Flavonoids are indispensable for complete male fertility in rice

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HIGHLIGHT

Complete male fertility in rice requires the presence of flavonoids and a combination of different classes are likely to be essential for pollen tube germination and growth inside pistils.

ABSTRACT

Flavonoids are essential for male fertility in some but not all plant species. In rice, the chalcone synthase mutant *oschs1* produces flavonoid-depleted pollens and is male sterile. The mutant pollens are viable with normal structure but they displayed reduced germination rate and pollen tube length. Analysis of pollens of *oschs1*/+ heterozygous lines showed that pollen flavonoid deposition is a paternal effect and fertility is independent from the haploid genotypes (*OsCHS1* or *oschs1*). To understand which classes of flavonoids are involved in male fertility, we further analyzed rice mutants for branch-point enzymes of the downstream flavonoid pathways, including flavanone 3-hydroxylase (OsF3H; flavonol pathway entry enzyme), flavone synthase II (CYP93G1; flavone pathway entry enzyme) and flavanone 2-hydroxylase (CYP93G2; flavone *C*-glycoside pathway entry enzyme). Rice *osf3h* and *cyp93g1 cyp93g2* CRISPR/Cas9 mutants, and *cyp93g1* and *cyp93g2* T-DNA insertional mutants showed altered flavonoid profiles in anthers but only *osf3h* and *cyp93g1 cyp93g2* mutants displayed reduction in seed yield. Our findings indicated that flavonoids are essential for complete male fertility in rice and a combination of different classes (flavanones, flavonols, flavones, and flavone *C*-glycosides) are likely to be important, as opposed to the essential role played primarily by flavonols as reported previously in several plant species.

KEYWORDS

Chalcone synthase, flavanones, flavones, flavone *C*-glycosides, flavonols, male fertility, rice (*Oryza sativa*)

INTRODUCTION

Flavonoids are a large group of plant secondary metabolites commonly distributed in plants with multiple roles in physiology, growth, and development. Together with co-pigments, flavonoids especially anthocyanidins account for the pigmentation of most flowers, fruits and seeds (Ferreyra *et al.*, 2012). Due to their antimicrobial activities, some flavonoids function as phytoalexins against pathogens. For example, sakuranetin accumulation in rice increased resistance to blast fungus infection (Kodama *et al.*, 1992; Hasegawa *et al.*, 2014). Sorghum (*Sorghum bicolor*) 3-deoxyanthocyanidins and luteolin induced by *Colletotrichum sublineola* exhibited toxicity to spores of the fungus (Lo *et al.*, 1999; Du *et al.*, 2010b). Insecticidal activities have been reported for maysin, apimaysin and methoxymaysin which are present in maize (*Zea mays*) silks (Waiss *et al.*, 1979; Elliger *et al.*, 1980; Snook *et al.*, 1994). In addition, flavonoids are involved in abiotic stress responses. For example, catechin and quercetin are released in an aluminum resistant maize cultivar, suggesting that they may enhance tolerance to aluminum toxicity (Kidd *et al.*, 2001). High-level accumulation of flavonols, flavones, *O*-glycosyl flavonols and *C*-glycosyl flavones occurred in plants exposed to elevated solar UV-B radiation, suggesting their roles in UV protection (Ryan *et al.*, 2002; Van de Staaij *et al.*, 2002; Agati *et al.*, 2011). Meanwhile, flavonoids are required for root nodulation in legume plants (Eckardt, 2006; Wasson *et al.*, 2006). Suppression of chalcone synthase (CHS), the committed enzyme for flavonoid biosynthesis, in *Medicago truncatula* results in reduced root nodulation which could be reversed by feeding of flavanones (Wasson *et al.*, 2006). Furthermore, some flavone or flavonol aglycones inhibit polar auxin transport to enhance localized accumulation of auxin in plants (Peer and Murphy, 2007; Kuhn *et al.*, 2011; Lewis *et al.*, 2011).

In several plant species, flavonoids also have demonstrated functional roles in male fertility. Early studies in petunia (*Petunia hybrida*) and maize reported that their *chs* mutant pollen grains are sterile but could be functionally complemented by exogenous application of the flavonol kaempferol (Mo *et al.*, 1992; Taylor and Jorgensen, 1992; Pollak *et al.*, 1993). RNA interference (RNAi)-mediated suppression of flavonol synthase (FLS) in tobacco (*Nicotiana tabacum*) led to reduced seed yield due to impaired pollen function which could be rescued by another flavonol quercetin (Mahajan *et al.*, 2011). Recently, flavonols were further demonstrated to reduce the abundances of reactive oxygen species (ROS) in tomato

(*Solanum lycopersicum*) pollens, especially during heat stress, which could otherwise compromise pollen viability, germination as well as germ tube functions (Muhlemann *et al.*, 2018). Interestingly, *CHS* RNAi downregulation was demonstrated to be a viable strategy for the generation of parthenocarpic tomato fruits as seed production is defected due to impaired pollen tube growth (Schijlen *et al.*, 2007). By contrast, the Arabidopsis (*Arabidopsis thaliana*) *transparent testa4* (*tt4*) mutant, which is defective in CHS and does not accumulate flavonoids, is completely fertile (Burbulis *et al.*, 1996). Apparently, flavonoids are not universally required for male fertility in flowering plants.

The core flavonoid biosynthetic pathway is initiated through CHS-catalyzed sequential condensation of three malonyl-CoAs with one *p*-coumaroyl-CoA to form naringenin chalcone (Fig. 1A). This is followed by isomerization by chalcone isomerase (CHI, encoded by a single-copy gene, *OsCHI*; Shih *et al.,* 2008) to produce naringenin (a flavanone) which is the precursor for different classes of flavonoids (Saito *et al.*, 2013). In rice, the predominant flavonoids in vegetative tissues (leaves and culms) are flavone *C*-glycosides and *O*-linked conjugates synthesized through separate pathways from flavanones. Accordingly, flavanone 2-hydroxylase (F2H; CYP93G2; encoded by a single-copy gene; Du *et al.,* 2010) produces 2-hydroxyflavanones from flavanones, followed by *C*-glycosylation and dehydration to generate flavone *C*-glycosides (Brazier-Hicks *et al.*, 2009; Du *et al.*, 2010a; Brazier-Hicks and Edwards, 2013). On the other hand, flavone synthase II (FNSII; CYP93G1; encoded by a single-copy gene; Lam *et al.,* 2014) converts flavanones directly to flavones, which are subjected to further modifications such as *O*-methylation and *O*-conjugation with sugars, monolignols or their derivatives (Lam *et al.*, 2014). Tricin are one of the most abundant soluble flavones in vegetative tissues of grasses. Formation of tricin aglycone requires an apigenin 3'-hydroxylase/chrysoeriol 5'-hydroxylase (A3'H/C5'H; CYP75B4; encoded by a single-copy gene; Lam *et al.,* 2015; Lam *et al.,* 2019) which is phylogenetically related to flavonoid 3'-hydroxylases in the cytochrome P450 (CYP) 75B subfamily (Lam *et al.*, 2015; Lam *et al.,* 2019). In the grass family, tricin also functions as a co-monomer with monolignols, leading to the formation of tricin-integrated lignin (del Río *et al.*, 2012; Lan *et al.*, 2015). Recently, it has been further demonstrated that FNSII and A3'H/C5'H are also indispensable for generating tricin for lignification in rice; enzymatic saccharification was enhanced in *fnsII* and *a3'h/c5'h* mutant biomass with tricin-depleted lignin (Lam *et al.*, 2017; Lam *et al.*, 2019). Meanwhile, due to the lack of flavanone 3-hydroxylase (*F3H*, a single-copy

gene) gene expression in rice vegetative tissues (Shih *et al.*, 2008), there is no accumulation of flavonols which are constitutively accumulated in many dicot plants.

Interestingly, several anther-specific CHS-like enzymes (belonging to the type III polyketide synthase superfamily which includes CHS) and dihydroflavonol 4-reductase (DFR)-like enzymes were reported to be essential for male fertility in Arabidopsis, rice, and tobacco (Dobritsa *et al.,* 2010; Tang *el al.,* 2008; Wang *et al.,* 2013). However, these enzymes do not possess CHS or DFR activities (Tang *el al.,* 2008; Dobritsa *et al.,* 2010; Zhu *et al.,* 2017; Wang *et al.,* 2013). Instead, the CHS-like enzymes, including Arabidopsis PKSB/LAP5 and PKSA/LAP6 (Dobritsa *et al.,* 2010; Kim *et al.,* 2010) and rice OsPKS2 (Zhu *et al.,* 2017), catalyze the condensation of different hydroxylated fatty acyl-CoAs with malonyl-CoA to generate tri- and tetraketide α-pyrones. Meanwhile, the DFR-like enzymes function as tetraketide α-pyrone reductases (TKPRs), such as rice OsTKPR1 (Wang *et al.,* 2013), that generate fatty-acyl monomers for sporopollenin formation in pollen wall. Hence, disruption of the tetraketide pathway resulted in defective pollen development with different levels of exine malformation. On the other hand, since OsPKS2 and OsTKPR1 are not *bona fide* flavonoid enzymes, the importance of flavonoids for male fertility in rice remains elusive.

Previously, we demonstrated that rice OsCHS1 is a functional CHS *in planta* by complementation of Arabidopsis *tt4* mutation, restoring flavonoid production in different tissues (Shih *et al.*, 2008). In this study, an *OsCHS1* T-DNA insertion mutant (*oschs1*) with no seed formation was characterized. Reciprocal crossing with wild-type plants demonstrated that OsCHS1 is essential for male fertility. Pollen formation was unaffected, pollen size and structure were normal, but pollen germination and tube growth were impaired, leading to complete sterility. Flavonoid profiling revealed the accumulation of flavonols, flavones, flavone *C*-glycosides and flavanones in mature anthers of wild type but not in *oschs1* mutant. Furthermore, mutations in genes encoding downstream flavonoid enzymes in rice resulted in altered flavonoid profiles in anthers and different levels of seed yield reduction, suggesting that maintaining a combination of flavonoid classes might be essential for full fertility in rice. This is in contrast to the critical roles of flavonols (i.e. kaempferol and/or quercetin) for proper pollen functions in several formerly analyzed plant species.

MATERIALS AND METHODS

Plant materials and growth conditions

Rice *oschs1* (accession RMD-03Z11BO45; cv. Zhonghua 11), *cpy93g1* (accession K-00244; cv. Kitaake) and *cyp93g2* (SHIP_ZSX0568; cv. Zhonghua 11) T-DNA insertional mutant seeds were obtained from National Centre of Plant Gene Research at Huazhong Agricultural University (Wuhan, China) (Zhang *et al.*, 2006), Crop Biotech Institute of Kyung Hee University (Yongin, Korea) and National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology at Chinese Academy of Sciences (Shanghai, China) (Fu *et al.*, 2009), respectively. For the generation of *osf3h* mutant, sgRNA targeting the first exon of *OsF3H* was cloned downstream of OsU6 promoter in CRISPR/Cas9 binary vector pYLCRISPR/Cas9-MH (Ma *et al.*, 2015; Ma and Liu, 2016). For the generation of *cyp93g1 cyp93g2* double mutant, independent sgRNA targeting the second exon of *CYP93G1* and the second exon of *CYP93G2* were linked to OsU3 and OsU6 promoter, respectively, in pYLCRISPR/Cas9-MH vector (Ma *et al.*, 2015; Ma and Liu, 2016). The primer sequences used for constructing the CRISPR/Cas9 vectors are listed in the Supplementary Table S3. The CRISPR/Cas9 binary vectors were transformed into embryonic calli developed from wild-type rice seeds (cv. Nipponbare) by *Agrobacterium* strain EHA105.

Fully-genotyped T_3 generation CRISPR/Cas9 mutants derived from homozygous T_2 plants were used for further analyses. All mutants and their isogenic wild-type controls were germinated and grown side by side in phytotron with temperature maintained at 28 ℃. The T-DNA insertional and CRISPR/Cas9 mutants used for analyses were genotyped by PCR and direct sequencing (Ma *et al.*, 2015), respectively (Supplementary Table S3).

Germination assays of mature pollen

For *in vitro* pollen germination assay, mature pollen grains collected from wild-type and *oschs1* rice plants were incubated in pollen tube germination medium (0.75 M maltose, 0.01% H_3BO_3, 0.03% $CaCl_2$, 10.6% PEG4000) with 100% relative humidity at 30 ℃. After incubation for 2 h, the pollen grains were observed and photographed by the Nikon 80i Fluorescent Microscope equipped with Nikon DS-Ri2 camera (Nikon, Japan) under bright field. Pollen germination rate and pollen tube length were analyzed by Image J software (Abràmoff *et al.*, 2004).

For *in vivo* pollen germination assay, pistils from various mutants and their isogenic wild-type controls were collected more than 3 h after self-pollination or crossing with donor pollens. The pistils were fixed in ethanol: acetic acid (3:1) for 1 h at 4 ℃ and softened in 1M KOH at room temperature overnight (Wang *et al.*, 2012). After washing three times with distilled water, the pistils were incubated in staining buffer (0.1% aniline blue in 0.05 M KPO_4, pH 8.5) at room temperature for 2.5 h in the dark. The stained pistils were then washed with 50% (v/v) glycerol and photographed by the Nikon 80i Fluorescent Microscope equipped with Nikon DS-Ri2 camera (Nikon, Japan) using a 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) filter.

Pollen staining

Mature pollen grains collected from *oschs1* and wild-type plants were used for different staining analyses. I_2-KI staining (Yang *et al.*, 2008), calcofluor white staining (Moon *et al.*, 2013), auramine O staining (Moon *et al.*, 2013), 4',6-diamidino-2-phenylindole (DAPI) staining (Huang *et al.*, 2013), diphenylboric acid 2-aminoethyl ester (DPBA) staining (Saslowsky and Winkel-Shirley, 2001) and 5-(and 6)-chroromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) staining (Muhlemann *et al.*, 2018) were carried out as described previously. The stained pollens were observed and photographed using Cytation™ 1 Cell Imaging Multi-Mode Reader (BioTek, USA) with appropriate filters.

Scanning electron microscopy

Mature pollen grains collected from *oschs1* and wild-type plants were fixed, dehydrated and gold-coated as described previously (Wang *et al.*, 2013). The samples were examined by Hitachi S-4800FEG Scanning Electron Microscope (Hitachi, Tokyo, Japan).

Gene expression analysis

Anthers with mature pollen (mature pollen stage, stage 5, lemma ~8 mm; Chhun *et al.*, 2007) were collected from wild-type plants for total RNA extraction using RNAiso Plus (TARAKA, Japan) according to the manufacturer's instructions. First strand cDNA was synthesized using *TransScript* One-Step RT-PCR SuperMix kit (Transgen Biotech, China). Relative expression levels of different flavonoid biosynthetic genes in the anther were

analyzed by RT-PCR using gene-specific primers (Supplementary Table S3). The qRT-PCR experiments were carried out as described previously (Koshiba *et al.*, 2013).

LC-MS/MS analysis of anther metabolites

Plant metabolites were extracted as described previously (Lam *et al.*, 2014). Briefly, stage-5 rice anthers (100 mg) collected from different mutants and their isogenic wild types were ground into fine powder by TissueLyzer (Qiagen) after frozen in liquid nitrogen. HPLC-grade methanol (300 µl) was added into the samples, mixed well and incubated at 4 ℃ overnight. To remove *O*-conjugates of different flavonoids, equal volume of 2 M HCl was added to the extracts, followed by incubation at 90 ℃ for 1 h. After filtration, the extracted metabolites were separated by a C18 column (Synergi™ 4 µm Fusion-RP 80 Å 50 × 2.0 mm, Phenomenex, USA) connected to SCIEX quadrupole time of flight mass spectrometer (QTOF-MS) X500R (AB SCIEX). A solvent system of 0.5% (v/v) formic acid/water (A) and 0.5% (v/v) formic acid/methanol (B) with a linear gradient of 10% to 65% B over 10 min was used. The HPLC flow rate was maintained at 0.5 mL min^{-1}. Detection of flavonoids was performed by information dependent acquisition. Compound identification was carried out by comparing retention times and fragmentation patterns of extracted ion chromatographs with authentic standards.

Accession numbers

Sequence data can be found in the EMBL/GenBank data libraries under accession number AB00801 (*OsCHS1*, LOC_Os11g32650), CI252891 (*OsF3H*; LOC_Os04g56700), AK100972 (*CYP93G1*; LOC_Os04g01140) and AK099468 (*CYP93G2*; LOC_Os06g01250).

RESULTS

Phenotypic characteristics of *oschs1* mutant

To examine the roles of flavonoids in growth and development of rice, a mutant line with T-DNA inserted in the intron of *OsCHS1* was obtained and analyzed (Fig. 2A). Genomic PCR genotyping has identified wild type (WT1; cv. Zhonghua 11) and several lines that are homozygous (*oschs1*) or heterozygous (*oschs1/+*) for the T-DNA insertion. qRT-PCR experiments showed that the T-DNA insertion leads to the absence of the intact transcript of *OsCHS1* in *oschs1* plants (Fig. 2B). Seed setting was normal for wild-type and heterozygous plants. However, no seed was formed in all the *oschs1* plants even they exhibit normal floral morphology including pistils and anthers (Fig. 2C–H). On the other hand, the heterozygous plants showed similar seed yield to that of wild-type plants (Table 1), indicating that fertility is not affected by the genotypes (*OsCHS1* or *oschs1*) of the microgametophytes and megagametophytes.

The fertility of the *oschs1* plants was further examined by crossing with wild-type plants (Table 2). Wild-type pollens could fertilize *oschs1* mutants, indicating *oschs1* mutation does not affect female fertility. However, no seed was formed when *oschs1* plants served as the pollen donor, indicating that their sterility is male-specific. We also used pollens collected from *oschs1/+* heterozygous plants to cross *oschs1* plants. Consequently, approximately half of the F1 progenies were *oschs1* and the other half were heterozygous (Table 2), further indicating that *oschs1* pollens from heterozygous plants are fertile even on *oschs1* pistils.

oschs1 pollen analyses

Pollens collected from *oschs1* plants were subject to different chemical and structural analyses. Mutant pollens were well stained by I_2-KI, hence starch accumulation was not affected and they were viable (Fig. 2I, J). In addition, they were effectively stained by auramine O (an exine stain) and calcofluor white (an intine stain), further indicating that the sporopollenin wall is well developed (Fig. 2K–N). After DAPI staining, mature mutant pollens showed two generative nuclei and a less condensed vegetative nucleus, indicating

normal nuclear division (Fig. 2O and P). Pollen wall structure was further analyzed by scanning electron microscopy (SEM) which revealed normal morphology in the mutant. Pollens appear regular, round in shape with well-defined tectum ornamentation on wall surface (Fig. 2Q–T). In addition, ROS accumulation in pollens was examined by 5-(and 6)-chroromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) staining. An increase in fluorescence intensity implicating the elevation of ROS accumulation was found in mutant pollens compared with wild-type pollens (Fig. 2U–V). Collectively, these results demonstrate that the pollens of the male sterile *oschs1* plants are viable with normal development but accumulate increased levels of ROS.

OsCHS1 was required for pollen tube germination and elongation

Functions of the mutant pollens were then investigated *in vitro* (Fig. 3) and *in planta* (Fig. 4). For *in vitro* pollen germination assay, pollens collected from anthers during anthesis were incubated in pollen germination medium for 2 h and then examined under microscope. The average germination rate for pollens from *oschs1* plants was appreciably lower than that for pollens from wild-type plants (Fig. 3C). In addition, mutant pollen tube length (~46.7 μm) was approximately half of the wild-type pollen tube length (~95.2 μm) (Fig. 3D). Hence, pollens from *oschs1* plants showed reduction in both germination rate and tube length. We also attempted to examine ROS accumulation in pollen tubes by CM-H_2DCFDA staining. However, the fluorescence intensities in pollen tubes of both *oschs1* and wild-type plants were too low to distinguish any differences between them.

To examine pollen tube growth inside pistils, aniline blue staining was performed after self-pollination. In wild-type plants, the majority of pollens that adhered on the stigma germinated successfully and grew inside the style, reaching the base of the pistil (Fig. 4A). In *oschs1* plants, pollens that landed on the stigma geminated normally, but pollen tube growth was arrested near the chalazal end of the embryo sac (Fig. 4B). These results indicated that the male sterility of *oschs1* plants is caused by precocious termination of pollen tube growth inside the pistils. To investigate possible female sterility in *oschs1* plants, their stigmas were pollinated by wild-type pollens. All of the pollens germinated and some of the pollen tubes reached to the micropylar region of the ovule in *oschs1* (Fig. 4C). On the other hand, when wild-type stigmas were pollinated with *oschs1* pollens, some of the

adhered pollens germinated but pollen tube growth was arrested shortly within the style (Fig. 4D), consistent with the lack of seed formation from this cross (Table 2).

Flavonoid analyses of *oschs1* mutant

Pollens collected from anthers during anthesis were examined for the presence of flavonoid by diphenylboric acid 2-aminoethyl ester (DPBA) staining. As shown in Supplementary Fig. S1, wild-type pollens were stained positively for flavonoids while pollens from *oschs1* plants are DPBA-negative, hence the mutant pollens do not contain flavonoids. Meanwhile, all pollens collected from heterozygous plants showed positive DPBA staining. As half of the haploid pollens generated from heterozygous plants are having the *oschs1* mutation, the presence of flavonoids is likely to be a consequence of paternal effect.

In addition, seeds harvested from heterozygous parents were germinated and stained for flavonoids using DPBA. Among 116 seeds germinated, 88 seeds showed strong fluorescence in the coleoptiles. The unstained germinated seeds are morphologically indistinguishable from the DPBA-positive germinated seeds (Supplementary Fig. S2). Subsequently, genomic DNA of the coleoptile and embryo was extracted from each germinated seed for genotyping. All the DPBA-positive seeds are either wild-type or heterozygous while the DPBA-negative seeds are all *oschs1*. The segregation ratio matched the expected wild-type:heterozygous:*oschs1* = 1:2:1 (33:55:28) for the F_1 generation of self-pollinated heterozygous parents (X^2 =0.741<$X^2_{0.05}$= 5.99, P>0.05). These results indicated that the fertility of *oschs1* pollens/ovules from heterozygous parents is similar to wild-type pollens/ovules. Meanwhile, the F_1 progeny with *oschs1* genotype showed normal seed germination, suggesting that flavonoids are not essential during this stage in rice.

Furthermore, metabolites were extracted from mature anthers of *oschs1* and wild-type plants to analyze their flavonoid profiles. Following acid hydrolysis to remove *O*-linked conjugates, purified metabolites were separated and analyzed by LC-MS/MS. In wild-type anther extracts, flavonols (kaempferol and quercetin) were accumulated as the major extractable flavonoids (Fig. 5A). In addition, flavones (apigenin, luteolin, chrysoeriol and tricin), flavone *C*-glycosides (vitexin, isovitexin, orientin and isoorientin) and flavanones

(naringenin) were also detected at relatively lower levels than flavonols, whereas the upstream flavonoid precursor, naringenin chalcone (Fig. 1) was not found (data not shown). By contrast, none of the flavonoid metabolites could be found in *oschs1* anthers, consistent with absence of *OsCHS1* function.

Downstream flavonoid genes function in fertility

To further investigate the function of flavonoids in rice fertility, expression of flavonoid structural genes downstream of *OsCHS1*, which serve as branch point enzymes for channeling the metabolite flux to different classes of flavonoids, in anthers was first analyzed using the microarray data available in the Rice Expression Profile Database (Sato *et al.*, 2012) (ricexpro.dna.affrc.go.jp) (Fig. 1B). OsF3H, CYP93G1, and CYP93G2 are entry enzymes for the productions of flavonols, flavones, and flavone *C*-conjugates, respectively (Fig. 1A; Shih *et al.,* 2008; Du *et al.,* 2010; Lam *et al.,* 2014). The investigated genes, *OsF3H*, *CYP93G1*, *CYP93G2* as well as *OsCHS1* displayed high expression levels in late developmental stages of rice anthers. We further confirmed that these genes were expressed in mature wild-type anther using RT-PCR experiments (Fig. 1C). Hence, they are potentially involved in the biosynthesis of different flavonoids accumulated in mature anthers (Fig. 5).

Analysis of fertility and flavonoid profiles of rice flavonoid mutants

As it is well established that flavonols are required for male fertility in maize (Pollak *et al.*, 1995), petunia (Mo *et al.*, 1992; Ylstra *et al.*, 1994), tobacco (Mahajan *et al.*, 2011) and tomato (Muhlemann *et al.*, 2018), we speculated that the male sterility in *oschs1* plants might be due to the absence of flavonol production in anthers. To test this hypothesis, we generated rice mutant of *OsF3H*, which encodes the first branch point enzyme for flavonol biosynthesis, using CRISPR/Cas9-mediated mutagenesis. A sgRNA targeting the first exon of *OsF3H* was designed with minimum off-target potential. Among 16 T_0 plants genotyped, 11 plants harbored mutations in *OsF3H*. Surprisingly, seed formation was found in all the mutant lines of all the generations, which is in large contrast with the complete sterility in the flavonoid depleted *oschs1* plants. A mutant line with a single base substitution and a single base deletion was selected for further cultivation (Supplementary Fig. S3A). Homozygous T_3 plants were subsequently isolated for further analysis. The deletion results in a frame shift mutation, leading to the formation of a premature stop codon (data not

shown). We also examined sequences of the top 3 ranked off target sites predicted by CRISPR-P (Liu *et al.*, 2017) and no off-target mutation was found (Supplementary Table S1).

To analyze the alteration of flavonoid profile in anthers of *osf3h* mutants, metabolites extracted from mature anthers were subjected to LC-MS/MS analysis after acid hydrolysis. In the *osf3h* mutant anther extracts, flavonol contents were substantially reduced compared with its isogenic wild type, WT2, of the same cultivar (cv. Nippobare) (Fig. 5B). In particular, levels of kaempferol and quercetin detected were decreased from 41.2 µg/g fresh weight (FW) to 4.2 µg/g FW and from 27.5 µg/g FW to 12.1 µg/g FW, respectively. On the other hand, enhanced levels of several flavones (apigenin, from 0.6 µg/g FW to 2.6 µg/g FW; chrysoeriol, from 0.7 µg/g FW to 7.8 µg/g FW; and tricin, from 0.8 µg/g FW to 18.3 µg/g FW), flavone *C*-glycosides (isovitexin, from 3.5 µg/g FW to 10.4 µg/g FW; vitexin, from 1.2 µg/g FW to 2.9 µg/g FW; isoorientin, from 5.4 µg/g FW to 20.7 µg/g FW; and orientin, from 0.7 µg/g FW to 1.8 µg/g FW;) and flavanones (naringenin, from 0.5 µg/g FW to 2.9 µg/g FW; and eriodictyol, from 0.0 µg/g FW to 10.2 µg/g FW) were recorded when compared to wild-type anthers (Fig. 5B). Complete depletion of flavonols was not observed in *osf3h* mutant suggesting the presence of redundant F3H activities in rice anthers. Nonetheless, the fertility rate of *osf3h* mutant (68.2%) was slightly decreased when compared to that of wild-type plants (90.0%) grown under the same conditions simultaneously (Table 1).

Furthermore, the fertility rates of rice mutants for CYP93G1 and/or CYP93G2, which channel metabolite flux to flavones and flavone *C*-glycosides, respectively (Fig. 1A), were investigated as these flavone-derived metabolites were also accumulated in wild-type anthers. As the accumulation of flavonoids varies among wild types of different cultivars (Dong *et al.,* 2014) and is also affected by growth conditions, the accumulation of flavonoids in each mutant was compared with its wild-type control of the same cultivar growing side by side. The *cyp93g1* (cv. Kitaake) and *cyp93g2* (cv. Zhonghua 11) T-DNA insertional single mutants were retrieved from our previous collections (Supplementary Fig. S3B, C) (Du *et al.*, 2010a; Lam *et al.*, 2014). In addition, *cyp93g1 cyp93g2* double mutants (cv. Nipponbare) were generated using CRISPR/Cas9-mediated mutagenesis. Among the 29 T_0 plants genotyped, 13 plants harbored mutations in both *CYP93G1* and *CYP93G2*. Homozygous T_3 plants with 1 bp insertion in both *CYP93G1* and *CYP93G2* were subsequently isolated for further analysis (Supplementary Fig. S3D).

Flavonoid profiles in anthers of these flavone mutants were first examined. In the *cyp93g1* mutant anther extracts, tricin content was reduced from 0.8 μg/g FW to 0.3 μg/g FW, whereas other flavones and another two flavonoid classes showed no significant changes except for a slight increase in isovitexin, quercetin and naringenin when compared with its isogenic wild type, WT3 (Fig. 5C). In the *cyp93g2* mutant anther extract, there was no accumulation of flavone *C*-glycosides but increased levels of flavones (apigenin, chrysoeriol, and tricin), flavonols (kaempferol and quercetin) and flavanones (naringenin and eriodictyol) were detected (Fig. 5D). In the *cyp93g1 cyp93g2* double mutant anthers, luteolin and flavone *C*-glycosides were undetectable. On the other hand, reduced quantities of flavones (apigenin, from 0.6 μg/g FW to 0.2 μg/g FW; chrysoeriol, from 0.7 μg/g FW to 0.3 μg/g FW; and tricin, from 0.8 μg/g FW to 0.3 μg/g FW) were found whereas various flavonols (kaempferol, from 41.2 μg/g FW to 119.8 μg/g FW; and quercetin, from 27.5 μg/g FW to 114.8 μg/g FW) and flavanones (naringenin, from 0.5 μg/g FW to 9.2 μg/g FW; and eriodictyol, from 0.0 μg/g FW to 94.1 μg/g FW) showed elevated accumulation when compared with its isogenic wild type, WT2 (Fig. 5E).

The fertility rates of *cyp93g1*, *cyp93g2* and *cyp93g1 cyp93g2* mutants were then compared to those of the respective wild-type cultivars (Table 1). There were no apparent changes in seed yields for the *cyp93g1* and *cyp93g2* single mutants. On the other hand, the fertility rates were significantly decreased in the *cyp93g1 cyp93g2* double mutant (53.4%) when compared with that of WT2 (90.0%), suggesting that the presence of both flavones and flavone *C*-glycosides is also required for male fertility in rice.

As the cause of male sterility in *oschs1* plants was the inability of the pollen tubes to elongate and reach the ovary, we further analyzed pollen tube elongation in the *osf3h* single and *cyp93g1 cyp93g2* double mutants, which displayed reduced fertility rates. The mutants and wild-type controls (WT2) were sampled after self-pollination and the percentages of elongated pollen tubes that could reach the bases of the ovaries were determined. In WT2, 89.3% of the pollen tubes from germinated pollens reached the ovaries (Supplementary Table S2). By contrast, the percentages were significantly reduced to 70.3% and 65.1% in the *osf3h* and *cyp93g1 cyp93g2* mutants, respectively. These data results suggested that the

379 alteration of anther flavonoid profiles in these mutants impacted negatively on pollen tube
380 proliferation *in planta*, resulting in reduced fertility.

381

382 **DISCUSSION**

383

384 This study first demonstrates that OsCHS1, the first committed enzyme in flavonoid
385 biosynthesis, is essential for complete male fertility in rice. There is no seed formation in the
386 *oschs1* homozygous T-DNA insertion mutant (Table 1). As the *oschs1/+* heterozygous plants
387 showed normal fertility, the *oschs1* mutant allele is recessive. Crossing of the *oschs1* mutant
388 by wild-type pollens restored fertility, hence female reproductive functions are not affected
389 by *oschs1* mutation. Interestingly, down-regulation of *CHS* in tomato resulted in impaired
390 female fertility and parthenocarpic fruit development (Schijlen *et al.*, 2007). Thus, seed
391 production in tomato *CHS* RNAi flowers fertilized by wild-type pollens was reduced. On the
392 other hand, normal seed yield could be achieved in wild-type pistils crossed with the *CHS*
393 RNAi pollens which could grow down through the style to reach the ovule.

394

395 In rice, complete male sterility in the *oschs1* mutant is most likely to be caused by
396 impaired pollen tube germination and/or elongation instead of defects in pollen maturation.
397 Pollen analyses revealed that the *oschs1* mutant pollens were viable (Fig. 2I, J), nuclear
398 division was normal (Fig. 2O, P) and pollen walls are well-structured (Fig. Q–T), hence the
399 pollen maturation process is apparently normal. Events taken place from pollination to
400 successful fertilization in grasses are divided into five phases (Dresselhaus and Franklin-
401 Tong, 2013), and failure in any steps would lead to sterility. Upon adhesion and hydration on
402 stigmas, pollens start to germinate (phase I) (Dresselhaus and Franklin-Tong, 2013). Pollen
403 tubes of the compatible pollens invade the stigma (phase II), grow along the transmitting
404 tract of the style and reach the ovular cavity (phase III) (Dresselhaus and Franklin-Tong,
405 2013). Afterwards, pollen tubes continue to grow along the ovule surface to micropyle
406 (phase IV), releasing sperm cells for double fertilization of megagametophyte (phase V)
407 (Dresselhaus and Franklin-Tong, 2013). Here, the flavonoid-depleted *oschs1* mutant pollens
408 are likely to be defective in phases I and III. Pollen germination rate *in vitro* is slightly lower
409 than wild type (Fig. 3C), implicating that flavonoids could enhance pollen germination in rice,
410 albeit not absolutely essential. While the pollen tubes could penetrate through the stigma in

411 *oschs1* mutant, their growth was arrested midway within the style before reaching the pistil
412 base (Fig. 4B). Consistently, *in vitro* germination assays showed that *oschs1* mutant pollens
413 produced shorter pollen tubes on average compared with wild-type pollens (Fig. 3D).
414 Similarly, when wild-type pistils were crossed with *oschs1* pollens, pollen tube growth was
415 arrested within in the style (Fig. 4D). These data strongly suggested that flavonoids are
416 required for pollen tube growth inside pistils. Previously, similar results were observed
417 in *CHS* antisense petunia (Ylstra *et al.*, 1994), tomato *CHS*-RNAi plants (Schijlen *et al.*, 2007)
418 and *FLS*-silenced tobacco (Mahajan *et al.*, 2011). In these examples, growth of mutant pollen
419 tubes was arrested without reaching the ovary, leading to impaired seed set and/or
420 parthenocarpy.

421

422 Following CHS, CHI is involved in the isomerization step for the generation of
423 naringenin in the central flavonoid biosynthesis pathway (Fig. 1). Mutation of *CHI* usually
424 resulted in substantial depletion of flavonoid accumulation in various plant species (Pelletier
425 *et al.,* 1999; Kang *et al.,* 2014; Clayton *et al.,* 2018). It was previously reported that mutation
426 of *OsCHI* in Nipponbare rice resulted in dramatic reduction in seed yield (Hirano *et al.,* 2017),
427 further suggesting that flavonoids are required for full fertility in rice.

428

429 The requirement of flavonoids for reproduction does not appear to be universal or
430 consistent in all flowering plants. The most notable exception is Arabidopsis fertility which is
431 not affected by the absence or deficiency of flavonoids (Burbulis *et al.*, 1996). In this study,
432 we have revealed several unique and new features regarding flavonoids and male fertility in
433 rice. First, flavonoid depletion in rice does not impact pollen viability and structural integrity.
434 By contrast, the tomato *anthocyanin reduced* (*are*) mutant pollen grains are reduced in
435 number and viability with collapsed pollen wall structure (Muhlemann *et al.*, 2018). In
436 addition, flavonoid production apparently occurs in sporophytic tissues of rice anthers. The
437 *osch1*/+ heterozygous mutant produces pollen grains which are all DPBA-positive although
438 half of them have the *oschs1* haploid genotype (Supplementary Fig. S1). Since *oschs1*
439 progenies (approximately ¼) were identified from self-fertilized heterozygous parents or
440 *oschs1* plants crossed with heterozygous pollens, the flavonoid-containing *oschs1* pollen
441 grains from heterozygous plants are fully functional. In maize and *Brassica* plants, flavonols
442 are detected exclusively in tapetum cells during anther development. Following lysis of
443 tapetum cells, flavonols are deposited onto pollen coat which is on the surface of

444 sporopollenin wall (Hsieh and Huang, 2007; Li *et al.*, 2012). Similarly, different flavonoid
445 derivatives may be produced in diploid tissues (such as tapetum) inside the heterozygous
446 anthers, followed by deposition in haploid pollens regardless of their *OsCHS1* genotypes.

447

448 CHS and CHI channel the metabolite flux to the production of naringenin which is the
449 common precursor for different flavonoid classes. In particular, the roles of flavonols (e.g.
450 kaempferol, quercetin) in male fertility have been explored extensively. They are necessary
451 for pollen viability, germination, and tube growth in maize, petunia, tomato and tobacco
452 (Mo *et al.*, 1992; Ylstra *et al.*, 1994; Pollak *et al.*, 1995; Mahajan *et al.*, 2011; Muhlemann *et*
453 *al.*, 2018). Among these species, maize, petunia and tomato predominately accumulate
454 flavonols in their anthers and/or pollens (Ceska and Styles, 1984; Pollak *et al.*, 1993; Li *et al.*,
455 2012; Žilić *et al.*, 2014; Muhlemann *et al.*, 2018). Exogenous application of flavonols rescued
456 seed formation in maize *chs* mutant and restored pollen tube growth in petunia *chs* mutant
457 (Mo *et al.*, 1992; Taylor and Jorgensen, 1992; Pollak *et al.*, 1995). Biosynthesis of flavonols
458 (e.g. kaempferol and quercetin) requires the sequential activities of F3H and flavonol
459 synthase (FLS). Consistently, RNAi suppression of *FLS* expression reduced pod number and
460 size as well as seed set in tobacco (Mahajan *et al.*, 2011). Recently, the tomato *are* mutant,
461 which is defective in F3H, was reported to produce fewer and collapsed pollen grains with
462 defective functions leading to reduction in seed formation (Muhlemann *et al.*, 2018).
463 Furthermore, flavonols were revealed to regulate the levels of ROS which would otherwise
464 impair pollen production, viability, integrity, germination and tube growth in tomato,
465 especially during high-temperature stress (Muhlemann *et al.*, 2018).

466

467 In rice, flavonols are not produced in vegetative tissues due to the absence of *OsF3H*
468 expression (Shih *et al.*, 2008). In this study, we found that *OsF3H* is expressed in maturing
469 anthers together with other flavonoid biosynthesis genes (Fig. 1B, C). Consistently, rice wild-
470 type anthers accumulate flavonols as the major soluble flavonoid metabolites together with
471 relatively lower levels of flavones, flavone *C*-glycosides and flavanones (Fig. 5). As *OsF3H*
472 mutation did not result in complete depletion of flavonols (Fig. 5B), redundant F3H activities
473 are likely present. In fact, it was reported that OsFLS and three other F3H homologs harbor
474 F3H activities *in vitro* (Kim *et al.*, 2008, Park *et al.*, 2019). Nevertheless, the considerable
475 reduction (kaempferol, 90% lower than WT2; quercetin, 56% lower than WT2) of flavonols in
476 *osf3h* mutant anthers only resulted in moderate seed yield reduction (i.e. ~22% lower than

WT2; Table 1). This is in sharp contrast to tomato *are* (*F3H*) mutant which displayed ~75% reduction in seed set, although the levels of flavonol derivatives were estimated to be reduced by only ~45% (Muhlemann *et al.*, 2018). Hence, our findings indicate that flavonols are less essential for male fertility in rice compared with tomato. Unlike tomato anthers which only accumulate flavonols (Muhlemann *et al.*, 2018), rice anthers also accumulate other classes of flavonoid derivatives which might partially compensate the impacts of flavonol reduction on fertility.

The depletion of flavone *C*-glycosides in *cyp93g2* mutant anthers did not influence fertility significantly, or the impact of their absence could be compensated by elevated levels of other flavonoid classes. On the other hand, since flavones were still detected in *cyp93g1* mutant anthers, albeit in reduced amounts (Fig. 5C), the necessity of this specific flavonoid group alone for male fertility in rice remains inconclusive. The residual flavone production may be resulting from redundant flavone synthase activities in anthers (Lee *et al.*, 2008) or a leaky *CYP93G1* mutation in the T-DNA line.

The absence of flavone *C*-glycosides along with moderately reduced levels of flavones in the *cyp93g1 cyp93g2* double mutant anthers led to considerable seed yield reduction (i.e. 37% lower than WT2) (Table 1), suggesting that flavones and *C*-glycosides play a role together in male fertility in rice. Meanwhile, the accumulation of flavonols was elevated by 2 to 3-fold in the double mutant anthers (Fig. 5E). Apparently, flavonols could only partially compensate the impact of the reduction of both classes of flavone derivatives on male fertility. By contrast, flavonol derivatives are the most essential or the only flavonoid class required for male reproductive functions in in tomato and other species reported previously (Mo *et al.*, 1992; Ylstra *et al.*, 1994; Mahajan *et al.*, 2011; Muhlemann *et al.*, 2018). For example, pollens of the *CHS*-deficient plants could only be rescued by various flavonols and partially by taxifolin but not by flavones, flavanones or chalcone (Mo *et al.*, 1992; Ylstra *et al.*, 1994). Exogenous application of the flavone apigenin even inhibited pollen tube growth in wild-type maize (Ylstra *et al.*, 1994). On the other hand, it is possible that the reduced male fertility observed in the *cyp93g1 cyp93g2* double mutant is due to altered flavonoid composition in anthers: substantially increased levels of flavonols and flavanones, depletion of flavones, and absence of flavone *C*-glycosides (Fig. 5E). It was recently reported that flavonols affect pollen tube growth and integrity, and in turn, fertility, by regulating ROS

homeostasis (Muhlemann *et al.*, 2018). As different flavonoids and their *O*-conjugated derivatives harbor different ROS scavenging activities (Pietta, 2000; Burda and Oleszek, 2001; Heim *et al.*, 2002), alteration of flavonoid profiles might lead to changes in ROS homeostasis, potentially impairing pollen tube growth and fertility. It is also worth noting that modified flavonoid profiles were also reported in pollens of rice mutants defective in PKS and TKPR enzymes. However, their male sterility is directly caused by disruption of the tetraketide pathway that generates fatty-acyl monomers for sporopollenin formation (Dobritsa *et al.,* 2010; Kim *et al.,* 2010; Wang *et al.,* 2013; Zhu *et al.,* 2017; Xu *et al.,* 2019).

In conclusion, male fertility in rice shows an absolute requirement for flavonoids which are most likely to be essential for pollen tube germination and growth inside pistils. In addition, flavonols do not appear to play a prominent role in male fertility, as opposed to maize, tomato, petunia, or tobacco. Instead, a combination of different flavonoid classes is probably necessary for the expression of complete male fertility in rice.

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TABLES

Table 1. Fertility rate of various flavonoid mutants compared with their isogenic wild types.

	fertility rate (%)
WT1	70.9 ± 18.5
oschs1/+	64.8 ±18.1
oschs1	0.0 ± 0.0***
WT2	90.0 ± 2.5
osf3h	68.2 ± 7.9**
cyp93g1 cyp93g2	53.4 ± 10.3*
WT3	92.9 ± 1.4
cyp93g1	92.9 ± 3.0
WT4	93.2 ± 4.5
cyp93g2	97.1 ± 3.6

The values are means ± standard deviation (SD) (n = 5, Student's t-test, *P < 0.05, ** P < 0.01, ***P < 0.001).

Table 2. Crossing of WT1, *oschs1/+* heterozygous and *oschs1* homozygous mutants.

	number of seeds formed	number of F1 plants of each genotype		
		WT1	*oschs1/+*	*oschs1*
oschs1 (♀) × WT1 (♂)	30	0	30	0
WT1 (♀) × *oschs1* (♂)	0	0	0	0
oschs1 (♀) × *oschs1*/+ (♂)	25	0	12	13

Number of seeds formed and genotypes of the progenies (F1) obtained from each cross are shown.

FIGURE LEGENDS

Fig. 1. Proposed flavonoid biosynthetic pathway in rice and gene expression analysis of target genes of this study.

(A) Proposed flavonoid biosynthetic pathway in rice. CHS: chalcone synthase. CHI: chalcone isomerase. F3H: flavanone 3-hydroxylase. FLS: flavonol synthase. F2H: flavanone 2-hydroxylase. CGT: *C*-glucosyltransferase. FNSII: flavone synthase II. A3'H/C5'H: apigenin 3'-hydroxylase/chrysoeriol 5'-hydroxylase. F3'H: flavonoid 3'-hydroxylase. FOMT: flavonoid *O*-methyltransferase. Glc: glucose. In red: the target enzymes investigated in this study. In grey: redundant enzyme with insignificant contribution. Purple arrows: flavonol specific pathway. Orange arrows: flavone specific pathway. Blue arrows: flavone *C*-glycosides specific pathway. Dotted arrow: proposed step.

(B) *In silico* gene expression analysis of *OsCHS1*, *OsF3H*, *CYP93G1* and *CYP93G2* at different developmental stages of anther in wild-type rice. Microarray data were retrieved from Rice Expression Profile Database (http://ricexpro.dna.affrc.go.jp/).

(C) RT-PCR gene expression analysis of *OsCHS1*, *OsF3H*, *CYP93G1* and *CYP93G2* in mature wild-type rice anther.

Fig. 2. Phenotypes of WT1 and *oschs1* homozygous mutants.

(A) Gene structure of *OsCHS1* and site of T-DNA insertion in *oschs1* mutant.

(B) Gene expression analysis of *OsCHS1* in WT1 and *oschs1* mutants by qRT-PCR. The values are means ± standard deviation (SD) ($n = 5$).

(C, D) Mature panicles of WT1 (C) and *oschs1* mutants (D) after ripping (~45 days after heading). Unfilled spikelets without seed formation remain green after ripening (Ding *et al.,* 2012; Ansari *et al,* 2017; Zhang *et al.,* 2017). Scale bars denote 1 cm.

(E, F) Mature pistils of WT1 (E) and *oschs1* mutants (F) at heading stage. Scale bars denote 500 µm.

(G, H) Anthers of WT1 (G) and *oschs1* mutants (H) before anthesis. Scale bars denote 500 µm.

(I, J) I_2-KI staining of mature pollens of WT1 (I) and *oschs1* mutants (J). Scale bars denote 50 µm.

(K, L) Pollen exine staining by auramine O for mature pollens of WT1 (K) and *oschs1* mutants (L). Scale bars denote 50 µm.

(M, N) Pollen intine staining by calcofluor white for mature pollens of WT1 (M) and *oschs1* mutants (N). Scale bars denote 50 µm.

(O, P) DAPI staining for nuclei for mature pollens of WT1 (O) and *oschs1* mutants (P). Scale bars denote 50 µm.

(Q–T) Pollen surface examination of mature pollens of WT1 (Q, S) and *oschs1* mutants (R, T) by scanning electron microscope (SEM). Scale bars denote 5 µm (Q, R) or 500 nm (S, T).

(U, V) ROS staining by CM-H_2DCFDA for mature pollens of WT1 (U) and *oschs1* mutants (V). Scale bars denote 20 µm.

Fig. 3. *In vitro* pollen germination assay WT1 and *oschs1* homozygous mutants.

(A, B) Germinated pollens of WT1 (A) and *oschs1* mutants (B). Scale bars denote 100 µm.

(C) Pollen tube germination rate of WT1 and *oschs1* mutants.

(D) Pollen tube length of WT1 and *oschs1* mutants.

The values are means ± standard deviation (SD) (n = 300 for the experiment of pollen germination rate; n = 60 for the experiment of pollen tube length, Student's t-test, ***P < 0.001).

Fig. 4. *In vivo* pollen germination assay of WT1 and *oschs1* homozygous mutants.

(A) Pollen tube germination and elongation in self-pollinated WT1 pistil.

(B**)** Pollen tube germination and elongation in self-pollinated *oschs1* mutant pistil.

(C) Pollen tube germination and elongation in *oschs1* mutant pistil pollinated by WT1 pollens.

(D) Pollen tube germination and elongation in WT1 pistil pollinated by *oschs1* mutant pollens.

Pollen tube germination and elongation were examined 3 h after pollination. Yellow arrows indicate pollen tubes that grow and elongate to the base of ovary. Red arrows indicate pollen tubes that are germinated but elongation stops in the middle of the pistils. Scale bars denote 100 μm.

Fig. 5. Flavonoid metabolites accumulated in anthers of various flavonoid homozygous mutants compared with their isogenic wild types.

Quantitation of flavanones, flavones, flavone *C*-glycosides and flavonols in anthers of *oschs1* (A), *osf3h* (B), *cyp93g1* (C), *cyp93g2* (D) and *cyp93g1 cyp93g2* (E) mutants and their isogenic wild types. The upstream flavonoid precursor, naringenin chalcone (Fig. 1), was not detected in any of the samples. The values are means ± standard deviation (SD) (n = 3, Student's t-test, *P < 0.05, ** P < 0.01, ***P < 0.001). FW: fresh weight. ND: not detected.

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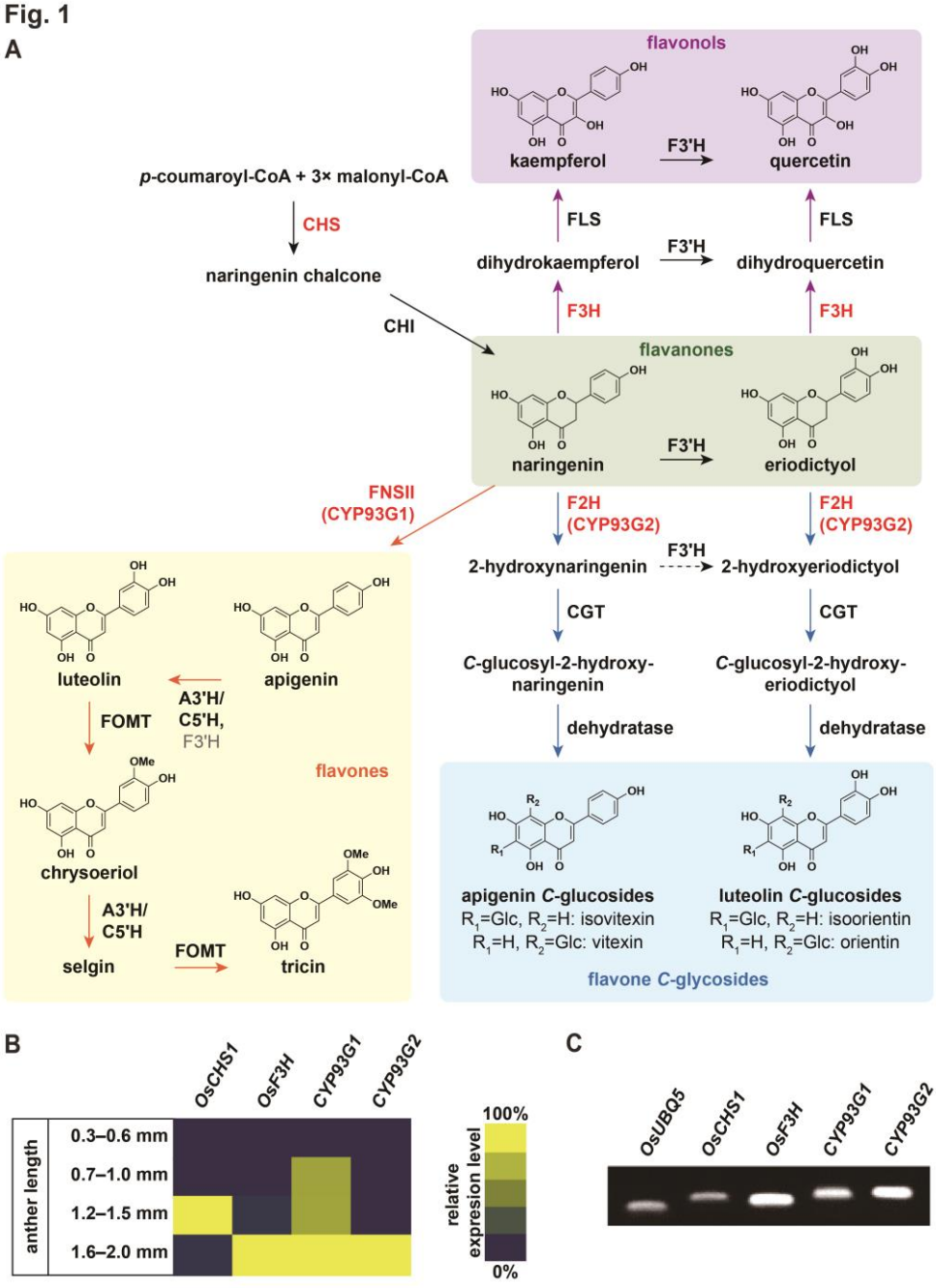
Fig. 1



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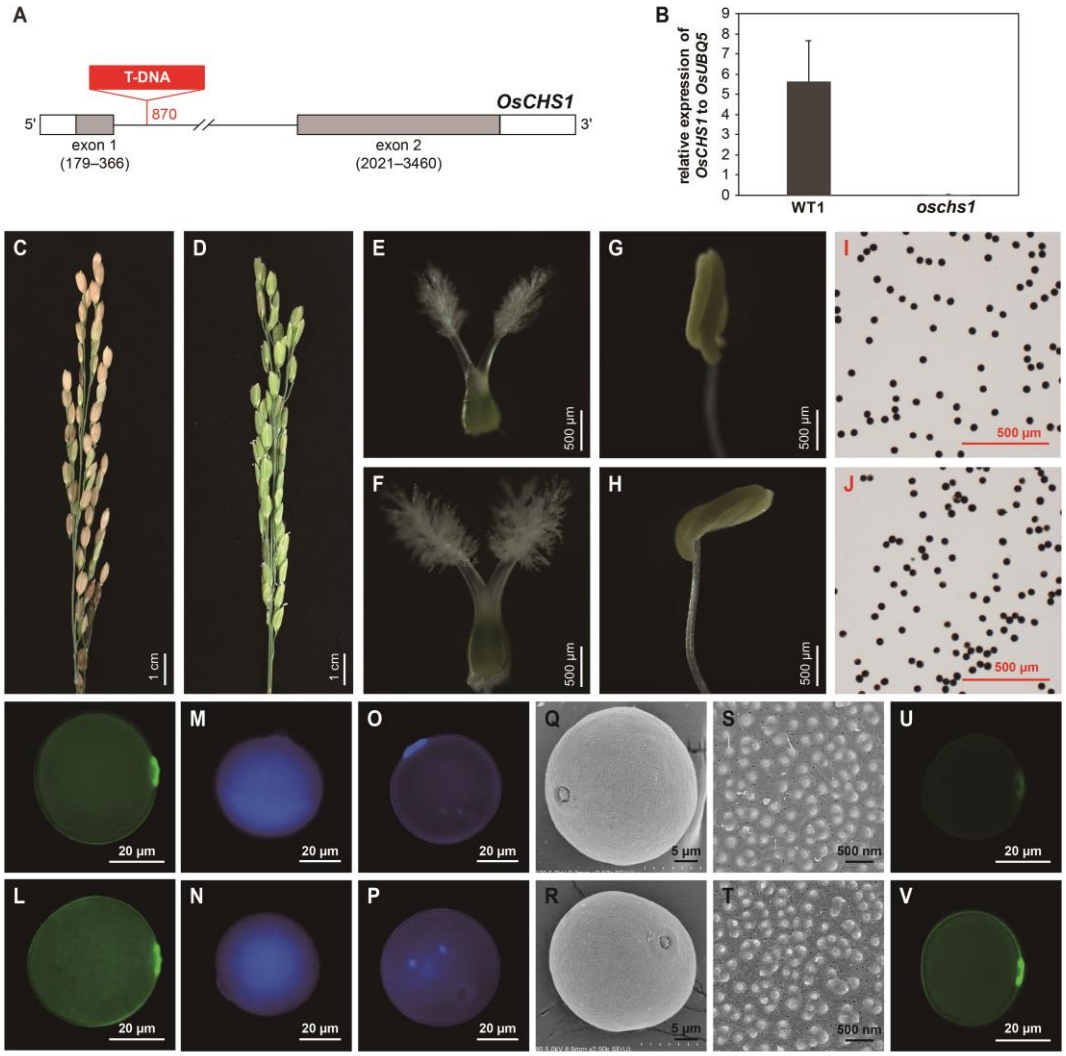

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Fig. 3

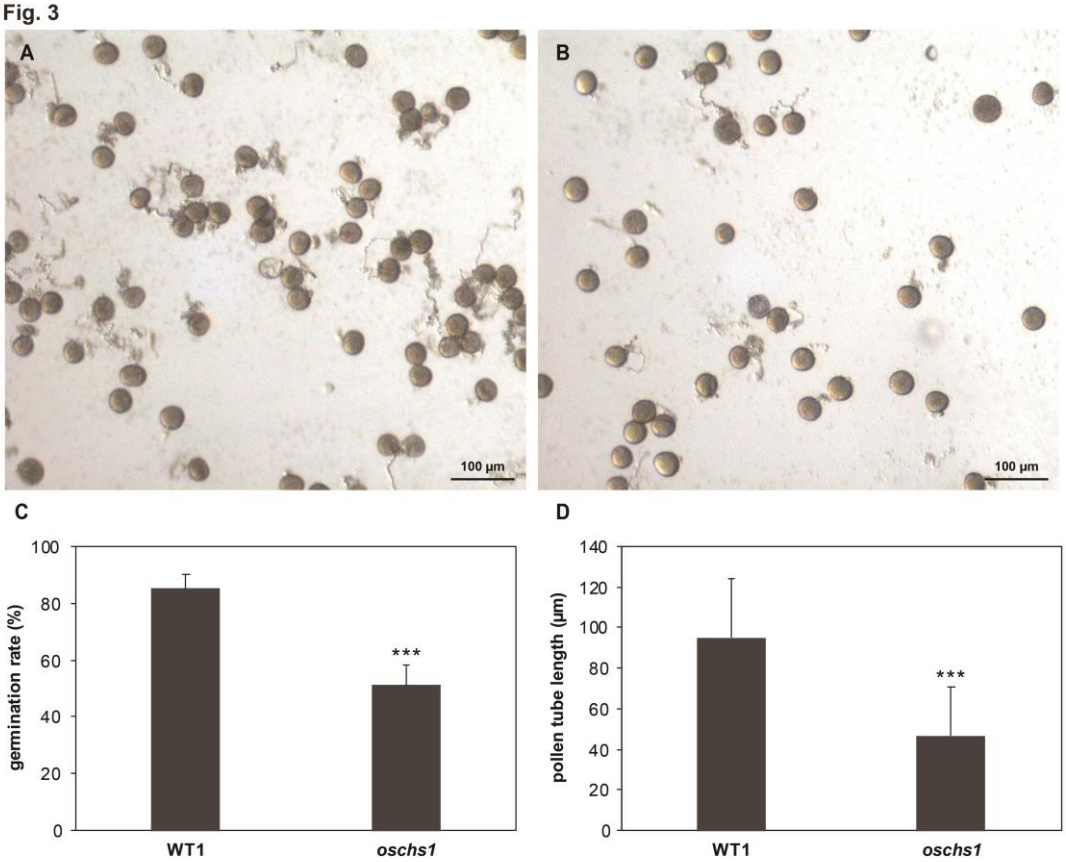

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Fig. 4

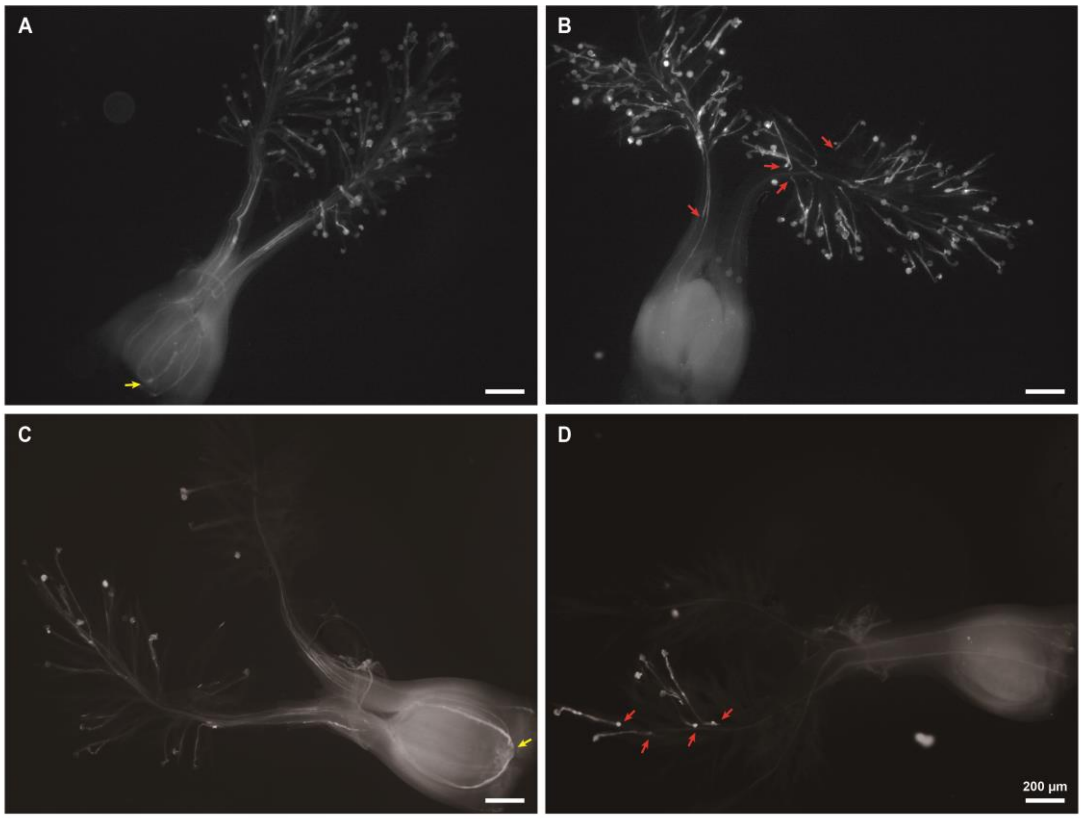

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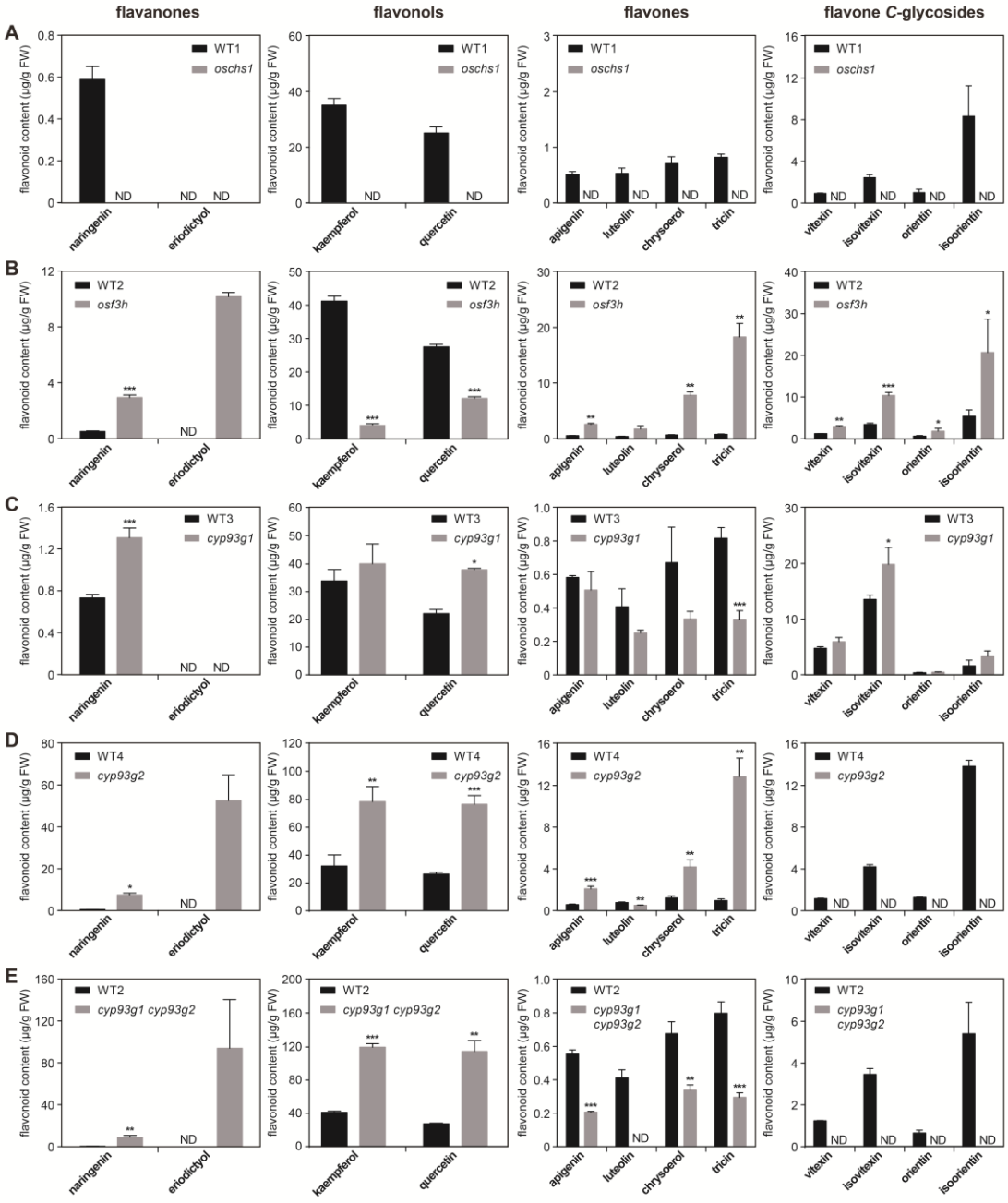
Fig. 5



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