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Research paper

Identification of *cyp703a3-3* and analysis of regulatory role of *CYP703A3* in rice anther cuticle and pollen exine development

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ABSTRACT

Anther cuticle and pollen exine are two elaborated lipid-soluble barriers protecting pollen grains from environmental and biological stresses. However, less is known about the mechanisms underlying the synthesis of these lipidic polymers. Here, we identified a no-pollen male-sterility mutant *cyp703a3-3* from the *indica* restorer line Zhonghui 8015 (Zh8015) mutant library treated with ^{60}Coγ-ray radiation. Histological analysis indicated that *cyp703a3-3* underwent abnormal tapetal cells development, produced few orbicules and secreted less sporopollenin precursors to anther locule, as well as cutin monomers on anther. Genetic analysis revealed that *cyp703a3-3* was controlled by a single recessive gene. Map-based cloning was performed to narrow down the mutant gene to a 47.78-kb interval on the chromosome 8 between two markers S15-29 and S15-30. Sequence analysis detected three bases (GAA) deletion in the first exon of *LOC_Os08g03682*, annotated as *CYP703A3* with homologous sequences related to male sterility in *Arabidopsis*, causing the Asparagine deletion in the mutant site. Moreover, we transformed genomic fragment of *CYP703A3* into *cyp703a3-3*, which male-sterility phenotype was recovered. Both the wild-type and *cyp703a3-3* mutant 3D structure of CYP703A3 protein were modeled. Results of qPCR suggested *CYP703A3* mainly expressed in anthers with greatest abundance at microspore stage, and genes involved in sporopollenin precursors formation and transportation, such as *GAMYB*, *TDR*, *CYP704B2*, *DPW2*, *OsABCG26* and *OsABCG15*, were significantly reduced in *cyp703a3-3*. Collectively, our results further elaborated *CYP703A3* plays vital role in anther cuticle and pollen exine development in rice (*Oryza sativa* L.).

1. Introduction

In higher plants, male reproduction development is a complex biological process which includes the formation of the stamen with differentiated anther tissues (Zhang and Wilson, 2009). In rice, each spikelet has six stamens and each stamen consists of an anther and a filament. Anther includes four somatic layers from outside to inside: epidermis, endothecium, middle layer, and tapetum, produced by the stamen primordium differentiation and division. Anther provides a safe compartment for pollen formation. Microsporocytes centrally locate to locus of anther and undergo meiosis producing microspores. Then microspores undergo twice asymmetric cell division generating the mature pollen grains, which contain three nuclei, namely two smaller sperm nuclei, and a larger vegetative nucleus (Ma, 2005; Zhang and Wilson, 2009; Zhang et al., 2011).

Pollen development is a critical step in plant reproduction development (Gomez et al., 2015). To ensure this process success, plants have evolved anther cuticle and pollen exine which comprises the outer sexine and the inner nexine to protect the pollen grains from environmental and biological stresses. The sexine contains the tectum and bacula, and underlying sexine, there are nexine I (foot layer) and nexine II (endexine) consisting bilayer nexine, these two portions sculpt the species-specific structure of the pollen grains. Besides exine, the last layer of pollen wall is intine underlying the exine (Li et al., 2010; Ariizumi and Toriyama, 2011). The research progress in biochemical composition of the anther surface and pollen exine is slow because of the insoluble and hard to degrade properties of sporopollenin, and the technical limitation of purifying and obtaining a large quantity of materials for analysis. Until recent several years, highly sensitive methods have been adopted to analyze sporopollenin and anther cuticle, such as Fourier transform-infrared (FTIR), nuclear magnetic resonance (NMR) spectroscopy, and gas chromatography–mass spectrometry (GC–MS).

Abbreviations: PCD, programmed cell death; bHLH, basic helix-loop-helix; LTP, lipid transfer protein; TEM, transmission electron microscope; SEM, scanning electron microscope; ORFs, open reading frames

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These techniques have demonstrated that anther cuticle is mainly composed of a cutin matrix with waxes embedded in and overloaded on the surface of the matrix (Jung et al., 2006; Morant et al., 2007; Pollard et al., 2008; Yang et al., 2014), while sporopollenin is thought to be composed of complex biopolymers derived mainly from saturated precursors such as long-chain fatty acids or long aliphatic chains, oxygenated aromatic rings and phenylpropanoic acids (Murphy, 1998; Bubert et al., 2002). All these precursors of anther cuticle and sporopollenin are synthesized by tapetum and then secreted to extracellular for anther and pollen architecture.

With the advance of genomics and genetics, mutants have become popular materials for elucidating how genes function in organisms. Several genes have been cloned according to defective phenotype of mutants, which involve in the biosynthesis, secretion, transportation and assembly of anther cuticle monomers and sporopollenin precursors. *GAMYB* encodes a R2R3 MYB transcription factor, which was first isolated as a positive transcriptional regulator of gibberellin (GA)-dependentα-amylase expression in barley aleurone cells. Mutations of *GAMYB* impair α-amylase expression in aleurone and flower development (Kaneko et al., 2004). Additionally, *GAMYB* has also been shown to be involved in programmed cell death (PCD) of tapetal cells and direct activation *CYP703A3* to regulate the development of exine and Ubisch bodies (Aya et al., 2009; Liu et al., 2010). TDR, *UDT1* and *EAT1*, encoding putative basic helix-loop-helix (bHLH) domain transcription factors, are mainly expressed in tapetal cells and involved in tapetum development and timely PCD. In mutants of them, tapetum exhibited abnormal phenotype and aborted pollen formation (Jung et al., 2005; Li et al., 2006; Zhang et al., 2008; Niu et al., 2013). *Wax-Deficient Anther1* (*WDA1*) gene has been shown to be strongly expressed in the epidermal cells of anther walls, and *WDA1* is proposed to participate in the biosynthesis of very-long-chain fatty acids for the establishment of the anther cuticle and pollen exine (Jung et al., 2006). *ACOS5* encodes a fatty acyl-CoA synthetase (ACOS) for medium-chain fatty acids synthesis in *Arabidopsis* (de Azevedo Souza et al., 2009). *OsACOS12*, an orthologue of *ACOS5*, is essential for sporopollenin synthesis in rice. *ACOS5* and *OsACOS12* are conserved for pollen wall formation in monocot and dicot species (Li et al., 2016). In addition, *CYP704B1*and *CYP703A2* belong to cytochrome P450s catalyzing the biochemical pathway of hydroxylation of fatty acids in *Arabidopsis* (Morant et al., 2007; Dobritsa et al., 2009). *CYP704B2*, the rice ortholog of *CYP704B1*, is preferentially expressed in tapetal cells and the recombinant CYP704B2 protein can catalyze ω-hydroxylation of long-chain fatty acids (Li et al., 2010). While in *Arabidopsis CYP703A2* catalyzes the conversion of medium-chain saturated fatty acids to the corresponding monohydroxylated fatty acids, with a preferential hydroxylation of lauric acid at the C-7 position (Morant et al., 2007). Furthermore, *MS2*, *DPW1* and *DPW2* encode fatty acid reductase participating in the synthesis of precursors for pollen, exine sporopollenin and anther epicuticle (Jung et al., 2006; Xu et al., 2016). After synthesized in tapetum, these precursors are secreted to outside of tapetal cells and transferred onto the wall surfaces to be polymerized into biopolymers of sporopollenin, wax, and cutin. Previous reports showed that, *OsABCG15* and *OsABCG26*, members of the G subfamily of ATP-binding cassette (ABC) transporters are involved in this metabolic process. Both these are preferentially expressed in tapetum and their proteins are localized to the plasma membrane. *osabcg15* exhibited an undeveloped anther cuticle, abnormal Ubisch body development, tapetum degeneration with a falling apart style, and collapsed pollen grains without detectable exine while *abcg26* tapetal cells accumulated numerous vesicles and granules. These results suggest that the two gens play a crucial role in the transfer of sporopollenin lipid precursors from tapetal cells to anther locules, facilitating exine formation on the pollen surface (Choi et al., 2011; Wu et al., 2014b). *OsC6*, a unique lipid transfer protein (LTP) encoding gene, is likely to transfer lipidic molecules from metabolically active tapetal cells to other anther cells for orbicule and pollen wall development (Zhang et al., 2010).

In this study, we isolated and characterized a new allele *cyp703a3-3* of the rice *CYP703A3* gene, from Zhonghui 8015(Zh8015) mutant library treated with ^{60}Coγ-ray radiation. *cyp703a3-3* displayed smaller and pale anthers and invisible pollen grains while normal female development was confirmed by cross analysis. Histological analysis indicated that microspores in *cyp703a3-3* became abnormal from tetrad and tapetal cells produced little orbicules and secreted less sporopollenin precursors to anther locule, and rarely detectable Ubish bodies on anther. Moreover, we transformed a wild type genomic fragment of *CYP703A3* into homozygous *cyp703a3-3* mutant calli, and rescued the mutant phenotype. Expression analysis suggested that genes involved in sporopollenin precursors formation and transportation, such as *CYP704B2*, *DPW2* and *OsABCG15*, were significantly reduced in *cyp703a3-3*. Our study confirmed the function of *CYP703A3* underlying the generation of sporopollenin precursors during pollen development in rice.

2. Materials and methods

2.1. Materials and planting

A *cyp703a3-3* mutant was found from the progenies of *indica* cultivar Zhonghui8015 (Zh8015) after treating with ^{60}Coγ-ray radiation. The *cyp703a3-3* as the pollen acceptor was crossed with the wild type Zh8015 and a *japonica* cultivar 02428 to generate F_1 and F_2 populations for genetic analysis and fine mapping. Zh8015 was crossed with 02428 to investigate the hybrid progeny fertility as the control. Meanwhile fertility was investigated in all populations.

All plants were grown in paddy fields in Lingshui, Hainan Province in the spring of 2014 and in Hangzhou, Zhejiang Province of China in the summer of 2014. The planting density was 20.0 cm × 23.0 cm. We grew rice seeding when 20–25 days sowing seeds later by single seedling. Crop management followed commercial rice production practices.

2.2. Phenotype survey

Plants and flowers at the heading stage were photographed with a Nikon E995 digital camera. The paleas and lemmas of wild-type and *cyp703a3-3* mutant spikelets were removed and photographed using OLYMPUS MVX10 stereomicroscope. Mature anthers were mashed and immersed into 1% iodine potassium iodide (I_2-KI) solution for a short time to make the I_2-KI solution enter into the anther for pollen starch staining, then observed with a 10 × 10 microscope to judge pollen fertility and photographed with a microscope (Leica DM2500).

2.3. Cytological observation

Semi-thin resin section was used to observe anther development. Spikelets at different developmental stages were collected, and were fixed overnight at room temperature with FAA, which contained 5% (v/v) formaldehyde, 5% (v/v) acetic acid, and50% (v/v) ethanol. Then samples were vacuumized and dehydrated with a 50–100% ethanol series, ultimately embedded in Technovit glycol methacrylate 7100 resin (Heraeus Kulzer). Leica RM2265 microtome was used to cut 2 mm sections, stained with 0.1% (w/v) toluidine blue in 0.1 M phosphate buffer (pH = 7.0). Specimens were photographed under bright-field illumination Leica DM2000 (Ito et al., 2007).

For transmission electron microscope (TEM) assay, spikelets at various developmental stages were fixed in 2.5% glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.0) and postfixed in 2% OsO_4 in PBS (pH 7.2). Following ethanol dehydration, samples were embedded in acrylic resin. Ultra-thin sections (50 to 70 nm) were double stained with 2% (w/v) uranyl acetate and 2.6% (w/v) lead citrate aqueous solution and examined with a JEM-1230 transmission electron microscope (Li et al., 2006). Scanning electron microscope (SEM) assay was also performed. The samples were fixed at 4 °C in 2.5% v/v