responses (Kuromori et al., 2010; Kuromori and Shinozaki, 2010). The functions of the other AtABCGs remain largely unknown. Here, we present evidence that one of the AtABCG genes, *AtABCG26*, encodes a protein that has a function required for post-meiotic pollen development and normal fertility in *Arabidopsis*.

Materials and methods

Plant materials and PCR-based genotyping

Arabidopsis thaliana (L.) Heynh., *atabcg26-1* (SALK_062317) and *atabcg26-2* (GABI_859F02), were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH, USA) and Nottingham Arabidopsis Stock Centre (University of Nottingham, Leicestershire, UK), respectively. The *Arabidopsis* Col-0 accession was used as a control plant. Plants were grown in soil in a growth chamber at 22 °C, under a 16 h light/8 h dark cycle.

Genomic DNA was prepared using an automatic DNA isolation system (Kurabo, Osaka, Japan). To determine the genotype of *atabcg26-1*, PCR-based genotyping was performed with the following primers: At3g13220F (5′-TCTCTGTTGCTTCTAGACGAGC-3′), At3g13220R (5′-AAAGAGTGATGACGATGTCCAG-3′), and LBa1 (5′-TGGTTCACGTAGTGGGCCATCG-3′). To determine the genotype of *atabcg26-2*, PCR-based genotyping was performed with the following primers: GABI_859F02_L (5′-CTTCGAGAAGATCTACCTGGTG-3′), GABI_859F02_R (5′-ATGGCAAATGCTTATTTTCTTG-3′), and GABI_o8409 (5′-ATATTGACCATCATACTCATTGC-3′).

Alexander staining and DAPI staining

To check pollen viability, anthers from mature flowers were stained with Alexander's solution (Alexander, 1969) and photographed under a BX60 upright microscope (Olympus, Tokyo, Japan) with a VB-7010 CCD camera (Keyence, Osaka, Japan).

To check the pollen nucleus, pollen grains were isolated by disrupting fixed anthers on a glass slide using needles under a dissecting microscope. The pollen grains were then gently squashed in 1 μg/mL 4′-6-diamidino-2-phenylindole (DAPI) solution under a coverslip and viewed by UV epi-illumination.

Toluidine blue (TB) staining

Microspore defects in mutant anthers were detected using TB staining, following previously described methods (Ito et al., 2007). Flower samples were fixed with 1% (v/v) formaldehyde, 2.5% (v/v) acetic acid, and 45% (v/v) ethanol. The fixed samples were either stained directly with a 0.05% TB solution or embedded in a Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany). The resin was then sectioned (4 μm) and stained with the TB solution.

Results

Isolation of a T-DNA insertional mutant of AtABCG26

Previously, we reported that a half-size type member of the ABCG subfamily of ABC transporters, *COF1*/*AtABCG11*, encoded a protein that functioned in lipid transport and cuticle formation in *Arabidopsis* (Ukitsu et al., 2007). More recently, another member, *AtABCG25*, was found to encode a protein that functions in abscisic acid transport and responses in *Arabidopsis* (Kuromori et al., 2010; Kuromori and Shinozaki, 2010). To investigate other genes of the half-size ABCG subfamily in *Arabidopsis*, we have collected homozygous insertion lines from T-DNA-tagged or transposon-tagged lines in which the T-DNA or *Ds* transposon is inserted in coding regions of half-size ABCG subfamily genes. In this mutant series, we found that a mutant line, SALK_062317, exhibited a sterile phenotype (Fig. 1A). According to the genomic sequences flanking the T-DNA insertion in the isolated line (SALK_062317), the T-DNA element was in the fifth exon of a predicted open reading frame of the At3g13220 gene. The At3g13220 gene encodes AtABCG26 (also known as AtWBC27), and therefore, this mutant was designated *atabcg26-1*. The mutation and the phenotype were closely linked because all T-DNA homozygote plants showed a sterile phenotype; however, no T-DNA heterozygote plants exhibited the sterile phenotype. Additionally, a second allelic mutant line of the At3g13220 gene, *atabcg26-2* (GABI_859F02), has been reported to show similarly sterile phenotype (Choi et al., 2011).

Observations in atabcg26 flowers and pollen grains

The *atabcg26-1* mutant plants showed basically no visible phenotypic differences compared to wild-type Col-0 plants until flowering. When the siliques ripened, however, mutant plants had very small siliques, showing few fertility (Fig. 1A). Other floral organs appeared to be normal, but the anther locules turned brown and released fewer pollen grains than wild-type plants (Fig. 1B and C). Alexander staining at flower stage 12 revealed anthers of *atabcg26-1* mutant plants that appeared to have very few viable but many burst pollen grains, whereas the anthers of wild-type plants had many viable pollen grains (Fig. 1D and E). TB staining of tetrads including microspores indicated that tetrads of *atabcg26-1* mutants appeared to be composed of four microspores enclosed in callose, as in wild-type plants (Fig. 1F and G). However, each microspore appeared to be more stuck than the wild-type microspores, indicating the possibility of an aberrant meiotic process in *atabcg26-1* mutant microspores (Fig. 1F and G). TB staining of pollen grains from a stage 10/11 flower revealed shrunken or missing cytosol of pollen grains in *atabcg26-1* mutant flowers (Fig. 1I, J), whereas the cytosol of pollen grains in wild-type flowers was normal (Fig. 1H). DAPI staining of pollen grains from stage 12 flowers revealed a pair of missing sperm nuclei in *atabcg26-1* pollen grains (Fig. 1K), whereas these nuclei were clearly observed in wild-type grains (Fig. 1K, inset). These results indicate that the pollen grains of *atabcg26-1* mutant plants have defects in pollen mitosis, even if mutant pollen grains survived pollen developmental stages. In addition, the mutant pollen wall was defective in exine structure (Fig. 1M) compared to wild-type pollen grains (Fig. 1L).