The Tapetal Major Facilitator NPF2.8 is Required for Accumulation of Flavonol Glycosides on the Pollen Surface in Arabidopsis thaliana

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Short title: Flavonol glycoside transport to the pollen surface

One-sentence summary: The tapetal major facilitator NPF2.8 is required for accumulation of flavonol glycosides on the pollen surface of Arabidopsis thaliana.

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ABSTRACT
The exine of angiosperm pollen grains is usually covered by a complex mix of metabolites including pollen-specific hydroxycinnamic acid amides (HCAAs) and flavonoid glycosides. Whereas the biosynthetic pathways resulting in the formation of HCAAs and flavonol glycosides have been characterized, it is unclear how these compounds are transported to the pollen surface. In this report we provide several lines of evidence that a member of the NTR/PTR nitrate/peptide transporter family is required for the accumulation and transport of pollen-specific flavonol 3-O-sophorosides, characterized by a glycosidic β-1,2-linkage, to the pollen surface of Arabidopsis. Ectopic, transient expression in Nicotiana benthamiana epidermal leaf cells demonstrated localization of this flavonol sophoroside transporter, termed FST1, at the plasmalemma when fused to green fluorescent protein (GFP). We also confirmed the tapetum-specific expression of FST1 by GFP reporter lines driven by the FST1 promoter. In vitro characterization of FST1 activity was achieved by microbial uptake assays based on \textsuperscript{14}C-labeled flavonol glycosides. Finally, rescue of an fst1 insertion mutant by complementation with an FST1 genomic fragment restored the accumulation of flavonol glycosides in pollen grains to wild-type levels, corroborating the requirement of FST1 for transport of flavonol-3-O-sophorosides from the tapetum to the pollen surface.

Keywords: Arabidopsis, flavonoid, transport, pollen, exine, major facilitator
INTRODUCTION

Angiosperm male gametophytes are protected by a complex, rigid, and most durable polymer, the pollen wall that is synthesized by the tapetum, the inner layer of the anther locule (Blackmore et al., 2007; Liu and Fan, 2013). This single cell layer provides metabolites and structural components to the protective shell of the male gametophyte (Hsieh and Huang, 2007, Quilichini et al., 2014a). The composition of the polymeric part of the pollen wall, termed sporopollenin, is composed of several layers of heterogeneous aliphatic and aromatic building blocks, of which the sculptured exine is most substantial (Ariizumi and Toriyama, 2011, Quilichini et al., 2015). Harsh chemical degradation of the pollen wall was only of limited value to resolve its monomeric composition (Wiermann and Gubatz, 1992), but genetic evidence allowed the dissection of the contribution of aliphatic and aromatic structures from the lipid and the polyketide biosynthetic pathways (Morant et al., 2007; Kim et al., 2010; Dobritsa et al., 2010, 2011; Grienenberger et al., 2010, Quilichini et al., 2014a). Sporopollenin is covered by a blend of compounds of aliphatic and aromatic origin including waxes, lipids, sterols, sugars and proteins, collectively termed pollen coat, pollenkitt or trypnine in the case of the Brassicaceae (Piffanelli et al., 1998; Pacini and Hesse, 2005; Ariizumi and Toriyama, 2011). The trypnine coating provides the actual interface between the pollen and the surrounding environment. Among the non-polymerized and soluble aromatic constituents deposited within trypnine, two-specific types of phenylpropanoids, hydroxycinnamoyl spermidine conjugates (HCAAs) and flavonol-3-O-glycosides were identified as characteristic and abundant compounds in dicots and monocots (Figure 1) (Meurer et al., 1988; Fellenberg and Vogt, 2015; Elejalde-Palmett et al., 2015).

In Arabidopsis (Arabidopsis thaliana), accumulation of tapetum-specific HCAAs requires a combination of two cytochrome P450s (CYP98A8 and CYP98A9) and an O-methyltransferase (TSM1 for TAPETUM-SPECIFIC METHYLTRANSFERASE) for decorations of common phenylpropanoid units that are attached via amide bonds to all three nitrogen atoms of spermidine by a BAHD-type spermidine hydroxycinnamoyl transferase (SHT) (Matsuno et al., 2009; Fellenberg et al., 2008; Grienenberger et al., 2009). The tapetum-specific UDP glycosyltransferase UGT79B6 catalyzes the formation of a specific β-1,2-linkage from UDP-glucose and flavonol 3-O-monogluco-sides (Yonekura-Sakakibara et al., 2014). This results in the formation of kaempferol- and quercetin-3-O-gluco-syl-(1→2)-glucosides (K3GG and Q3GG), termed sophorosides. No information on the subcellular localization is currently available for the tapetum-specific HCAAs and the flavonoid pathways. However, the enzymes required for
sporopollenin accumulation are co-expressed and act synergistically, presumably in a metabolon localized at the endoplasmic reticulum (Lallemand et al., 2013).

In addition to the unknown compartmentation, how precisely the soluble constituents of trypchine are deposited onto the developing exine is still a matter of debate. Consistent with the observation that the assembly of the pollen wall before degeneration of the tapetum is a tightly controlled process, a coordinated transport of different metabolite classes is anticipated (Blackmore et al., 2007). This may be achieved by fusion of vesicles with the tapetal plasmalemma and subsequent release onto the maturing exine (Bedinger, 1992; Wu and Cheung, 2000; Wilson and Zhang, 2009). Even active secretion of enzymes involved in sporopollenin biosynthesis into the anther locule has been suggested to accelerate exine formation (Wang et al., 2018). Many genes are transcriptionally active during anther development and microgametogenesis, with more than 1,000 transporter candidates described at individual stages of gametophyte development (Honys and Twell, 2004; Bock et al., 2006). Therefore, initial discoveries of transporters were based on co-expression analysis with already established pathways for exine-specific polyketides and soluble HCAAs (Matsuno et al., 2009; Dobritsa et al., 2010, Grienenberger et al., 2009; 2010). Several ATP-BINDING CASSETTE (ABC) transporter mutants in Arabidopsis and rice were characterized with impaired deposition of exine building blocks. A mutant deficient in ABCG26 has severely reduced fertility and exine formation; the wildtype gene therefore appears central to the transport of sporopollenin precursors (Quilichini et al., 2010; Choi et al., 2011). When investigated by two photon laser microscopy, abcg26 mutants accumulated fluorescent microbodies in the tapetum that were missing in the wild-type tapetum. These could be linked to sporopollenin polyketides but were independent from HCAA and flavonoid transport (Quilichini et al., 2014b). Small lipid transfer proteins were proposed to be involved in the translocation of soluble compounds via the ER-trans-Golgi-network as an alternative to explain the highly coordinated assembly of these metabolites into the pollen exine (Huang et al., 2013). Other ABC transporters, namely ABCG1 and ABCG16, were reported to affect the transport of precursors of intine biosynthesis and development, but it remains unclear which exact components are targeted (Yadav et al., 2014). The transporters ABCG9 and ABCG31 are also highly expressed in tapetal cells and are presumably involved in the deposition of steryl glycosides on the pollen surface (Choi et al., 2011, 2014). Several putative orthologous ABC transporters were also identified from rice, consistent with the observation that pollen development in rice and Arabidopsis is fairly similar at the molecular level (Wilson and Zhang, 2009). OsABCG26 and OsABCG15 are required for the transport of components forming the anther cuticle, specifically wax and cutin monomers, whereas OsABCG15, an orthologue of
Arabidopsis ABCG26, is essential for sporopollenin precursor transport (Zhao et al., 2015; Shi et al., 2015). Therefore, the whole array of ABCG-type transporters described so far for the tapetum appears involved in the transport of lipids or sporopollenin precursors rather than the translocation of flavonoids or HCAAs to the pollen tryphine.

Experimental evidence for the participation of multidrug and toxin extrusion (MATE) proteins in the transport of sporopollenin and tryphine components is still missing. The classical MATE transporter, TRANSPARENT TESTA 12 (TT12), was identified nearly two decades ago (Debeaujon et al., 2001; Marinova et al., 2007). TT12 is essential for the translocation and deposition of proanthocyanidins to the Arabidopsis seed coat and works in cooperation with an H⁺-ATPase proton pump (Baxter et al., 2005). tt12 mutants exhibit reduced proanthocyanidin accumulation and a less intense coloration of the seed coat. Since then, only a few of the 58 MATE transporter genes in Arabidopsis have been functionally characterized. Thompson et al. (2010a) identified a MATE-type transporter, termed FLOWER FLAVONOID TRANSPORTER (FFT) or DETOXIFYING EFFLUX CARRIER 35 (DTX35), presumably localizing to the tonoplast of root cells and guard cells in floral tissues, with a role in plant fertility and floral flavonol glycoside accumulation. A series of similar and partially redundant DTX proteins were proposed for various organs in Arabidopsis and shown to be involved in flavonoid transport, although not characterized in functional detail (Thompson et al., 2010b). A DTX35 homolog from grapevine (Vitis vinifera) partially complemented the low anthocyanin phenotype seen in the Arabidopsis pale banyuls 1 (pab1) mutant (Kitamura et al., 2016). This observation suggested a role for DTX35 in anthocyanin transport in seeds. However, DTX33 and DTX35, both highly expressed during pollen germination, were identified as chloride anion channels, indirectly questioning the involvement of this subclade of transporters in flavonoid translocation (Zhang et al., 2017).

Interestingly, in the case of HCAAs, a MATE-type transporter from Arabidopsis epidermal cells contributes to the pathogen defense response against the potato late blight fungus Phytophthora infestans by translocation of 4-coumaroylagmatine to the plant surface (Dobritzsch et al., 2016).

Two types of nitrate (NTR) and peptide (PTR) transporters have been described in plants and show partially overlapping specificities (Tsay et al., 2007). They are members of the universal major facilitator superfamily (MFS) of transporters (http://pfam.xfam.org/clan/MFS; Marger and Saier Jr., 1993; Yan, 2015, Niño-Gonzáles et al., 2019). A more universal nomenclature and rigid classification of the substrate-based nomenclature of this nitrate and peptide family (now termed NPF) was proposed by Léran et al. (2014), identifying eight subfamilies in 31 genomes at the time. In Arabidopsis, an astonishing number of 60 NPF-type transporters were identified in three families within the MFS superfamily (Corratgé-Faillie and Lacombe, 2017; Niño-Gonzáles et al.,
Using a functional genomics approach, Nour-Eldin et al. (2012) demonstrated that the two plasma membrane-localized and proton gradient-driven NTR-type GLUCOSINOLATE TRANSPORTER GTR1 and GTR2, also termed NPF2.10 and NPF2.11, respectively, were required for long-distance translocation of aliphatic and indole glucosinolates, while GTR3 (NPF2.9) was characterized as an indole glucosinolate transporter (Jørgensen et al., 2017). The NPF orthologue from cassava (Manihot esculenta), MeCGTr1 (CYANOGENIC GLUCOSIDE TRANSPORTER1), was the first cyanogenic glycoside transporter to be functionally annotated (Jørgensen et al., 2017). NPF2.9 from periwinkle (Catharanthus roseus) acts in vacuolar export of the indole-terpene precursor strictosidine, pointing to a physiological role of this family in the translocation of specialized metabolites to the tonoplast (Payne et al., 2017).

Information garnered in Arabidopsis indicated that individual NPF-like proteins modulated auxin-transport, affected stress tolerance, and acted as plant hormone transporters for gibberellins, jasmonoyl isoleucine, and abscisic acid (Tal et al., 2016; Saito et al., 2015; Ishimaru et al., 2017).

In this report, we present evidence that among a number of putative candidates tested, neither anther-specific ABC-transporters nor MATE efflux carriers had any effect on the accumulation of flavonol glycosides in Arabidopsis pollen trypbine. Instead, an insertion mutant in a tapetum-specific member of the NPF-type transporter gene family resulted in a drastically reduced accumulation of flavonol sophorosides on the pollen surface. Neither the amount, nor the pattern of HCAAs or other hydrophobic trypbine compounds was affected. No ultrastructural changes of the tapetum or the pollen grains and no significant effects on pollen fecundity or pollen germination were observed.

RESULTS
Arabidopsis Pollen Deficient in a single NPF Transporter Displays Strongly Reduced Accumulation of Flavonol Sophorosides

Based on the assumption that deposition of trypbine compounds is a tightly controlled process and that any transporter associated with the deposition should be co-expressed with the genes encoding reactions of the biochemical pathways, we screened published microarrays for candidates expressed during pollen and anther development (https://www.arabidopsis.org; https://www.genevestigator.com; Klepikova et al., 2016). This initial focus was combined with information from previous reports suggesting that ABC-type (Quilichini et al., 2014b) and MATE-type transporters (Hsieh and Huang, 2007; Thompson et al., 2010b) as candidates for flavonoid and/or HCAA transport. A total of nine Arabidopsis insertion mutants, deficient in seven MATE and ABC transporters were obtained from the Arabidopsis Stock Center. Pollen was collected
from all homozygous mutants and tested for effects on the pollen phenylpropanoid pattern by high performance thin layer chromatography (HPTLC) and high performance liquid chromatography combined with UV and mass detection (HPLC-MS), and compared to their wild-type parental line (Arabidopsis accession Col-0). Among all lines, mutants in the putative MATE transporters DTX34 and TT12 were initially thought to be the most promising to affect flavonol sophoroside content. Information provided by Genevestigator (https://www.genevestigator.com) indicated that the DTX34 gene was co-expressed with the tapetum-specific gene encoding UGT79B6, catalyzing the β-1,2 linkage in flavonol diglucoside formation (Yonekura-Sakakibara et al., 2014) and also with SHT, required for amide bond formation in major Arabidopsis HCAAs (Grienenberger et al., 2009). TT12 was suggested by Hsieh and Huang (2007) to support accumulation of flavonoids in Arabidopsis anthers.

When methanolic extracts of pollen from both homozygous mutants generated during this investigation were analyzed for phenylpropanoids by HPTLC, neither the abundance of HCAAs nor the pattern of flavonol sophorosides were affected in either mutant lines. No changes were observed when we analyzed pollen from mutants lacking those ABC and MATE transporters that are reportedly expressed in Arabidopsis anthers (www.arabidopsis.org; Klepikova et al., 2016), including ABCG17, ABCG18, DTX27, DTX33, and DTX36) (Figure 2). Therefore, our focus turned to candidates from other transporter families that showed any co-expression with the key flavonoid-encoding genes identified. The SUBA4-database (Hooper et al., 2017) revealed a single gene, At5g28470, encoding an MFS-type transporter showing some weak expression correlation with UGT79B6 (http://SUBA.live). This gene was not expressed in germinating pollen upon entering the stigma and was severely down-regulated compared to non-germinated, fresh pollen (Qin et al., 2009). We termed this gene FST1 (for FLAVONOL SOPHOROSIDE TRANSPORTER 1). According to the recent nomenclature of the NPF family (Léran et al., 2014), it is identical to NPF2.8 referred to by Jørgensen et al. (2017) and Niño-Gonzáles et al. (2019).

A mutant line (SALK_027288C) was obtained from the Arabidopsis collection, from which a homozygous line was generated. Upon reverse transcription followed by quantitative PCR (RT-qPCR) analysis with RNA isolated from Arabidopsis young flower buds, no FST1 transcript was detected in the SALK_027288C line (Supplemental Figure 1). The T-DNA insertion in SALK_027288C was localized to the 3rd exon, and is thus considered a null allele. A second independent T-DNA insertion line (SALK_113184C) with a 99 bp insertion downstream in the 3´-UTR was also obtained but showed transcription of the FST1 gene (Supplemental Figure 1). This line did not show reduced flavonoid accumulation in tryphine. Therefore, this insertion line was not analyzed in further detail.
We prepared pollen extracts from fst1 and wild-type (Col-0) pollen. As shown in the HPTLC profile, homozygous mutant plants displayed a strong decrease in both flavonol sophorosides Q3GG and K3GG, whereas the HCAAs were virtually unaffected (Figure 2). The pattern of reduced flavonol sophoroside formation in this line relative to wild-type was further confirmed by HPLC-MS (Figure 3). Q3GG and K3GG eluted at retention times of 3.5 min and 4.0 min, respectively, with calculated masses of m/z 626 and m/z 610, and corresponding signals of (625.3 m/z) and (609.3 m/z), respectively, in negative ionization mode. The two major tri-substituted HCAAs eluted at 5.8 min and 6.2 min, with calculated m/z ratios of m/z 721 and m/z 735 as previously reported (Fellenberg et al., 2008). In the fst1 mutant, the level of Q3GG and K3GG was reduced by 90% compared to wild-type pollen, whereas no significant changes were evident in the case of the pattern and quantity of HCAAs (Table 1). No additional UV-absorbing peaks characteristic for phenylpropanoid-like derivatives were detected in the extracts, and the ratio of Q3GG to K3GG remained constant (Figure 3). This indicated that the compounds were not further or differentially modified in the fst1 mutant. Interestingly, virtually no difference between wild-type and the fst1 mutant was observed when total anthers were analyzed by HPLC-MS for the content of phenylpropanoids (Supplemental Figure 2). This surprising observation supports the result that only transport is affected and that there is no detectable effect or any negative feedback loop on biosynthesis. All other mutants were also investigated by HPLC-MS. Consistent with the HPTLC pattern, neither the total amount of phenylpropanoids nor the composition of individual metabolites differed in any of these lines.

In addition, we checked pollen hexane extracts for changes in lipophilic compounds including waxes and lipids. No changes in hexane-soluble metabolites could be detected by gas chromatography coupled to mass spectrometry (GC-MS). The pattern of major C-29 hydrocarbons, long chain alcohols or aldehydes in wild-type and in the fst1 mutant was virtually identical (Supplemental Figure 3).

**The fst1 Chemotype Can be Rescued by a Copy of the AtFST1 Gene**

Since we had a single mutant allele in the FST1 gene, we sought to verify the successful identification of the FST1 candidate by introducing a 2.55 kbp copy of its wild-type allele under the control of its tapetum-specific promoter in the homozygous fst1 mutant background. The T1 generation clearly showed the presence of the At5g28470 transcript (Supplemental Figure 4). Methanolic pollen extracts from several independent T2 complementation lines indicated that the accumulation of flavonol sophorosides on pollen trypine was restored to wild-type levels without affecting the accumulation of HCAAs (Figure 4). In summary, the complementation of the
homozygous SALK_027288C mutant confirmed that FST1 was indeed solely required for the accumulation of flavonol sophorosides in pollen tryphtine. No additional gene product, neither enzymatic, nor structural or regulatory, is responsible for the drastically reduced flavonol sophoroside accumulation.

**FST1 is Specifically Expressed in Arabidopsis Anthers**

The initial selection of FST1 as a potential transporter candidate was based on in silico data that suggested it was specifically expressed in Arabidopsis anthers (https://www.arabidopsis.org; https://www.genevestigator.com). These data were confirmed by RT-qPCR of total RNA isolated from various Arabidopsis organs using PROTEIN PHOSPHATASE 2A (PP2A) as a reference gene (Fellenberg et al., 2008). The FST1 expression pattern in floral organs of Arabidopsis wild-type plants is shown in Figure 5.

Relative expression levels of FST1 are high in young flower buds, especially in anthers isolated from closed flower buds (1-2 mm diameter), consistent with the data from the Arabidopsis eFP browser. These data confirm that FST1 is specifically expressed in anthers at developmental stages 6-11 (Klepikova et al., 2016). In contrast, transcription levels are already reduced in open flowers post-anthesis and are barely detected in other tissues, including carpels and siliques. The data clearly substantiate the observation that FST1 is expressed anther-specifically during the maturation of the grains. Although our HPLC-MS data already suggested that levels of flavonoids were not reduced in the tryphtine of pollen grains, as reported in a different experimental setup by Hsieh and Huang (2007), we also checked the expression pattern of TT12, encoding the Arabidopsis MATE-type cyanidin-3- O-glucoside transporter for comparison (Supplemental Figure 5). In contrast to FST1, TT12 transcripts were virtually absent from anthers but were detected in female organs, open flowers, and at the highest levels in young siliques, consistent with their role in anthocyanin deposition during early seed development (Marinova et al., 2007).

**Expression of FST1 is Restricted to the Tapetum**

Tryphine-localized flavonol sophorosides are synthesized by the tapetum in monocots and dicots (Hsieh and Huang, 2007, Li et al., 2012). If FST1 is involved into translocation of these compounds to the outer surface, then this transporter should be expressed in the tapetum. To test this hypothesis, we cloned the FST1 promoter and used it to drive GFP expression in stable, transgenic Arabidopsis lines (Figure 6).

In transgenic plants, GFP signal localized to the anthers. GFP fluorescence surrounded the pollen and the locule space and fit perfectly to the innermost cell layer of the anthers, the tapetum...
(Figures 6A-6C). GFP signal was not observed in Arabidopsis wild-type plants (Figure 6B). These results are consistent with the RT-qPCR analysis and corroborate the tapetum-specific expression of FST1. The moderate signal intensity and the low transcript levels compared to inducible biosynthetic genes like the tapetal methyltransferase TSM1 (Fellenberg et al., 2008) suggests that FST1 is constitutively expressed throughout tapetum development. We also confirmed that the accumulation of the FST1 transcript resulted in a tapetum-specific synthesis of the corresponding protein by analyzing FST1 localization, fused to GFP, in fst1 mutant lines expressing a FST1\textsubscript{pro}:FST1-GFP reporter construct (Supplemental Figure 6A and 6B). Again, a GFP signal was detected in the tapetal cell layer surrounding the pollen grains. Pollen grains were harvested from these lines: the level of Q3GG and K3GG was fully restored to wild-type levels. These data corroborate that the GFP fusion was functional and was expressed in the correct cell layer (Supplemental Figure 6C).

**FST1 Localizes to the Plasmalemma**

In previous reports, it was suggested that a transporter required for tapetal flavonoid transport was localized to the tapetosomes, which are derived from the endoplasmic reticulum (Hsieh and Huang, 2007; Li et al. 2012). The key enzymes for flavonoid biosynthesis are presumably located in the ER network (Saslowsky and Winkel-Shirley, 2001) and it was proposed that a terminal UDP-dependent glycosyl transferase involved in flavonol sophoroside formation could also be membrane associated (Vogt and Taylor, 1995; Yonekura-Sakakibara et al., 2014). To investigate the subcellular localization of FST1, GFP was fused in frame to FST1 at the C-terminal end (FST1-GFP) and the fusion protein was transiently expressed in tobacco (Nicotiana benthamiana) leaves (Figure 7). We detected GFP fluorescence at the plasma membrane of leaf epidermal cells. The GFP signal was typically very narrow and smooth in comparison to the diffuse signal seen with cytosolic, unfused GFP (Figures 6A and 6B). Co-expression with mCherry fused to the plasma membrane marker CD3-1007 (Nelson et al., 2007) showed the expected co-localization with FST1-GFP (Figures 7B and 7C). The plasmalemma localization of FST1 was further verified by plasmolysis (Figure 6D): In these experiments, reduced turgor in a hypertonic solution resulted in the partial detachment of the plasma membrane from the cell wall. When the plasmalemma became detached, both fluorescence signals (FST1-GFP and the mCherry plasma membrane marker) remained perfectly attached to the plasmolyzed epidermal protoplasts (Figures 7D and 7E). The characteristic formation of Hechtian strands during plasmolysis was observed: under conditions of concave plasmolysis, the protoplast is connected to neighboring cells by narrow cytoplasmatic strings only (Figure 7E). These data confirm that FST1 indeed is a plasma
membrane-localized putative transporter, consistent with reports for most members of the NPF family with the exception of NPF2.9 from periwinkle (Nour-Eldin et al., 2012; Niño-Gonzáles et al., 2019).

In a second, independent set of experiments, we confirmed the membrane association in mammalian cells with a FLAG-tagged FST1 construct transiently and ectopically expressed in primary human fibroblasts and also in colorectal cancer cell lines (Supplemental Figure 7). In both cases, FST1 co-localizes to cellular vesicles and to membrane structures. This experiment shows that this transporter can also be expressed in eukaryotic cell lines, as demonstrated for glucosinolate transporters functionally expressed in frog oocytes (*Xenopus laevis*) (Jørgensen et al., 2017).

**No Ultrastructural Changes are Observed in the *fst1* Tapetum**

ABCG transporter mutants involved in the exine formation show morphological abnormalities in the sporopollenin secreting tapetum or uncomplete exine formation (Choi et al., 2014; Quilichini et al., 2014b). Based on the observed reduction of the flavonol sophoroside content in *fst1* pollen extracts (Figure 3), we further analyzed the mutant line for ultrastructural changes. Direct imaging of the tapetum and pollen is challenging due to their deep localization inside anther tissues. In previous reports, transmission electron microscopy was established as a valuable method for the direct study of these tissues (Choi et al, 2011; Quilichini et al., 2014a). The *fst1* mutant was therefore compared to wild-type anthers for changes in the tapetal cell layer and microspore development by transmission electron microscopy (Figure 8).

In the case of the *fst1* mutant, both the tapetum and the locule space with its surrounding cell layers were normally developed when compared to wild-type (Figures 8A, 8B, 8E, 8F). Tapetal cells from the *fst1* mutant were also normally vacuolated and did not show signs of subcellular abnormalities. This is in contrast to mutants in genes controlling multiple aspects of tapetum development and pollen germination, like the *BRI1* (*BRASSINOSTEROID INSENSITIVE1*) receptor gene or transcription factor genes like *MALE STERILITY 1* (*MST1*) or dysfunctional *tapetum* (*Dyt1*), which not only affected sporopollenin formation and pollen development, but also result in altered deposition of tryphine metabolites (Yang et al., 2007; Ye et al., 2010; Feng et al., 2012). In this case, tapetal cells were described to be vacuolated and degraded at an early stage. In the *fst1* mutant, the polymeric structures structure of the baculae and the deposition of tryphine appeared unchanged (Figures 8E and 8F, with insert) compared to wild-type pollen grains (Figures 8B and 8C, with insert). Accumulation of starch granules indicated that wild-type pollen (C) was somewhat older than the *fst1* pollen but that this did not affect tryphine deposition. Small
oribules in the locule were visible in wild-type as well as in the *fst1* mutant (Figures 8B and 8E, with insert). No abnormalities were observed in *fst1* microspores compared to the wild-type when microgametogenesis was compared from the early bicellular stage, marked by an asymmetric cell division to the mature tricellular stage (Figures 8B and 8D). This indicated that earlier microsporogenesis, microspore release, and the following pollen mitoses were not impaired by this mutation. In summary, all results indicate that the tapetal FST1 transporter affects neither the formation of intact pollen grains including exine formation, nor modifies any morphological structures of the mature tapetum.

**FST1 Shows Flavonol 3-O-sophoroside Transport Activity**

To investigate transport activity of FST1, several C-14-labeled flavonol glycosides were synthesized by recombinant UGT79B6 and UGT78D2, whose genes were cloned and functionally expressed in *Escherichia coli* (Supplemental Figure 8). Both recombinant enzymes were of sufficient stability and purity to produce the required radiolabeled substrates without the formation of side products. UGT79B6 was used to synthesize Q3GG and K3GG from quercetin-3-O-glucoside (Q3G) and kaempferol-3-O-glucoside (K3G) with specific activities of 130 MBq/mmol and 190 MBq/mmol, respectively. Recombinant UGT78D2 enabled the production of C-14-labeled que-3-O-glc with a specific activity of 71 Mbq/mmol.

Transport studies with 14C-labeled Q3GG and K3GG revealed that *E.coli* Rosetta cells expressing *FST1* accumulated substantially more label over time when compared to non-induced or empty vector control cells (Figure 9A and 9B). Moreover, the corresponding uptake reached saturation at around 20 to 30 min. These import data suggested that FST1 was capable of transporting flavonol sophorosides. Cis-inhibitory assays were conducted to gain deeper insights into the substrate specificity of FST1 (Figure 9C). Interestingly, excess of unlabeled Q3G and of several unlabeled quercetin-diglycosides (quercetin-3-O-rhamnosido-glucoside (rutin), -gentiobioside, -arabinoglucoside, or 3,7-O-diglycoside), decreased Q3GG uptake by more than 50%. The observed import reduction was indicative of a possible competition between unlabeled flavonol glycosides and 14C-labeled Q3GG during binding and translocation. To test this hypothesis, we conducted a representative transport assay with 14C-labeled Q3G (Figure 9D). Indeed, cells expressing *FST1* showed much higher uptake rates over control. Thus, Q3G can be considered as being a true substrate of this transporter. The transport data, in sum, imply that the transporter does not have an absolute specificiticy for the β-1,2-linked sophorosides but may tolerate several closely related flavonoid glycosides. In the case of quercetin-3,7-O-diglycoside, the sugars are even attached at different positions of the flavonoid molecule. It is important to
note that excess of non-labeled UDP-glucose did not hinder the accumulation of label. Therefore, we can conclude that 1) UDP-glucose does not represent a substrate of FST1, and 2) FST1 translocates intact glycosides.

In order to check whether the proton gradient across the bacterial membrane influences transport via FST1, we analyzed the effect of protonophore addition on radiolabeled Q3GG and Q3G uptake (Supplemental Figure 9). Import into the induced cells in absence of the protonophore m-chlorophenylhydrazone (CCCP) was set to 100% and the remaining rates were normalized to this value. Disruption of the proton gradient of induced cells stimulated the uptake of Q3GG slightly and of Q3G substantially. However, CCCP addition also led to a measurable accumulation of radioactivity in the non-induced cells. While Q3G uptake into the induced cells was slightly higher, the Q3GG accumulation of non-induced cells reached values similar to those of the induced cells. Because of this fact, we were not able to clarify the transport mode of FST1.

fts1 Shows Limited Effects on Pollen Viability and Does not Impair Pollen Germination Even When Exposed to High UV Irradiance

Defects in the formation of the pollen coat have often been reported to result in reduced pollen viability. Alterations in the pollen coat composition can result in increased sensibility to water stress, as is the case in transporter mutant lines abcg9 and abcg31 (Choi et al., 2014). In the dtx35 mutant investigated by Thompson et al. (2010a), an influence of this transporter on the pollen viability was discussed. To investigate if pollen viability was reduced in the fts1 mutant, we performed a classical pollen viability assay based on staining with fluorescein diacetate. In this assay, the relative pollen viability of the wild type amounted to about 77%, compared to 68% for fts1 (Figure 10A). This 10% reduction in fts1 pollen viability showed just a weak significance (p-value = 0.018). This result clearly indicates that pollen viability appears only slightly affected by the loss of FST1 function despite the significant reduction of flavonol-3-O-sophorosides on the pollen surface.

Flavonoids have been known for a long time to be required for pollen tube germination, growth, and fertility (Mo et al., 1992; Ylstra et al., 1992), but so far have been investigated in detail only in the Solanaceae. When the fts1 mutant was tested for pollen germination, the relative in vitro germination rate of the fts1 pollen grains was comparable to that of wild-type, both about 44% after 6 h under the conditions tested (Figure 10B). No obvious changes in pollen tube length or shape was observed between wild-type and the mutant. These results are consistent with previous reports indicating that pollen development and male fertility in Arabidopsis are independent of flavonoids (Burbulis et al., 1996). To check whether the reduced levels of Q3GG
and K3GG might have any impact on pollen germination rates upon UV-treatment, we treated wild-type and fst1 pollen with high energy UV-irradiance emitted by a Xenon-lamp (Supplemental Figure 11) for 1 or 2 h. The total photon flux of 262 µmol m$^{-2}$ s$^{-1}$ in the UV- and blue light spectrum (between 300 nm to 400 nm) was much higher for a short time period when compared to the total photon flux of 1,500 µmol m$^{-2}$ s$^{-1}$ full of photosynthetic radiation PAR of a bright, sunny day (Caldwell et al., 1983; Ibdah et al., 2002) not to mention the ambient 100 µmol m$^{-2}$ s$^{-1}$ PAR in greenhouse-grown Arabidopsis plants. Subsequent pollen germination was compared to untreated pollen that had been kept in the dark for the same time period. Even under these extreme UV-stress conditions, no differences in germination rates were observed between either line (Figure 10C). This indicates that the flavonoid sophorosides deposited on the pollen surface are not primarily required for protecting the male gametophyte from high solar UV-radiation. One should consider that UV-B absorbing HCAAs and about 10% of the flavonoids are still present in the tryphine of the fst1 mutant.

**FST1 is a Member of the NPF Family of Transporters**

FST1 is a protein of 559 amino acids with a calculated molecular mass of 61.6 kDa. Based on sequence identity and the predicted 12 transmembrane helices, FST1 is clearly annotated as a member of the NPF transporter family within the MFS superfamily of proteins (Patil et al., 2019). In Arabidopsis, this family comprises 218 transmembrane proteins, classified into 22 subfamilies. FST1, termed NPF2.8 was placed into the heterogeneous NPF2 subfamily and clusters with the glucosinolate transporters NPF2.9, NPF2.10 and NPF2.11 (Jørgensen et al. 2017; Niño-Gonzáles et al., 2019) but showed only weak glucosinolate transport activity. Using the amino acid sequence of FST1 in a BLAST (Basic Local Alignment Search Tool) against the protein database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the sequence identity of NPF2.8 to the characterized glucosinolate transporters was only 38% and thus less significant than anticipated. Arabidopsis NPF2.9, NPF2.10, and NPF2.11 belong to a clade of glucosinolate transporters among the Brassicaceae in line with previous reports (Jørgensen et al. 2017). FST1 shares a conserved N-terminal EXXEK-motif, reported by Jørgensen et al. (2015; 2017) to be indispensable for function, presumably for proton coupling and the transport activity of NPF-type transporters. FST1 characterizes a different clade of NPF transporters from subfamily 2 putatively ubiquitously present in vascular plants (Figure 11, Supplemental Data Set 1). Within this clade, very high sequence identities of up to 92% characterize orthologues from the Brassicaceae, like Arabidopsis lyrata or Brassica napus. We observed a distinct separation (100% bootstrap value) between FST1-like sequences and the subclade of glucosinolate transporters (GTR1, GTR2 and
GTR3). Within the Brassicaceae, FST1-like amino acid sequences are at least 80% identical, while they show only 40% identity to sequences within the glucosinolate clade. At least one putative FST1 homologue with an identity between 50% and 60% can be detected in major eudicots including Fabales, Rosales, Solanales and Asterales to name the most prominent taxa first. Solanaceae are somewhat overrepresented due to the large sequence pool provided by the Solgenomics database (https://solgenomics.net/tools/blast/). Similar sequences were also detected in Malvales (cotton, Gossypium sp.), Sapindales (Citrus sp), and Ranunculales (e.g. Aquilegia sp.). In contrast, only rather distant sequences were obtained in the genomes of monocots like Lilium brownii, Iris japonica, and Yucca filamentosa, or Amborella trichopoda, considered a sister lineage of extant angiosperms. Among these taxa, sequence identities were ≤ 30%. No orthologues were identified from gymnosperms (Pinus pinaster) or from the moss Physcomitrella patens. Considerable sequence identities of 45% and clustering of the well-characterized and crystallized Arabidopsis nitrate transporter (At1g12110; pdb 5A2N; Parker and Newstead, 2014) in close proximity to the carboxylate transporter from Alnus glutinosa (Jeong et al., 2004) do not necessarily mean functional similarity. This indicates simply that there are many MFS-like transporters in plants and that we know very little about their sequence-based specificity and function. In summary, the conserved NPF2.8-like sequences show a uniform presence in all major eudicot families and support an essential role of the gene and the corresponding transporter in flowering plants.

DISCUSSION

The Identification of FST1 (NPF2.8) Was “Finding a Needle in a Haystack”

The almost canonical focus on ABC- and MATE-type transporters as sole candidates for the transport of specialized metabolites was questioned by Nour-Eldin et al. (2012), proposing a more general role of NPF-type transporters that was not limited to long-distance translocation of glucosinolates, but also involved in the transport of numerous specialized metabolites. Currently, more examples for such neofunctionalization of transporters that were originally considered as exclusive primary metabolite transporters have been published. For example, besides the NPF2 subfamily, members of the purine uptake permease (PUP) family were demonstrated to be specific transporters of nicotine (Hildreth et al., 2011) or benzylisoquinoline alkaloids (Dastmalchi et al., 2019). In the case of microgametogenesis and anther development, Thompson et al., (2010) overruled earlier suggestions on apoptosis and simple release of trypine compounds to the locule and the pollen exine that had postulated transport mechanisms by Lipid Transfer
Proteins (LTPs), ABC(G)- or MATE-type of transporters (Huang et al., 2013; Quilichini et al., 2010). In light of this emerging functional diversification of plant transporters, it is conceivable that a member of the NPF2 subfamily might be required for the transport of flavonoids. The reported classification of At4g28470, encoding FST1 (NPF2.8), alongside the NPF-glucosinolate transporters NPF2.9, NPF2.10, and NPF2.11, initially excluded NPF2.8 from our list of candidates, although this study already indicated that glucosinolates were not accepted as substrates by NPF2.8 (Jørgensen et al., 2017). FST1 is neither co-expressed with genes encoding enzymes required for tapetum-specific flavonoid biosynthesis nor is it up-regulated with genes encoding enzymes of sporopollenin biosynthesis like ACYL-COA SYNTHETASE 5 (ACOS5), or the cytochromes P450 CYP704B1 and CYP703A2 (Quilichini et al., 2010; Choi et al., 2011; Dobritsa et al., 2011). Mutants lacking function in the MALE STERILITY transcription factors like MS1, MS188, or MYB99, all reportedly controlling the expression of phenylpropanoid genes encoding enzymes of tapetal flavonol or sporopollenin biosynthesis, did not result in an altered expression of FST1 or any other transporter candidate (Ito et al., 2007; Wang et al., 2018; Battat et al., 2019). The experimentally confirmed anther-specific expression of FST1 and its weak co-expression with UGT79B6 pointed to its possible role as a flavonol sophoroside transporter. The new functional annotation of FST1 presented here was based on a combination of biochemical, analytical, and genetic tools. The availability of 14C-labeled physiological flavonoid glycosides for transport assays, synthesized by the recombinant glucosyltransferases UGT79B6 and UGT78D2, overcame one bottleneck, considered to be crucial for the characterization of transporters of any kind (Larsen et al., 2017; Lefèvre and Boutry, 2018). Quantitative analysis of purified pollen (Johnson-Brousseau and McCormick, 2004), membrane-specific localization of GFP fusion constructs in combination with complementation of the mutant line corroborates that FST1 (NPF2.8) is a tapetal transporter sufficient to restore flavonol glycoside levels on the pollen surface.

**Distribution of the FST1 Clade in the Plant Kingdom**

The specific expression in the tapetum and its lack of co-expression with biosynthetic genes of phenylpropanoid and exine biosynthesis indicates that the FST1 transporter developed independently of flavonol and HCAA biosynthesis. It was likely derived from an ancestral protein with a different specificity by neofunctionalization. This might have been a low affinity peptide, nitrate, or nutrient transporting ancestor during angiosperm evolution, as discussed previously for glucosinolate transporters (Jørgensen et al., 2017). In their structural analysis of the Arabidopsis nitrate transporter NRT1.1, Parker and Newstead (2014) had pointed out that some minor
changes in the binding site of this transporter were sufficient to switch from peptide to nitrate transport. The resulting and emerging multi-substrate nature of the NPF-type transporters (Corratgé-Faillie and Lacombe, 2017) confirmed by our work is certainly linked to the diversification of plant specialized metabolism in angiosperms and the requirements to not only synthesize the metabolites in specialized organs, but also to evolve mechanisms for efficient translocation. Based on a phylogenetic analysis of the NPF-type of transporters in plants, algae and animals, von Wittgenstein et al. (2014) had concluded that this large family was monophyletic, with a limited distribution of individual members in algae, 18 members in the moss Physcomitrella, followed by the reported extreme diversification in land plants. This diversification is largely unexplored in terms of precisely characterizing the domains or individual amino acids required for substrate specificity. Sequence alignment of FST1 and the Arabidopsis nitrate transporter NRT1.1 shows an identity of 22% at the amino acid level, although the overall structure of 12 transmembrane helices appears conserved. His 356 of NRT1.1, highlighted by Parker and Newstead (2014) to be required for nitrate binding, is replaced in FST1 by Thr and is also not conserved in the complete NPF2 clade. In contrast, the Thr 101 residue that is phosphorylated in NRT1.1 in response to low nitrate and switches it to a high-affinity transporter is highly conserved; phosphorylation might therefore be a mechanism in the regulation of the transporter activities of FST1 and other NPF2-type members.

FST1 defines an independent clade within the NPF2 family. Putative orthologous genes identified from tomato (Solanum lycopersicum), potato (Solanum tuberosum), and poplar (Populus trichocarpa) are expressed and localized exclusively in reproductive organs (http://bar.utoronto.ca/eplant/), as is FST1. The specific location in reproductive organs is different from the root and shoot localization associated with the NPF transporters involved in the translocation of nitrate, glucosinolates, cyanogenic glucosides, gibberellins, or abscisic acid (Taochy et al., 2015; Jørgensen et al., 2017, Tal et al., 2016). It is tempting to speculate that expression of most putative orthologues of the FST1 clade will be observed in reproductive tissues, where identical or similar anther-specific flavonoid glycosides will be the target of these types of transporters. However, the nodular expression of an orthologous NPF2 candidate in soybean (Glycine max) (http://bar.utoronto.ca/eplant/ - GLYMA.5G030300) already challenges the hypothesis that these transporters are exclusively expressed in reproductive tissues.

Based on the current data, additional MFS family members required for flavonoid or anthocyanin transport are likely present in different Arabidopsis organs but, as in the case of FST1, the apparent lack of co-expression, their functional redundancy, and potentially promiscuous substrate profiles, may limit their identification and appropriate annotation. The
plasma membrane localization of FST1 and related transporters (Nour-Eldin et al., 2012) also requires individual confirmation since tonoplast association might be more common than we currently think, as in the case of Catharanthus roseus NPF2.9 (Payne et al., 2017).

**Transport Mechanism and Specificity**

At this point, we can only speculate about the driving force of flavonoid export mediated by FST1 into the locule. NPF-type transporters are reported to act via proton symport (Nour-Eldin et al., 2012; Niño-Gonzáles et al., 2019). Usually, a proton gradient is generated by a plasma membrane H^+-ATPase to a more acidic outside, in this case the locule. A loss of function mutation of the H^+-ATPase AHA10 (also known as TT13) led to a reduction of proanthocyanin levels in Arabidopsis seeds by two orders of magnitude, presumably by impacting TT12 transporter activity (Baxter et al., 2005). This example vividly illustrates the sensitive interaction between proton gradient and transport of specialized metabolites. The plasma membrane H^+-ATPase AHA9 is expressed specifically in anthers (Houlné and Boutry, 1994) and may contribute to the acidic locular outside. In addition, the MALE GAMETOGENESIS IMPAIRED ANther (MIA) genes encoding intracellular P5-type ATPase cation pumps are also essential for cation homeostasis and male fertility (Jakobsen et al., 2005). How may FST1 catalyze flavonol glycosides export against this proton gradient? Intense biosynthesis of cytosolic phenylpropanoids during a short time of tapetum maturation confirmed substantial levels of flavonoid and HCAAs on the pollen surface (Fellenberg et al., 2008; Grienenberger et al., 2009; Yonekura-Sakakibara et al., 2014), likely resulting in a steep product gradient between the tapetum and the locule. Since the exported compounds are deposited at the pollen trypoline and thereby removed from the equilibrium, the flavonol glycoside gradient might be sufficient to drive or at least facilitate an anticipated proton-symport even against a gradient of presumably higher proton concentrations outside the plasma membrane in the locule versus the inside of the tapetal cells. Moreover, it is plausible that during evolution of the new substrate spectrum, the proton symport activity disappeared or even changed into an antiport process. Similar sequences (compared to the glucosinolate transporters) do not necessarily indicate the same transport mechanism. Chlamydial and plastidial nucleotide transporters (NTTs) function as ATP/ADP-antiporters, yet the very similar NTTs in diatoms function as symporters (Ast et al., 2009). An antiport mechanism would be consistent with the observed slight increase of transport upon addition of CCCP in experiments performed in *E. coli*. The higher proton concentration (outside versus inside) may then even inhibit import activity, while addition of CCCP would minimize this negative influence. Further experimental evidence,
including ectopic expression in Xenopus oocytes and yeast, or transport studies with plant protoplasts are required to decipher the mechanism.

Usually flavonols present at the surface of cells are not glycosylated but rather methylated or acylated (Wollenweber and Dietz, 1981). In the case of pollen flavonoids translocated into the locular fluid, glycosylation ensures water solubility and increases the stability of the aglycones that would otherwise be rapidly oxidized (Kummer et al., 2016). Since heterologously-expressed FST1 transports Q3GG, K3GG, and Q3G and since several unlabeled flavonol glycosides compete with the transport, FST1 appears quite promiscuous and apparently accepts various flavonoid mono- and diglycosides, regardless of the type of sugar attached. Even the linkage of individual sugars, $\beta$-1,2 versus $\beta$-1,4 or $\beta$-1,6 resulting in quite different confirmation of individual glycosides, appears irrelevant. This promiscuity appears to be in line with the observed multi-substrate translocation properties of NPF- and MFS-type proteins in general (Yan et al., 2015) and confirms the functional independence of the conserved flavonol-$\beta$-1,2-linked sophoroside pattern observed in many angiosperms (Fellenberg and Vogt, 2015). At some point during evolution, any type of phenylproanoid might have been translocated to the developing microgametophyte by an ancient MFS-type transporter required for nutrient supply from a secretory tapetum, also present in *A. trichopoda* (Furness and Rudall, 2001). However, no FST1 orthologue was identified from the Amborella genome database. An FST1-like transporter therefore likely developed after Amborella diverged from all extant flowering plants some 130 million years ago (Soltis and Soltis, 2004).

No effect in the *fst1* mutant was observed for HCAAs, the second class of phenylpropanoids. There was no correlation between sporopollenin formation and deposition of HCAAs on the pollen surface (Quilichini et al., 2014b). Loss of function alleles of the potential MATE candidates *DTX34* or *DTX27*, both co-expressed with the decisive *SHT* in Arabidopsis anthers, failed to show any effect on deposition of these compounds. It is tempting to speculate that some unknown NPF candidate may also be involved in the transport of these metabolites. However, in contrast to glucosinolates, cyanogenic glucosides, and the tapetal flavonols, HCAAs are quite hydrophobic and usually not glycosylated. Therefore, they may require a different type of transporter. Association of HCAAs with LTPs might be considered as a plausible alternative, and a phenolic profiling of the corresponding LTP-type III mutants should be undertaken (Huang et al., 2013). A cartoon summarizing the current status of tapetal transporters required for assembly of the developing Arabidopsis macrogametophyte exine is shown in Figure 12.

**Functional Aspects of FST1 and its Flavonol Substrates**
The apparent functional integrity of \textit{fst1} pollen is in contrast to other transporter mutants like \textit{abcg26}, which impaired pollen development and plant fecundity (Quilichini et al., 2010; Choi et al., 2011), or \textit{abcg9} and \textit{abcg31} affecting drought resistance of isolated pollen grains due to altered lipid composition (Choi et al., 2014). The \textit{fst1} mutant has no obvious phenotype beyond the absence of a single type of metabolites, with no effect on the plant performance under greenhouse conditions, including pollen fitness and plant fecundity (Figure 10). Given the relevance of flavonoids for UV-protection (Agati and Tattini, 2010), it is surprising that even under the strongly enhanced UV-irradiation in our experiment no effect on pollen germination was observed. This contrasts with experiments where growing pollen tubes from flavonoid-deficient mutants were exposed to UV-stress (Hsieh and Huang, 2007). One should keep in mind however that tri-substituted HCAAs, which show absorbance maxima in the UV-B range between 318 nm to 325 nm, were not reduced in the \textit{fst1} mutant (Figures 2 and 3). High levels of 4-coumaric acid and traces of the flavanone naringenin, covalently attached to aliphatic units, were recently identified in the sporopollenin structure of pollen from pitch pine (\textit{Pinus rigida}) by NMR, where they were covalently attached to aliphatic units (Li et al., 2019). Sporopollenin, in addition to its mechanical properties, therefore contributes to protect the male gametophyte from damaging UV-radiation. The residual 10\% flavonol glycosides that are still present in the \textit{fst1} mutant may also contribute some protective effect. The combination of polymeric and monomeric phenolics in \textit{fst1} ensures similar pollen germination rates in \textit{fst1} and wild-type, even upon elevated radiation. Double mutants where both HCAAs and flavonoids are reduced or eliminated should be compared in terms of pollen germination to define the respective contribution of sporopollenin, flavonoids, and HCAAs in photoprotection of the gametophyte.

If we assume that UV-protection of the mature pollen grain is not the major, or at least not the only function of flavonols and of FST1, what else is there? The widespread occurrence of the members of the FST1 clade in flowering plants together with the conserved presence of the specific $\beta$-1,2-linked flavonol sophorosides of the pollen surface leaves room for further interpretations and speculations as to why these metabolites and a putatively conserved transporter have been maintained during the evolution of flowering plants. Of note, the \textit{tt4} null allele in purple morning glory (\textit{Ipomoea purpurea}) is reported to be self-fertile but showed decreased fertility under heat stress (Coberly and Rausher, 2003). Interestingly, it was also found in tomato that heat stress may lead to the accumulation of flavonoids in pollen, indicating an alternative physiological function of these specialized metabolites for plant fitness (Paupière et al., 2017). The conserved pollen flavonoid pattern of both entomophilous and anemophilous species therefore may eliminate reactive oxygen species (ROS) during growth of the pollen tube
generated by abiotic stressors, such as UV-light or high temperatures (Hsieh and Huang, 2007; Muhlemann et al. 2018). This general sensitivity towards ROS might explain the observed lack of pollen germination resulting in male sterility in petunia (*Petunia hybrida*), tobacco (*Nicotiana tabacum*), and maize (*Zea mays*) (Mo et al., 1992; Ylstra et al., 1992). Arabidopsis *tt4* mutants, which carry a null allele of the chalcone synthase gene, are devoid of flavonols but are still fertile without an effect on pollen germination or pollen tube growth (Burbulis et al., 1996). The proposed loss of function of flavonols in Arabidopsis pollen (Taylor and Hepler, 1997) might theoretically be compensated by other factors, like brassinosteroids which were also shown to promote pollen tube growth in petunia and are present on the pollen grains of many species, including Arabidopsis (Ylstra et al., 1995; Vogler et al., 2014). The controlled growth under ambient greenhouse conditions without extensive ROS-mediated stress may quench any phenotype in the *fst1* and flavonoid deficient Arabidopsis mutants.

Finally, how stringent is the connection between *FST1* and tapetal flavonol glycosides? Petunia and Arabidopsis share the tapetum-specific β-1,2-linkage forming glucosyltransferases encoded by the genes *UGT79B6* (in petunia) and *UGT79B31* (in Arabidopsis) (Yonekura-Sakakibara et al., 2014; Knoch et al., 2018). UGT79-like sequences with > 50 % sequence identity can be identified for all families listed in the FST1 clade (Figure 11), although anther-specific expression is not confirmed, except for some Solanaceae. Pollen from the monocot saffron (*Crocus sativus*) reportedly contains identical flavonol-β-1,2-linked sophorosides synthesized by UGT707B1 from a different glucosyltransferase subfamily that shares only 20% sequence identity with UGT79B6 (Trapero et al., 2012). Database searches failed to identify any FST1 transporter orthologue in saffron, although this might also be due to low sequence coverage of this clade in the current datasets available for this species. Flavonol and flavone glycosides were also reported from rice anthers (Zhu et al., 2017) and several MFS-type transporters with roughly 40% sequence identity to FST1 can be found in rice and many other monocots. Their identity is higher than the identity of FST1 to the Arabidopsis NPF2.9, NPF2.10, and NPF 2.11 glucosinolate transporters. Therefore, at least functionally, orthologous transporters with somewhat different substrate specificity may be required for translocation of flavonoids to the pollen surface of all angiosperms. We hope that our investigation will initiate a more detailed view on the evolution and diversification of the FST1 clade of major facilitators from low affinity nitrate or nutrient transporters to specialized metabolites, specifically flavonoid transporters in the reproductive tissues of angiosperms.
METHODS

Plants and Growth Conditions

Arabidopsis plants were grown under long day conditions (16 h light: 8 h dark) at an average temperature of 24°C (day) and 19°C (night) in a greenhouse. Light was provided by LED-lamps (R-SB-IPB002, RHENAC Green Tec AG, Hennef, Germany), at a irradiation of 100 µmol m² s⁻¹. Both light fluence and spectral distribution were recorded and controlled by a Yeti Specbos 1211UV (Jeti, Jena, Germany). The humidity in the fully climatized greenhouse was 55-65%. All plants used here were in the Columbia-0 accession, except the tt12-1 mutant (W10067), which was isolated in the Wassilewskija (Ws2) accession.

Isolation of T-DNA Insertion Mutant Lines

All T-DNA-insertion lines were obtained from the European Arabidopsis Stock Center (http://arabidopsis.info) and were considered by the SALK institute to be homozygous lines (Alonso et al., 2001). The T-DNA insertion site of each mutant was verified by PCR on genomic DNA using primers recommended by the SALK primer design tool (http://signal.salk.edu/tdnaprimers.2.html) to confirm homozygosity. All primers used are listed in Supplemental Table 1. PCR amplification of full-length cDNA from young buds using primers specific for each mutant verified that the tested T-DNA-insertion lines were deficient for the accumulation of the transcript of each candidate gene, which is illustrated for the FST1 mutant as an example (Supplemental Figure 1). The COMT1 and UGT79B6 genes were used as controls. The tt12 mutant alleles tt12-1 (W10067) and tt12-2 (GK797D03) showed the characteristic transparent testa phenotype of the seed coat and have been described before (Kitamura et al., 2016). The dtx33 mutant (SALK_131275C) has also been reported elsewhere as dtx33-2 (Zhang et al., 2017).

Reverse-Transcription Quantitative PCR

Total RNA was extracted from different plant organs with the NucleoSpin® RNA Plus Kit (Macherey Nagel, Düren, Germany) according to the manufacturer’s instructions. Total RNA from anthers and stigma including the carpels was isolated with the NucleoSpin® RNA Plus XS Kit. Impurities of genomic DNA were removed with the included NucleoSpin® gDNA removal columns. The quantity and quality of extracted RNA was determined photometrically by the 260 nm/280 nm ratio and electrophoresis, respectively. cDNAs were reverse-transcribed from isolated total RNA with the Maxima H Minus First Strand cDNA Synthesis Kit from Thermo Scientific (Dreieich, Germany). The resulting cDNAs were used as template in qPCR. In parallel, isolated
RNA not subjected to reverse transcription was used as control. The qPCR primers were designed to overlap on an exon-intron overhang to be highly specific for the targeted cDNA; non-specific product formation was excluded by the determination of melting curves. Relative expression levels were recorded for the \textit{FST1} (At5g28470) and \textit{TT12} (At3g59030) genes. As an internal control, we used the constitutively expressed gene encoding the 65-kDa subunit of the protein phosphatase 2A, \textit{PP2A} (At1g13320). All qPCR primers are listed (Supplemental Table 2). Quantitative PCR analysis was performed using the 5x Eva Green qPCR Mix II (BIO & SELL). qPCR reactions were run on the CFX Connect™ Real-Time System (BIO-RAD) with the following protocol: denaturation (95°C for 15 min), amplification (40 cycles of 95°C for 15 sec and 60°C for 30 sec), and melting curve (95°C for 10 sec, 65°C heating up to 95°C with a heating rate of 0.05°C sec\(^{-1}\)). The resulting data were analyzed using the CFX Manager Software (BIO-RAD). The relative expression levels were measured in three independent experiments and each reaction was performed as technical triplicates.

\textbf{Collection of Pollen Material from Arabidopsis}

The collection of pollen material from 5 to 6 week-old Arabidopsis plants was performed by the vacuum-cleaner method published by Johnson-Brousseau and McCormick (2004). Two nylon mesh layers with a respective pore size of 40 \(\mu\)m and 11 \(\mu\)m (Neolab, Heidelberg, Germany) were used to obtain highly purified pollen grains (Supplemental Figure 10). Pollen was removed from the 11 \(\mu\)m mesh and directly used for analysis or was stored at \(-80^\circ\)C without any change in the metabolite pattern.

\textbf{Preparation of Methanolic Pollen Extracts}

Pollen material from wild-type and transgenic Arabidopsis lines were collected as described above by the vacuum-cleaner method (Johnson-Brousseau and McCormick, 2004). 10 mg of freshly harvested pollen was degreased with 500 \(\mu\)l hexane. The suspension was centrifuged at 4\(^\circ\)C, 15 min, 20,817 g. The hexane supernatant was removed and used for additional gas chromatography-mass spectrometry (GC-MS) analysis (Supplemental Figure 3). The pellet was completely dried at 30\(^\circ\)C for 10 min to evaporate the remaining hexane and resolved in 200 \(\mu\)l of 90\% methanol. The samples were incubated for 15 min in a sonication bath (Transsonic 460 Elma, Germany) at 35 kHz. Afterward the samples were centrifuged for 15 min at 20,817 g at 4\(^\circ\)C to separate the residual pollen material. These methanolic extracts were further directly used for liquid chromatography-mass spectroscopy (LC-MS) and high-performance thin-layer chromatography (HPTLC) analysis.
**High Performance Thin-Layer Chromatography, HPTLC**

For HPTLC analysis, 20 µl of the methanolic pollen extracts were loaded on a 20 cm x 10 cm pre-coated silica NanoAdamant UV254 thin-layer chromatography plate (Macherey-Nagel) (stationary phase). As a reference, 10 µl of Q3G (0.5 mM) and Q3GG both (each standard 0.5 mM) were spotted onto the HPTLC-plate. As a mobile phase, a system of ethylacetate, formic acid, acetic acid, and water was used in a ratio of 100:11:11:27 in a closed glass tank. The separated product mix was subsequently stained with 1% DPBA (2-aminoethyl diphenylborate). The phenylpropanoid pattern was recorded at 254 nm and 366 nm before and after derivatization with DPBA by a TLC Visualizer (CAMAG, Muttern, Switzerland).

**Liquid Chromatography-Mass Spectroscopy, HPLC-MS**

Phenylpropanoid metabolite profiles were analyzed from defatted 90% methanolic pollen extracts of Arabidopsis wild-type and T-DNA insertion lines by HPLC-MS. Pollen extracts were separated on a 5 cm RP$_{18}$ Nucleoshell column (Macherey-Nagel) using the e2695 HPLC system (Waters, Eschborn, Germany) at a flow rate of 0.6 ml min$^{-1}$, with a gradient from 10% (v/v) B in A to 45% (v/v) B in A within 14 min (with solvents 0.1% formic acid [A] and acetonitrile [B]). UV-absorbance was detected by a Waters 2998 PDA detector at 354 nm, and between 280-380 nm. The injection volume was 10 µl from a 1:20 diluted methanolic extract. Quantification was based on a standard of Q3G. Eluted compounds were detected by the Acquity QDA-mass Detector (Waters) in a range from m/z 150 to 800 Da using negative mode/15 V cone voltage. To analyze the phenylpropanoid profile of complete anthers, 200 intact anthers each from wild-type and fst1 plants were randomly picked from plants grown at the same time and harvested on the same day. The harvest was performed for three consecutive days, resulting in a single experiment measured in triplicates. Whole anthers were immediately dissolved in 200 µl 90% methanol, ultrasonicated for 10 minutes, and centrifuged for one min at 20,000 g. 10 µl of supernatant was analyzed on a 25 cm RP$_{18}$ Nucleosil column (Macherey-Nagel) with a gradient from 10% (v/v) B in A to 50% (v/v) B in A within 20 min. Data are the means of

**Gas Chromatography-Mass Spectrometry, GC-MS**

1 µl of hexane washes of pollen surface metabolites was analyzed by GC-MS for potential changes in apolar metabolites between wild-type and the fst1 mutant. The extract was analyzed by a Trace GC Ultra gas chromatograph (Thermo Scientific) coupled to ATAS Optic 3 injector and an ISQ single quadrupole mass spectrometer (Thermo Scientific) with electron impact ionization.
Chromatographic separation was performed on a ZB-5ms capillary column (30 m × 0.32 mm, Phenomenex, Aschaffenburg, Germany) using splitless injection. The injection temperature rose from 60°C to 250°C with 10°C s⁻¹ rate, and the flow rate of helium was 1 ml min⁻¹. The GC oven temperature ramp was as follows: 50°C for 1 min, 50 to 300°C with 7°C min⁻¹, 300–330°C with 20°C min⁻¹, and 330°C for 5 min. Mass spectrometry was performed at 70 eV, in a full scan mode with m/z from 50 to 450. Data were analyzed with the device-specific software Xcalibur (Thermo Scientific).

Pollen viability and pollen germination assays

Pollen viability was performed by the fluorescein diacetate (FDA) staining method (Heslop-Harrison and Heslop-Harrison, 1970). Pollen grains were dissected from anthers of 5 to 6-week old plants and suspended in a staining solution containing 10% (w/v) sucrose and 0.1 mg ml⁻¹ FDA. Pollen grains were monitored by a Nikon AZ100 microscope (Zeiss, Jena, Germany). The number of the total pollen was determined in the bright field and the living pollen grains (stained by FDA) were counted separately. The green fluorescence from fluorescein was excited at 490 nm and detected at 520 nm. Pollen viability was determined in three independent experiments and for every trial at least 600 pollen grains were counted. In the assay the number of living pollen is shown as a percentage of the total number of pollen (sum of living and dead pollen grains).

Freshly harvested pollen material collected by the vacuum cleaner method (Johnson-Brousseau and McCormick, 2004) was used to examine in vitro pollen germination. The pollen germination medium described by Li et al. (1999) was prepared as follows: 18% (w/v) sucrose, 0.01% (v/v) boric acid, 1 mM MgSO₄, 1 mM CaCl₂, 1 mM Ca(NO₃)₂ and 0.8% (w/v) low melt agar, pH 7. The medium was carefully heated and slowly cooled down. Collected pollen grains were dispensed in the medium on an object slide and incubated for 6 hours in a humid chamber at room temperature. The germination rate was examined by counting the total number of mature pollen grains and germinating pollen under a light microscope. At least 300 pollen grains were counted per trial and three trials were performed for each genotype.

UV-irradiation of freshly harvested wild-type and fst1 pollen grains was performed with a high energy Philips Xenon HPR400 lamp for 1 or 2 h. Pollen, collected on a 10 µm filter, was positioned at a distance of 60 cm from the light source. Total irradiance of 55 W m⁻², from 300 nm to 780 nm was recorded by a Yeti Specbos 1211UV. Photon flux between 315 nm and 780 nm was 532 µmol m⁻² s⁻¹ and was 17.8 µmol m⁻² s⁻¹ between 315 nm - 340 nm. (for spectral detail see Supplemental Figure 11). At least 300 pollen grains were counted from two large pools of wild-
type and fst1 plants grown under identical greenhouse conditions and four replicates were analyzed.

**Promoter-GFP Expression Assay**

For the FST1 promoter assays, a GFP reporter construct was cloned using the Golden Gate cloning method (Engler et al., 2014). Full length GFP cDNA was cloned under the control of a 2.3 kbp FST1 promoter fragment in the reporter construct FST1_{pro}:GFP. The final level 2 Golden Gate construct was transformed into *Agrobacterium tumefaciens* (Agrobacterium) strain GV3101 and then introduced into Arabidopsis wild-type plants accession Col-0 using the floral dipping method described by Clough and Bent (1998). To investigate the organ-specific GFP signal, anthers from five independent transgenic T1 plants were isolated using forceps and observed by Confocal Laser Scanning Microscopy (CLSM) with a LSM780 (Zeiss). The microscope setting for the GFP signal were 488 nm for excitation by an Argon diode laser and the emission was recorded at 497 nm - 542 nm, and chlorophyll autofluorescence was detected at 651 nm – 717 nm. Images obtained at the two spectral settings were overlaid together with the transmission image.

**Generation of Complementation Lines**

A 2.55 kbp fragment including the full genomic FST1 (At5g28470) coding sequence was amplified in several fragments from genomic DNA of young Arabidopsis (Col-0) wild-type flower buds using the Phusion high fidelity DNA polymerase (Thermo Scientific) and assembled into level zero by Golden Gate Cloning as described. To control the expression of the genomic FST1 coding sequence, 2,245 bp of FST1 promoter region upstream of the ATG start codon was cloned, which was also used for the FST1 promoter GFP expression assay. Further, a 719 bp FST1 terminator was cloned. All cloning cassettes were assembled into the level 2 vector pAGM37443, generating the FST1_{pro}:FST1:FST1_{term} complementation construct. Additionally, a FST1_{pro}:FST1-GFP construct was cloned were genomic FST1 is in frame with GFP for an N-terminal GFP fusion reporter. All primer pairs are listed (Supplemental Table 2). The binary vectors were transformed into Agrobacterium GV3101 and finally transformed into the fst1 mutant (SALK_027288C) by the floral dipping method (Clough and Bent, 1998). The transcription of the FST1 complementation constructs was verified by qPCR (Supplemental Figure 4) from cDNA of young buds from individual T1 plants.
Subcellular Localization of FST1

To investigate the subcellular localization of FST1 in plant cells, the reporter construct \(35S_{pro}:FST1\)-GFP was cloned by the Golden Gate Cloning method (using the genomic sequence of \(FST1\) with an in frame \(GFP\) coding sequence cloned at its 3’ end and placed under the control of a 35S promoter in a level 1 vector (for primers see Supplemental Table 2). The construct was transformed into Agrobacterium strain GV3101:pMP90 (Leuzinger et al., 2013) before transient expression in \(Nicotiana benthamiana\) leaves by infiltration of leaves. For co-localization studies, the mCherry-tagged plasma membrane marker CD1007 (Nelson et al., 2007) was co-infiltrated. Images were taken 3 d after infiltration by CLSM with a LSM780 laser scanning confocal microscope (Zeiss). Upon excitation of the 488 nm GFP fluorescence, chlorophyll autofluorescence, and excitation of 561 nm for the mCherry tagged plasma membrane marker CD1007, signals were recorded at 497 nm – 551 nm (GFP), 651 nm – 717 nm (chlorophyll) and 589 nm – 651 nm (mCherry). Images obtained at the three spectral settings were overlaid.

Additionally, localization of FST1-GFP during plasmolysis was recorded by CLSM with a LSM780 (Zeiss) using the spectral settings described above. The plasmolysis of epidermal leaf cells was induced by putting leaf discs in hypertonic medium containing final concentrations of NaCl from 2 M NaCl to 5 M NaCl. To visualize the characteristic movement of the plasma membrane, the loss of turgor, and the formation of the Hechtian strands during the plasmolysis, a time series was recorded over 16 min with an image rate of 1 image every 50 sec, available upon request as a short movie.

For the subcellular localization of FST1 in anthers, inflorescences at different stages from transgenic complementation lines expressing the \(35S_{pro}:FST1\)-GFP construct were dissected. The anthers were isolated and the GFP signal was observed using CLSM with a LSM700 (Zeiss) with spectral settings for GFP (excitation, 488 nm; emission 520 nm). For a better visualization of the anther cells, chlorophyll autofluorescence was also monitored.

Ectopic FST1 Expression in Human Cell Lines

To investigate the ectopic expression in a eukaryotic system, we cloned the \(FST1\) full-length cDNA into pCS2+ (RZPD) at the EcoRI and XbaI restriction sites. A FLAG epitope tag was added to the C-terminus of the encoded protein using the reverse cloning primer FST1FLAGpCS2+rw for further immunolocalization studies. The colorectal cancer cell line HCT-8 was cultured at 37°C and 5% CO\(_2\) in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% (v/v) fetal calf serum (FCS) and antibiotic-antimycotic (Thermo Fisher Scientific). Primary human fibroblasts were cultured at 37°C and 5% CO\(_2\) in Dulbecco’s Modified Eagle Medium
(DMEM)/Hams F12 supplemented with 8% (v/v) FCS, 2% Ultroser G (PALL Life Sciences) and 2 mM L-glutamine. Transfections were carried out using Lipofectamine2000 (Thermo Fisher Scientific). 2 µg DNA of the eukaryotic FST1 pCS2+ expression vector was added to 1 x 10^5 cells (12-well plates) according to the manufacturer’s instructions. For immunofluorescence, transfected cells were fixed with 3.7% formaldehyde in PBS for 15 min, permeabilized with 0.2% (v/v) Triton X-100 in PBS for 10 min and blocked in 10% FCS/1% bovine serum albumin/0.05% Triton X-100 (v/v) in PBS for 30 min. Anti-FLAG M2 antibody (from Sigma-Aldrich) was diluted 1:200 and incubated for 1 h at room temperature. Alexa Fluor488-conjugated secondary antibody was diluted 1:200 and applied for 1 h at room temperature. F-actin was visualized with Atto546 Phalloidin (Thermo Fisher Scientific). DNA was stained with DAPI. Samples were covered with ProLong Gold antifade reagent (Life Technologies) and imaged using an Apotome-containing Axio Observer.Z1 equipped with a 63x oil objective and a monochrome Axiocam MRm camera (Zeiss). Representative images are shown.

Production of 14C-Labeled Flavonol-3-O-Sophorosides

The 14C-labeled flavonol-3-O-glycosides used as substrates in the uptake assays were enzymatically synthesized. The flavonol-specific glycosyltransferase UGT79B6 encoded by At5g54010 (Yonekura-Sakakibara et al., 2014) was used to produce labeled Q3GG and K3GG from Q3G (Roth, Karlsruhe, Germany). UGT78D2, encoded by At5g17050 (Lim et al., 2004), was used to produce 14C-labeled Q3G from quercetin (Roth). The UGT79B6 sequence was codon-optimized for heterologous expression in E. coli (Eurofins, Hamburg, Germany). Full length sequences of UGT79B6 and UGT78D2 were cloned into pETDuet-1 (Merck, Darmstadt, Germany) and pQE70 (Qiagen, Hilden, Germany), respectively. The expression constructs were transformed into compatible E. coli strains Rosetta (DE3) pLys (Novagen) and M15[Rep4] (Qiagen), respectively, for recombinant protein expression. Cells were grown in 1.2 L terrific broth medium, until the OD reached 0.4, at 20°C (UGT79B6) or 30°C (UGT78D2). Heterologous protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM to the culture. The cultures were further cultivated for 16 h at 20°C (UGT79B6) or 30°C (UGT78D2), and cells were collected by centrifugation (10,000 g, 15 min, 4°C), suspended in a buffer containing 50 mM potassium phosphate (KPi), pH 7.5 and 10% (v/v) glycerol. Cells were disrupted as reported (Fellenberg et al., 2008). Partial purification of recombinant enzymes was performed by Ni-NTA-affinity chromatography on a 5 ml Protino® Ni-NTA Column (Machery-Nagel). Subsequently, the partially purified UGTs were desalted using a
Sephadex G-25M PD-10 Column (GE Healthcare, UK). Fractions were monitored for proteins by SDS-PAGE and western blot analysis (Supplemental Figure 8).

Partially purified recombinant UGTs were used to produce radiolabeled glycosides at 30°C in 5 ml preparative assays. Assays included 200 - 400 µg of enzyme, 10 mM KPi buffer (pH 7.5), and final concentrations of 200 µM ascorbic acid, 100 µM of unlabeled sugar donor UDP-glucose and 50 µM of the individual sugar acceptor quercetin (UGT78D2) and Q3G or K3G (UGT79B6), respectively. For the production of 14C-labeled flavonol-3-O-glycosides, 90 kBq 14C-labeled UDP-glucose (300mCi/mmol, Hartmann Analytics, Braunschweig, Germany) was included in the assay. In parallel, a cold reaction was performed to monitor the success of the labeling by LC-MS and TLC without inclusion of radiolabeled UDP-glucose until the total substrate was converted into product (6 hours). In order to optimize the specific activity of radiolabeled substrates, in each case, only a 2-fold excess of UDP-glucose concentrations compared to the acceptor flavonol was used although the reaction would have run considerably faster with a larger excess of the sugar donor. Labeled and non-labeled products were purified by solid phase extraction using an Oasis® HLB3cc column (Waters, Milford, USA). 98% purity was achieved in the case Q3GG and K3GG and 89% purity in the case of Q3G. The incorporated radioactivity of all fractions was measured using a Hidex 300 SL liquid scintillation counter (Hidex, Mainz, Germany). Enzymatically produced flavonol glucosides were stable for months when stored frozen at -20°C.

Heterologous Expression in E. coli and Uptake Assays

The full-length FST1 (At5g28470) cDNA was obtained by PCR using the Phusion high fidelity DNA polymerase (Thermo Scientific), using cDNA from young Arabidopsis (Col-0) wild-type buds as template and the primers AtFST1fw and AtFST1rw (Supplemental Table 2). The FST1 fragment was ligated by T4 DNA ligase (Thermo Scientific) into the pET16b expression vector using the XhoI restriction site according to the manufacturers’ instruction (Merck, Darmstadt, Germany). The inserted sequence was confirmed by sequencing (MWG Eurofins, Europe). For recombinant protein synthesis, the expression vector construct was transformed into E. coli strain Rosetta II (DE3) pLys (Merck). The cells were grown at 37°C under aerobic conditions to an optical density (OD600) of 0.5, and then the heterologous expression was induced by adding 0.8 mM IPTG. The cells were harvested 1 h later by centrifugation (5 min, 5,000 g, 4°C). Non-induced cells harboring the expression construct as well as induced cells harboring the empty vector pET16b were used as controls. Radioactive uptake assays were performed as described by Klein et al. (2018) with slight modifications. For the analysis of the transport activity of the recombinant FST1 protein, E. coli cells were directly suspended in 50 mM KPi buffer (pH 7.0) adjusted to an
OD$_{600}$ of 5.0. 100 µl of cells were mixed in a 1:1 ratio with 100 µl transport medium and KPi buffer supplemented with $^{14}$C-labeled flavonol sophorosides to a final concentration of 10 µM. The samples were incubated at 30°C and transport was terminated after the indicated time by removal of the external substrate via vacuum infiltration and washing with KPi buffer (3 times 4 ml). The accumulated radioactivity in the cell samples on the filters was measured by scintillation counting (Beckman LSC6500). All unlabeled substrates used in competition experiments were applied at a final concentration of 100 µM. The ionophore carbonyl cyanide m-chlorophenyl hydrazine (CCCP) was diluted to 100 µM in 50% (v/v) dimethylsulphoxide (DMSO) shortly before adding to the transport assays.

**Sequence Alignment and Phylogenetic Analysis**

The complete set of protein sequences included in the dendrogram of Figure 11 was obtained by BLAST (Basic Local Alignment Search Tool) using the FST1 protein sequence as a query against the databases and selecting the sequences with highest sequence identity. Protein sequences were aligned, with accession numbers included directly in the phylogenetic tree constructed by MegAlign (DNA Star, Madison, Wi, USA) based on the Clustal W algorithm. For the cladogram a bootstrap analysis was performed with 1000 replicates. All proteins sequences and accession numbers are given in Supplemental Data Set 1.

**Electron Microscopy**

Anthers were fixed with 3% glutaraldehyde (Sigma) in 0.1 M sodium cacodylate buffer pH 7.2 (SCB) for 4 h, washed in SCB, post-fixed for 1 h with 1% osmiumtetroxide (Carl Roth) in SCB, dehydrated in a graded series of ethanol and embedded in epoxy resin according to Spurr (1969). Ultrathin sections (80 nm) were observed with an EM 900 (Zeiss) transmission electron microscope (acceleration voltage 80kV). Electron micrographs were taken with a slow scan camera (Variospeed SSCCD camera SM-1k-120, TRS, (Moorenweis, Germany).

**DATA AVAILABILITY**

NCBI accession numbers and gene identifiers are listed in the text and Supplemental data. Sequence information is summarized in Supplemental Data Set 1. All primary data are summarized in the public repository RADAR (www.radar-service.eu) under the following DOI: 10.22000/276. Raw data of the HPLC-runs were stored and secured on local IPB-hard drives and can be accessed from the corresponding author (T.V.).
SUPPLEMENTAL DATA

**Supplemental Figure 1.** RT-PCR analysis of two T-DNA insertions lines in the At5g28470 gene.

**Supplemental Figure 2.** HPLC-profile of Arabidopsis wild-type and fst1 anthers.

**Supplemental Figure 3.** GC-MS profile of wild-type (A) and fst1 (B) Arabidopsis pollen hexane extracts.

**Supplemental Figure 4.** Expression of the FST1 complementation construct in Arabidopsis T1 plants.

**Supplemental Figure 5.** Organ-specific expression profile of TT12 in Arabidopsis thaliana.

**Supplemental Figure 6.** FST1 localization in anther tissues and functional complementation of the fst1 mutant expressing the FST1pro:FST1-GFP reporter construct.

**Supplemental Figure 7.** FST1 localization in primary human fibroblasts and HCT8 cells.

**Supplemental Figure 8.** Partial purification of UGT79B6 and UGT78D2.

**Supplemental Figure 9.** Effect of protonophore addition on the in vitro transport of flavonol glycosides.

**Supplemental Figure 10.** Arabidopsis pollen grains purified by the vacuum cleaner-method (Johnson-Brosseau and McCormick, 2004).

**Supplemental Figure 11.** Original spectral data of the Xenon 400 XR bulb.

**Supplemental Table 1.** Primers used for genotyping and confirmation of homozygous lines.

**Supplemental Table 2.** PCR and sequencing primers.

**Supplemental Data Set 1.** Sequences and accession numbers for all proteins used to build the cladogram.

**Supplemental Data Set 2.** Sequence alignment for all proteins used to build the cladogram.

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AUTHOR CONTRIBUTIONS

S. G. performed research, designed the study, analyzed data, and wrote the paper; S. M. contributed analytical tools; G.H. performed research; I.H. performed and designed research;
H.E.K. analyzed data; A.V. performed research; T.H. designed research; T.V. performed and designed research and wrote the paper.

Table 1. Quantification of quercetin- and kaempferol 3-O-sophorosides (Q3GG and K3GG) in methanolic extracts of wild-type and fst1 pollen.
Quantification is based on UV$_{354}$ nm-absorption of commercially available Q3G and K3G with identical UV-absorbance characteristics compared to the corresponding flavonol 3-O-sophorosides. Pollen was collected from three independent experiments each.

| Compound                     | wild type [pmol/mg pollen] | fst1 [pmol/mg pollen] |
|------------------------------|----------------------------|-----------------------|
| Quercetin-3-O-sophoroside    | 383 +/- 38                 | 41 +/- 7              |
| Kaempferol-3-O-sophoroside   | 844 +/- 51                 | 93 +/- 6              |

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Figure 1. Structures of the pollen-specific phenylpropanoids in Arabidopsis.

(A) Flavonol-3-O-diglucosides: R1 = H, kaempferol-3-O-sophoroside (K3GG) and R1 = OH, quercetin-3-O-sophoroside (Q3GG). The characteristic β-1,2 glycosidic linkage of the sophorose group is marked.

(B) Structure of the dominant HCAA-conjugate N',N'-bis-(5-hydroxyferuloyl)-N''-sinapoylspermidine.
Figure 2. The phenylpropanoid pattern of Arabidopsis pollen grains from different T-DNA-insertion lines as analyzed by HPTLC. Methanolic extracts of pollen from wild-type and mutant lines were analyzed by high-performance thin-layer chromatography (HPTLC). WT, wild-type (Col-0); dtx27 (At5g65380), dtx33 (At1g47530), dtx34-1/2 (At4g00350) (two independent lines), dtx36 (At1g11670), abcg17 (At3g55100), abcg18 (At3g55110) fst1 (At5g28470), tt12-1/2 (At3g59030), standards: quercetin-3-O-glucoside (Q3G) and quercetin-3-O-sophoroside (Que3GG). Kaempferol-3-O-sophoroside (K3GG), Q3GG and as well as hydroxycinnamic acid amides (HCAAs) are marked. Individual lanes represent methanolic extracts from 1 mg of pollen grains. Pictures were taken under UV light illumination at 254 nm.
Figure 3. HPLC-MS-analysis of pollen from the *fst1* mutant.
Phenylpropanoid composition of methanolic pollen extracts from (A) Arabidopsis wild-type (Col-0) and (B) the *fst1* mutant in comparison to (C) a flavonoid standard of (1) quercetin-3-O-sophoroside recorded at 354 nm. The flavonols (1) quercetin-3-O-sophoroside, Q3GG (625.3 m/z) and (2) kaempferol-3-O-sophoroside, K3GG (609.3 m/z) are highlighted in grey. The major hydroxycinnamic acid amides (HCAAs) with 720.4 m/z (3) and 734.4 m/z (4) (structure in Figure 1B) are unmarked.
Figure 4. HPLC-MS analysis of wild-type, fst1 mutant and complementation lines. Phenylpropanoid composition of methanolic pollen extracts from (WT) Arabidopsis wild-type (Col-0) and fst1 (fst1), in comparison to three (out of eight individual lines, see Supplemental Figure 4) independent fst1 / FST1 complementation lines (A to C). The flavonol-3-O-diglycosides are underlined in grey (1) quercetin-3-O-sophoroside, Q3GG (625.3 m/z) and (2) kaempferol-3-O-sophoroside, K3GG (609.3 m/z) are restored to wild type levels. Major HCAAs (3 and 4) are virtually unchanged in all lines.
Figure 5. Organ-specific transcription profile of FST1 in Arabidopsis.
qPCR analysis of At5g28470, encoding FST1, in floral organs of the Arabidopsis wild type (Col-0). The expression of FST1 was normalized PP2A (At1g13320). Data are mean relative expression values ± se of three independent experiments in triplicates (n = 3).
Figure 6. Tapetal expression of the FST1 promoter
Representative confocal fluorescence images of anthers from stable Arabidopsis lines expressing the FST1pro::GFP reporter construct (A) in comparison to untransformed wild type (Col-0) (B). Bars = 100 µm. (C) Enlarged confocal image of the tapetum-specific GFP signal in FST1pro::GFP anthers. Shown are the GFP channel (GFP), chlorophyll autofluorescence, the transmission image (TM) and the merged image of all three channels (merge). Bar = 50 µm.
Figure 7. Subcellular localization of FST1 in epidermal cells
Representative confocal fluorescence images of GFP fluorescence in epidermal cells of *Nicotiana benthamiana* leaves expressing 35Spro:FST1-GFP (A) and 35Spro:GFP control (B). Bars = 200 µm. PM: plasma membrane marker; plastids: chlorophyll autofluorescence.
(C) Enlarged image of epidermal cells, the arrowheads indicate plasma membrane localization, the cell wall appears as dark interspace. Bars = 5 µm.
(D) Plasmolysis of the epidermal leaf cells in hypertonic medium after 10 min. Bars = 50 µm.
(E) Detailed image of a single plasmolyzed cell, which shows the characteristic concave detachment of the cytoplasm from the cell wall, the formation of the Hechtchian strands (indicated by arrows). Bar = 10 µm.
Figure 8. Identical morphology of microspore/pollen between wildtype fst1 mutants by transmission electron microscopy.

(A to C) Wild-type Col-0.
(D to F) fst1 mutant.

(A), (D): Overview of locules (l) covered by the tapetum (t) and the anther wall (a) during microspore development; scale bars 10 μm.

(B), (E): Morphology of tapetal cells during microgametogenesis; bars 2 μm.

(C), (F): Pollen grains at the tricellular stage. The large vacuole is greatly decreased and the vegetative nucleus (v) and the sperm cells (arrow) are in a central position; scale bars 5 μm. The inserts in (C) and (F) show baculae (arrows) filled with tryphine; scale bars 1 μm. Wild-type pollen (C) shown is slightly older than fst1 pollen (F), as seen by inclusion of starch granules, but does not affect the structure of baculae or deposition of tryphine.
Figure 9. FST1 transports flavonol glycosides in vitro.

Time course of the uptake of 10 µM ^14^C-labeled quercetin-3-O-sophoroside (Q3GG) (A) or kaempferol-3-O-sophoroside (K3GG) (B) by Rosetta II cells producing FST1 (black squares). Non-induced cells (grey squares) or induced cells with the empty vector (white circles) represent the control.

(C) Cis-inhibitory assays. Transport medium contained 10 µM ^14^C-labeled Q3GG without supplement (−) or with 10 x excess (100 µM) unlabeled que-3-O-gentiobioside (Q-3-Gent); que-3-O-glucoside (Q3G); que-3-O-arabinogluicoside (Q-3-Ara-Glc); que-3,7-O-diglucoside (Q-3,7-diGlc), que-3-O-rhamnosido-glucoside (rutin) and UDP-glucose (UDP-Glc). Transport without supplement was set to 100% and the remaining rates were normalized according to this value.

(D) Time course of the in vitro uptake of 10 µM ^14^C-labeled quercetin-3-O-glucoside (Q3Glc). Data represent the mean of at least three independent replicates. Standard errors are given.
Figure 10. Classical pollen assays of wild-type and FST1-deficient pollen grains.
(A) Viability rate of pollen grains from wild-type Col-0 and fst1 mutant assayed with fluorescein diacetate. Four replicates were analyzed, for each trial 600 pollen grains were counted to determine the pollen viability.
(B) Pollen tube in vitro germination rate of Col-0 and fst1 pollen grains after 6 h.
(C) Pollen germination with and without UV-treatment for 2 h. Percentage of pollen germination is shown and wild-type Col-0 results are set to 100%. Triplicates were measured and at least 300 pollen grains were counted per trial. Shown are the mean relative values ± se from independent experiments (n = 4; n = 3). Significance analysis was performed by t test, (* means P < 0.05 and n.s. means no significance).
Figure 11. FST1 (NPF2.8) defines a new subclade of transporters among the NPF family. A bootstrapped cladogram of FST1 (NPF2.8) most similar putative orthologues from various plant families across the plant kingdom determined from a BLAST-search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) based on sequence comparison to non-redundant protein sequences of individual taxa aligned by MegAlign and bootstrapped. Alignments illustrate two subclades of NPF-type transporters characterized by at least 50% sequence identity: the FST1 clade and the glucosinolate transporter subclade (functionally characterized members are in bold). Transporters of Solanum spec. within the FST1 clade and poplar (Populus trichocarpa) are marked for their reported expression in reproductive organs. Both show > 55% sequence identity to FST1. The crystallized Arabidopsis nitrate transporter (pdb: 5A2N) NRT1.1/NPF6.3 (Parker and Newstead, 2014) is marked in bold. It clusters close to the dicarboxylate transporter from alder (Jeong et al., 2004) and many NPF-type proteins from monocots and dicots with roughly 30% sequence identity, but unknown function. NCBI or TAIR-accession numbers of all protein sequences are indicated. All sequences are listed in Supplemental Data Set 1.
Figure 12. Schematic overview of currently confirmed and putative transporters for exine compounds in Arabidopsis tapetal cells. 
Schematic tapetal cell imbedded in a TEM image of the Arabidopsis anther wall consisting of the endothecium (Ed), the middle layer (Ml) and the tapetum (T), surrounding the locule space (L) which contains the microgametophyte (M) covered by the sculptured exine (E). Three transporter-based routes, which contribute to exine formation, are shown. ABCG31 and ABCG9 paths are required for translocation of sterol glycosides (brown path). ABCG26 is required for polyketide precursor deposition for sporopollenin formation (black path). FST1 (NPF2.8) is required for deposition of flavonol sophorosides for tryphine formation (red path). ER-derived tapetosomes may contribute to flavonol glycoside formation (Hsieh and Huang, 2007). The transport mechanism of the HCAAs is still unknown. The contribution of lipid transfer proteins (LTPs) or small vesicle-based translocation (blue path) is speculative and remains to be proven.
The Tapetal Major Facilitator NPF2.8 is Required for Accumulation of Flavonol Glycosides on the Pollen Surface in Arabidopsis thaliana

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