RESEARCH PAPER

An *Arabidopsis* flavonoid transporter is required for anther dehiscence and pollen development

Elinor P. Thompson, Christopher Wilkins, Vadim Demidchik[†], Julia M. Davies and Beverley J. Glover*

Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, UK

[†] Present address: Department of Biological Sciences, University of Essex, Colchester, UK.

* To whom correspondence should be addressed. E-mail: bjg26@cam.ac.uk

Abstract

FLOWER FLAVONOID TRANSPORTER (FFT) encodes a multidrug and toxin efflux family transporter in *Arabidopsis thaliana*. FFT (AtDTX35) is highly transcribed in floral tissues, the transcript being localized to epidermal guard cells, including those of the anthers, stigma, siliques and nectaries. Mutant analysis demonstrates that the absence of FFT transcript affects flavonoid levels in the plant and that the altered flavonoid metabolism has wide-ranging consequences. Root growth, seed development and germination, and pollen development, release and viability are all affected. Spectrometry of mutant versus wild-type flowers shows altered levels of a glycosylated flavonol whereas anthocyanin seems unlikely to be the substrate as previously speculated. Thus, as well as adding FFT to the incompletely described flavonoid transport network, it is found that correct reproductive development in *Arabidopsis* is perturbed when this particular transporter is missing.

Key words: Anther, fertility, flavonoid, MATE transporter, nectary, pollen.

Introduction

Flavonoids are phenolic secondary metabolites. Among the molecules sharing the same chemical backbone are three major classes, the pink and purple anthocyanins, the pale yellow flavonols, and the proanthocyanidins (PAs) of seed-coats (Fig. 1). Various '*tt*' (*transparent testa*) mutants have provided a route for the study of flavonoid metabolic pathways, particularly the stages involving PAs. When these condensed tannins are perturbed in *Arabidopsis thaliana*, the seedcoat is paler than normal, giving rise to the name of the mutant series (Koornneef, 1990).

The presence of anthocyanins in *Arabidopsis* vegetative tissues is easily observed since stress often results in purple-pigmented rosette leaves. The main flavonoids in *Arabidopsis* leaves, however, are normally the paler-coloured kaempferol and quercitin glycosides (Pelletier *et al.*, 1999). Most of the aglycone flavonols become glycosylated in the C-3 and C-7 positions, and the principal sugar substitutions are glucose and rhamnose (Kerhoas *et al.*, 2006). Literature reports identify kaempferol as the origin of most vegetative flavonol glycosides, and kaempferol-3-*O*-rhamnoside-7-*O*-

rhamnoside as a major component (Routaboul *et al.*, 2006, and references therein). Flowers have also been reported to contain predominantly kaempferol compounds, but quercitin derivatives are known to accumulate in stamens (Shirley *et al.*, 1995; Burbulis *et al.*, 1996; Routaboul *et al.*, 2006). Among the quercitin glycosides identified in inflorescence tissue are quercetin–glucoside–rhamnoside, quercetin–glucoside–rhamnoside–rhamnoside, and quercetin–rhamnoside–rhamnoside (Jones *et al.*, 2003). Numerous others, including flavonol dimers and oligomers, have been identified in *Arabidopsis* seeds (Routaboul *et al.*, 2006).

The roles of flavonoids in plants range from protection from ultraviolet (UV) light to the pigmentation of flowers to attract pollinators (Shirley, 2006). Evidence is also accumulating that these compounds are involved in regulating auxin transport since the flavonols quercitin and kaempferol, in particular, can displace synthetic auxin transport inhibitors *in vitro* (Jacobs and Rubery, 1998). Indeed, some flavonoid mutants have phenotypes suggestive of altered auxin transport (Shirley *et al.*, 1995; Peer *et al.*, 2001, 2004;
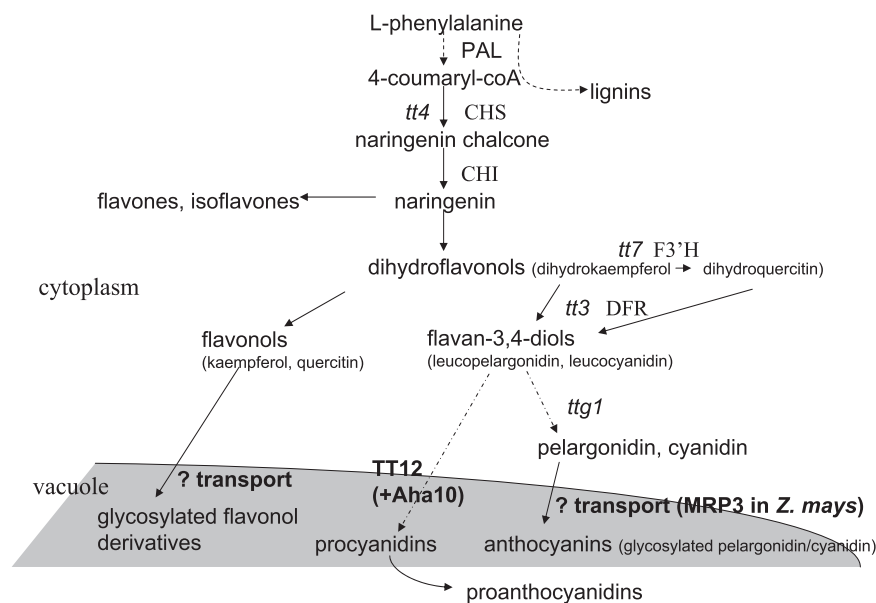
Fig. 1. Flavonoid biosynthesis pathways and transport steps, in outline. Enzymes and mutants are marked if mentioned in the text. PAL, phenylalanine ammonia lyase; CHS, chalcone synthase; CHI, chalcone isomerase; F3'H, flavonoid 3' hydroxylase; DFR, dihydroflavonol 4-reductase; tt, transparent testa.

Peer and Murphy, 2007). For example, the *Arabidopsis* chalcone synthase (CHS) *tt4* mutant has delayed gravitropism and, although flavonoids are classically thought of as localized in the vacuole, the root tissue flavonols involved here may be cytosolic (Buer and Muday, 2004). Meanwhile, control of bud outgrowth is affected in the *Arabidopsis max1* mutant, in which this regulator of flavonoid pathway genes is missing and which consequently has altered levels of auxin transporters (Lazar and Goodman, 2006).

Given the many roles of flavonoids, their transport from the site of synthesis (primarily the cytosol) to the correct cell compartment, and between tissues (Buer *et al.*, 2007), is obviously important and complex. Recently, Kitamura (2006) and Marinova *et al.* (2007) have summarized the known steps for flavonoid synthesis and transport in *Arabidopsis*, clearly showing the gaps in our knowledge (Fig. 1): whereas flavonoid biosynthesis is well understood, the proteins transporting anthocyanins and glycosylated quercitins and kaempferols remain little known. Characterization of *tt* mutants, however, has provided some insight into flavonoid transport. The product of *TT12* (At3g59030) in *Arabidopsis* is a vacuolar flavonoid/H$^+$ antiporter expressed in seeds (Debeaujon *et al.*, 2001, 2003; Marinova *et al.*, 2007). TT12 is a member of the <u>m</u>ultidrug <u>a</u>nd <u>t</u>oxin <u>e</u>fflux (MATE) transporter family, a group of proteins thought likely to fill some of the other missing steps (Kitamura, 2006; Fig. 1). These eukaryotic and prokaryotic transporters are one of the five groups that together comprise the multidrug transporter superfamily (MTS). MATE proteins typically contain 12 transmembrane helices, and are characterized by the absence of the signature sequences found in their four sister groups—the ATP-binding cassette, major facilitator, multidrug resistance (MRP), and resistance–nodulation–cell division families

(Omote *et al.*, 2006). *Arabidopsis* MATE proteins have been found in the tonoplast (Debeaujon *et al.*, 2001, 2003; Jaquinod *et al.*, 2007; Marinova *et al.*, 2007), as was an acylated anthocyanin transporter in recent work in grape (Gomez *et al.*, 2009), but one family member from lupin was identified in the plasma membrane (Uhde-Stone *et al.*, 2005). The sequestration of anthocyanin and other flavonoids in the vacuole is already linked to other MTS members: Goodman *et al.* (2004), using antisense technology to investigate a *Zea mays* (maize) MRP (ZmMRP3), found perturbed anthocyanin accumulation. *Arabidopsis* MRP may also be responsible for vacuolar uptake of glutathione–anthocyanin conjugates (Kitamura, 2006).

In *Arabidopsis*, there is a large group of >50 MATE proteins (Kitamura, 2006) of which only a few have been characterized. There are two subgroups, the smaller one being most similar to the bacterial MATE proteins. One of these has been studied: FRD3 (ferric reductase defective 3, At3g08040) is involved in root iron metabolism (Rogers and Guerinot, 2002; Green and Rogers, 2004). The second subgroup contains the majority of *A. thaliana* members, is similar to human and yeast MATE proteins, and includes the protein described here, a flower flavonoid transporter (FFT). Three proteins from this group have been studied: the product of *TT12*, mentioned above; ALF5 (aberrant lateral root formation 5, At3g23560; Diener *et al.*, 2001), thought to be involved in formation of lateral roots and toxin sensitivity; and EDS5 (enhanced disease susceptibility 5, At4g39030; Nawrath *et al.*, 2002).

The MATE family member under study here, FFT, is the product of At4g25640 [named, amongst a family of so-called <u>det</u>oxifying efflux carriers, as AtDTX35 by Li *et al.* (2002)] in *A. thaliana*. It is most similar to putative *Solanum lycopersicum* (tomato), *Vitis vinifera* (grape) and *Oryza*

sativa (rice) MATE proteins, and four other *Arabidopsis* proteins (Supplementary Fig. S1 available at *JXB* online). Its existence was noted previously in work on tomato describing a MYB overexpression line with altered anthocyanin regulatory pathways (Mathews *et al.*, 2003). Because of the sequence similarity, these authors speculated that FFT might be involved in anthocyanin sequestration in vegetative tissues. Now identified as a possible vacuolar membrane protein (in a mass spectrometry study of vacuoles isolated from *Arabidopsis* cell culture; Jaquinod *et al.*, 2007), loss of FFT could have profound effects.

It was found that *FFT* expression was widespread but particularly high in inflorescence tissues, especially in floral epidermal guard cells and those of the anther and nectary. Mutant analysis confirmed that abolishing *FFT* expression affects flavonoid levels in the plant, also altering root growth, seed development and germination, and pollen development and release. The FFT substrate is not established here, but the data suggest it is more likely to be a glycosylated flavonol than an anthocyanin as previously speculated. FFT can be added to the incomplete flavonoid transport network, and the results also show that correct reproductive development in *Arabidopsis* requires this putative transporter.

Materials and methods

Plants and growth conditions

Wild-type (WT) and mutant lines were grown in compost in greenhouses maintained at 20 °C. For growth assays and transformed plants, half-strength Murashige and Skoog (1/2MS) agar (pH 5.7; Duchefa, Haarlem, The Netherlands) was used, and plates or pots were incubated in a growth room at 20 °C with light of 10 µmol m^{-2} s^{-1} (16 h light cycles). For high-intensity light assays when testing photosynthetic parameters, a Fitotron growth chamber (Weiss-Gallenkamp, Loughborough, Leicestershire, UK) was used. N552331 and N604224 T-DNA insertion lines were purchased from the Nottingham Arabidopsis Stock Centre (NASC; Nottingham, UK) and segregated until homozygous. The presence and location of single T-DNA insertions were confirmed by Southern analysis (according to Church and Gilbert, 1984) and the absence of transcript by RT-PCR. Growth assays of *fft-1* were conducted on plates in which one half of each plate was sowed with mutant and the other half with WT seed, with 6–11 seeds on each half for germination counts.

Molecular biology

DNA manipulation was performed essentially according to Sambrook *et al.* (1989). Primers for cloning the *FFT* full-length cDNA were 5′-CCCTGCAGATGGATCCGACGGCGCCGTT-3′ and 5′-CCGAATTCTCACGCAAGTATATCCTTGGATGTC-3′. Primers for the *FFT* promoter were 5′-CGAGCTCTCTATTTTAT-CCTCCCGAACA-3′ and 5′-CATCTTCTGAAAATGATTAACC-3′. For transcript analysis via RT-PCR, primers were used that would generate product across introns to differentiate any genomic contamination in template from transcript. To investigate transcript levels in specific tissues, RT-PCR was carried out using primers to *actin8* (5′-AGAAAGATGCGTATGTTGGTGA-3′ and 5′-CTGCTGGAAAGTGCTGAGGGAA-3′), and the number of cycles of amplification determined that would produce the exponential phase of product amplification. The amount of template cDNA required to give this phase of PCR at the same cycle number in all tissue samples was also determined, and then the appropriate template concentration was used for RT-PCR with primers to *FFT* (5′-GGATGATAGCTGCTCCTGTG-3′ and 5′- CCGAATTCT-CACGCAAGTATATCCTTGGATGTC-3′).

Binary vectors and plant transformation

Vectors for β-glucuronidase (GUS)–promoter lines and complemented mutant were made using pGreenII0029-based constructs (John Innes Centre, Norwich, UK) and *Agrobacterium tumefaciens*-mediated transformation via floral dipping (Gilmartin and Bowler, 2002). Transformed plants (T₁) were grown on 1/2MS plates as above, supplemented with antibiotic as appropriate, and antibiotic-resistant plants grown on in compost.

Complemented mutant

The *fft-1* mutant was complemented to ensure a return of WT characteristics by transforming it with a WT copy of the transcript under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Effects on growth, seed and floral phenotypes were checked in three independent complemented transformant lines.

GUS staining

For promoter–GUS localization, tissues were stained according to Jefferson *et al.* (1987). Tissues fixed in 90% (v/v) ice-cold acetone for 20 min were rinsed twice in GUS working solution [0.1 M sodium phosphate buffer, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 10 mM Na_2EDTA, 0.1% (v/v) Triton X-100; pH 7.0] and stained by adding 40 mg ml^{-1} stock 5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexamine salt at 12.5 µl ml^{-1} buffer, vacuum infiltrating tissue, and incubating for 12–48 h as required at 37 °C. Tissues were cleared with 70% (v/v) ethanol. Three independent lines were examined to ensure reproducibility.

Microscopy of inflorescence tissues

Pollen and inflorescence tissues were examined using cryo-scanning electron microscopy (SEM) (Oxford Instruments CT 1500, Abingdon, Oxfordshire, UK). The Alexander stain was carried out essentially according to Pline *et al.* (2002). For a general screen of phenylpropanoid and flavonoid compounds in the *fft-1* mutant, the method of Hutzler *et al.* (1998) was followed, in which secondary fluorescence was observed following UV excitation of tissues stained with 0.1% (w/v) DPBA [diphenylboric acid 2-aminoethylester; in 0.1 M potassium phosphate buffer pH 6.8, 1% NaCl (w/v) (Hutzler *et al.*, 1998), since alkaline conditions induce fluorescence in flavonoids (Pfundel *et al.*, 2006)]. For both epifluorescence and confocal microscopy, tissues were excited at the peak of flavonoid absorbance, at ~350 nm; specifically, this was 330–380 nm (Nikon Eclipse 50i, epifluorescence attachment visualized using the UV-2A filter block) or, by confocal microscopy [Leica (Milton Keynes, UK) using TCS-SP1 equipped with Coherent Enterprise (Santa Paula, CA, USA) UV laser], selected areas were irradiated with the 351 nm laser line, and lambda scans were collected over 400–730 nm. Samples were examined with a ×63 oil immersion objective lens with a numerical aperture of 1.3. Fluorescence intensity was measured in 10 nm windows and displayed in the TCS (Leica) software as spectra.

Liquid chromatography–mass spectrometry (LC-MS)

Samples were run on a Thermo Finnigan (Thermo Fisher, Waltham, MA, USA) DecaXP+ ion trap LC-MS and an Agilent (Santa Clara, CA, USA) 1100 single quadrupole LC-MS system. Tentative quantification by mass spectrometry was checked against UV absorbance to help confirm identities. Flavonols were measured using the Agilent system as follows: analytes were separated on a 100×2 mm 3 µm Luna C18(2) column (Phenomenex, Torrance, CA, USA) at 23 °C using the following gradient of
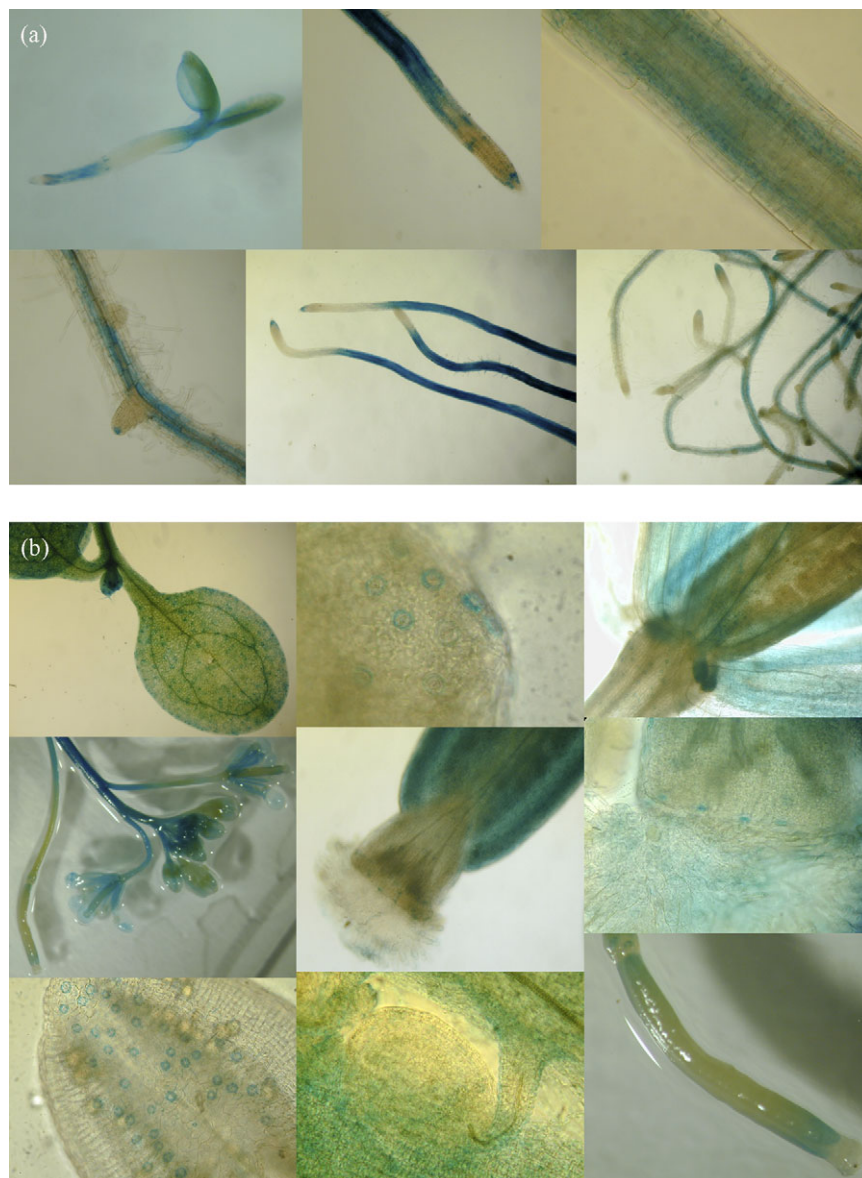
Fig. 2. X-gluc staining of GUS–*FFT*–promoter-transformed plants (each row, left–right). (a) Seedling roots: day 4 post-stratification; day 7 root tip, elongation zone, and developing lateral root; day 12; day 17. (b) Aerial tissues: (row 1) cotyledon guard cells (GC); mature leaf hydathode GC; nectaries; (row 2) inflorescence apex; silique apex; close-up of papillae and stigma GC; (row 3) anther GC; developing seed in siliques; developing silique.

solvent A [0.1% (v/v) formic acid in water] versus solvent B (methanol) at 230 µl min^{-1} (0 min, 2% B; 30 min, 40% B; 40 min, 70% B; 42 min, 70% B; 43 min, 2% B; 51 min, 2% B; all v/v). Detection was by UV absorbance at 254 nm and 325 nm, and by positive electrospray mass spectrometry from 100 to 1500 amu. Spray chamber conditions were 172 Pa nebulizer pressure, 11.0 l min^{-1} drying gas at 350 °C, 4000 V capillary voltage, and 70 V fragmentor. Selected peaks in extracted ion chromatograms were integrated automatically using Chemstation software. Anthocyanins were analysed on a Surveyor LC system (Thermo Fisher) attached to a DecaXPplus ion trap mass spectrometer. Samples were acidified by addition of HCl to 0.1 M and separated on a 100×2 mm 3 µm Luna C18(2) column at 30 °C using the following gradient of solvent A [1% (v/v) formic acid in water] versus solvent B (methanol) at 230 µl min^{-1} (0 min, 2% B; 40 min, 70% B; 41 min, 2% B; 50 min, 2% B; all v/v). Anthocyanins were detected by UV absorbance at 520 nm and by positive electrospray mass spectrometry with spray chamber conditions of 350 °C

capillary temperature, 50 units of sheath gas, 5 units of auxiliary gas, and a source voltage of 5.2 kV. Identification of anthocyanins was confirmed from MS2 and MS3 spectra collected at an isolation width of 4 amu and 35% collision energy. For ion-trap analysis of flavonols and glucosinolates, the identification of flavonols was confirmed by analysis in a Surveyor LC system attached to a DecaXP+ ion trap mass spectrometer. The gradient, column, and solvents were as used in the Agilent system, and spray chamber conditions were identical to those used for anthocyanins. Glucosinolates were identified using the same gradient, and negative electrospray ionization, with the same spray chamber conditions except that the source voltage was 5.0 kV.

Photosynthesis

Pulse amplitude modulation fluorimetry was used to measure the quantum yield of photosystem II (Blankenship, 2002) according to the manufacturer's instructions (Hansatech, King's Lynn, Norfolk,
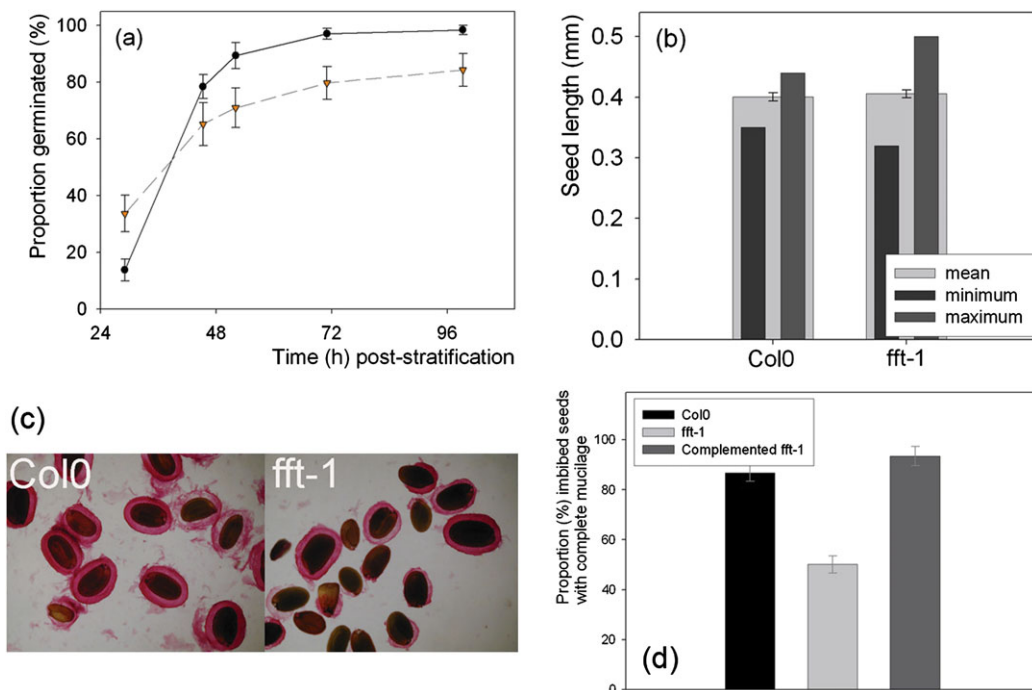
Fig. 3. (a) Germination of the *fft-1* (- -) mutant versus Col0 (—). (b) Seed size in Col0 and the mutant. (c) Ruthenium red staining of imbibing seed mucilage. (d) Proportion of imbibing Col0, *fft-1* and complemented *fft-1* seeds showing complete mucilage when stained with ruthenium red.

UK). Estimation of chlorophyll content was carried out according to Hipkins and Baker (1986) and Porra *et al.* (1989), extracting pigment with TRIS-Cl (pH 8.0)-buffered 80% (v/v) acetone.

Results

FFT *encodes a MATE protein*

The 488 amino acid protein (AtDTX35; estimated mol. wt, 53.2 kDa) is classified as a MATE protein (see TAIR; www.arabidopsis.org/abrc/). The most similar proteins to FFT in BLAST searches are an anthocyanin permease in tomato (Mathews *et al.*, 2003; 72% identity at the protein level), an unknown protein in grape (70% identity), a putative ripening-regulated protein in rice (DDTFR18, Os08g0562800; 61% identity), and four other *Arabidopsis* MATE proteins (48–67% identity; Supplementary Fig. S1 at *JXB* online). As expected, hydropathy plots (TMPred; www.cbs.dtu.dk/services/TMHMM-2.0/) predict 12 transmembrane spans for the protein encoded by At4g25640. There is no recognizable signal peptide to predict targeting of the translated protein (SignalP, PredoTar; urgi.versailles.inra.fr/predotar/predotar.html). Glutamate residues may be important for activity in MATE proteins, and one such, found in almost every eukaryotic and prokaryotic MATE protein's seventh transmembrane domain, is also seen in FFT (Supplementary Fig. S1). Site-directed mutagenesis of this amino acid in the human MATE1 protein severely affected expression and prevented uptake of tetraethylammonium substrate in human embryonic kidney-293 cells (Matsumoto *et al.*, 2008). Consistent with a likely role in

flavonoid metabolism, a search for *cis* regulatory elements (PLACE database; Higo *et al.*, 1999) finds MYB recognition sequences upstream of At4g25640 including two MYB-binding sites commonly found in flavonoid pathway genes such as *CHS* (consensus MACCWAMC; Sablowski *et al.*, 1994), situated at −99 nt and −23 nt relative to the start codon.

FFT *is most strongly expressed in floral tissues and guard cells*

RT-PCR indicates that *FFT* is highly transcribed in floral organs and is present to some degree in most tissues, including young seedlings, senescent leaves and roots (Supplementary Fig. S2 at *JXB* online). To investigate further the spatial and temporal expression of *FFT*, a 1.4 kb genomic fragment from immediately upstream of the coding regions was amplified and fused to the *uid*A reporter gene. Floral tissues of plants transformed with this construct again showed the strongest activity (Fig. 2). Notably, GUS expression directed by the *FFT* promoter was almost exclusively found in guard cells in aerial tissues, including the specialized guard cells of nectaries and the hydathodes of leaves. Anther, stigma and silique guard cells were strongly stained, whereas the activity in mature leaf guard cells was reduced compared with those of the inflorescence and of the cotyledon (Fig. 2b). In newly germinated seedlings, as well as strong staining in leaf guard cells, there was GUS activity in the roots (Fig. 2a). By day 4 post-stratification (ps), on 1/2MS plates (*t*=0 at transfer of plants to growth chamber), staining was found in two discrete
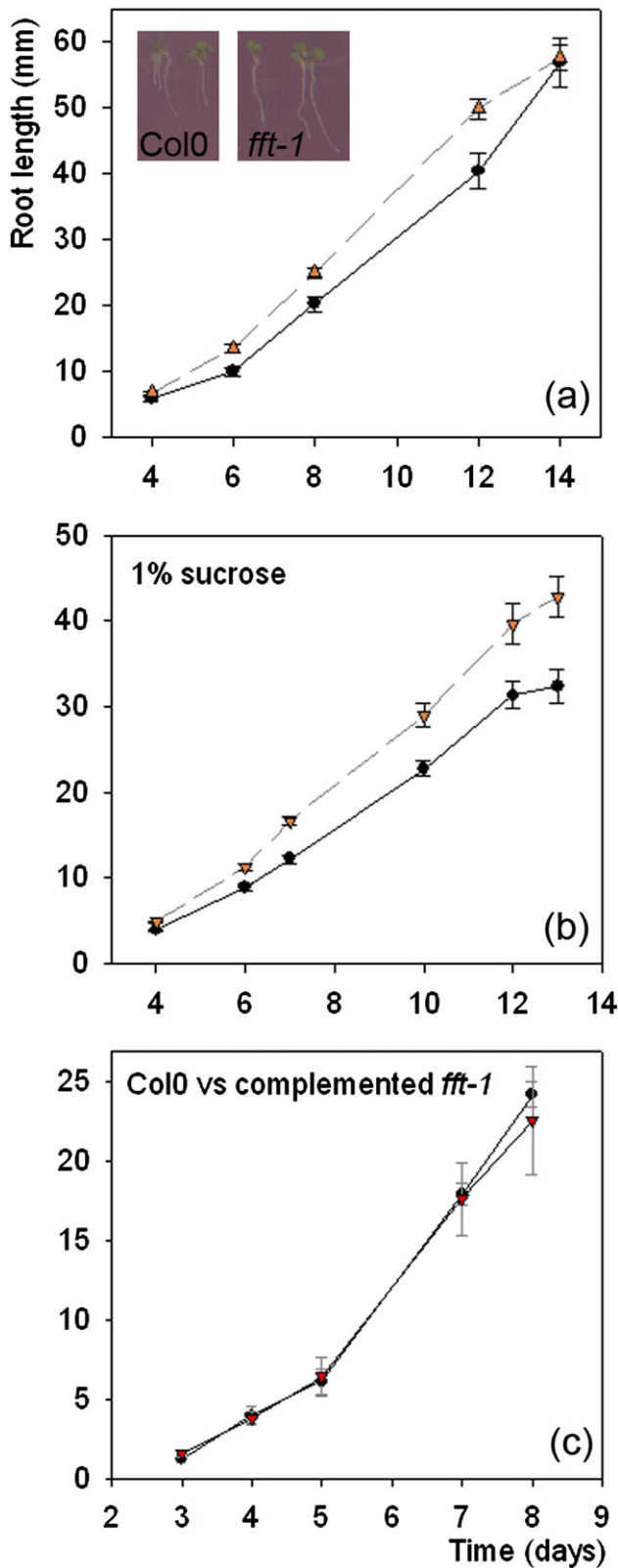
Fig. 4. Mutant *fft-1* (- -) roots grow more quickly than Col0 (—) up to ~2 weeks. (a) No sucrose: day 6–12, *P*=0.002–0.003 (*n*=70–81 for each time point). Inset: Col0 and *fft-1* seedlings at day 4 post-stratification. (b) 1% sucrose: day 6–12, *P* <0.0001–0.001 (*n*=60–97 for each time point). (c) The *fft-1* mutant complemented with 35S-At4g25640 returns to wild-type growth characteristics.

areas of the roots, namely at the root apex (meristematic zone) and in the elongation zone through to the fully expanded cells of the differentiation zone. The transition zone showed little staining. GUS activity in the elongation–differentiation zone was strongest internal to the epidermis, apparently in the endodermis and cortex. The tips of lateral roots were also stained blue (Fig. 2a).

Isolation of the fft-1 null mutant

Seeds of two mutants with T-DNA insertions in the At4g25640 open reading frame (ORF) were obtained from the NASC. Both were segregated to homozygosity. The insertion in line N552331 was situated ~200 bp into the 3′-untranslated region: this mutant (*fft-2*) was not null so was not studied further. Transcript was abolished in line N604224 (*fft-1*), in which the T-DNA was inserted into the third intron (Supplementary Fig. S3 at *JXB* online). Having established that only one T-DNA was present in this line (Supplementary Fig. S3), phenotypic analyses were conducted on the homozygous mutant *fft-1*.

Several differences were observed between *fft-1* and WT (Col0) plants in terms of growth and fertility. To ensure these phenotypic effects were the consequence of loss of function of *FFT*, genetic rescue was carried out, re-introducing a WT cDNA into the mutant line. The ectopic copy of the WT gene restored normal growth and fertility: root growth, seed and pollen development phenotypes were tested, and it was found that the *FFT* cDNA complemented all aspects of the mutation (Fig. 3–5; Supplementary Figs S4, S5 at *JXB* online).

Germination and seed morphology is perturbed in fft-1

Of plate-grown plants, >30% of the *fft-1* mutant seeds [standard error of the mean (SE), 6%] had germinated by 29 h ps whereas in the WT this was <14% (SE, 4%; *t*-test *P* = 0.01, 14 df; Fig. 3a]. Fewer mutant seeds in total, however, germinated successfully in the mutant by 99 h [WT, 100% (SE, 2%); *fft-1*, 84% (SE, 6%)]. Because flavonoid mutants often show altered seedcoats and, because germination in *fft-1* was variable, seeds were examined for physical abnormalities. The seedcoat of *fft-1* was brown pigmented like that of the WT, but ruthenium red (RR) staining showed altered seed mucilage. RR stains acidic polysaccharides such as pectic polysaccharides: with imbibing *Arabidopsis* seeds, RR densely stains an inner layer of mucilage plus an outer, more diffuse (and easily disrupted) layer (Macquet *et al.*, 2007). This staining of imbibing *fft-1* seeds showed that the surrounding mucilage layer was absent or disrupted in the mutant. In the WT, 87% (SE, 3%) had complete mucilage compared with only 50% (SE, 4%) in the mutant (*t*-test, *P* <0.0001, 44 df; Fig. 3c, d). Seed mucilage contributes to seed dormancy and, thus, heterogeneous mucilage release in *fft-1* mutant seeds is consistent with the abnormal germination rates. In addition, seed size was significantly more variable in the
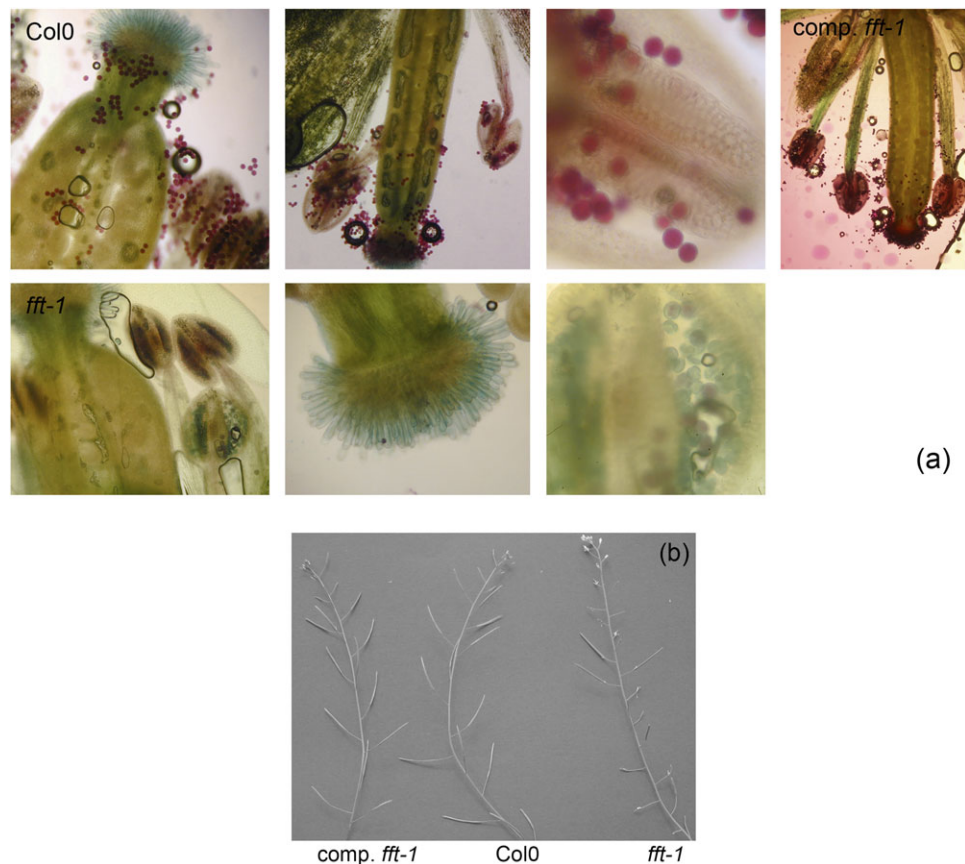
Fig. 5. (a) Alexander's staining showing (left–right) lack of dehiscence, no pollen on the stigma, and reduced proportion of viable pollen in anthers in *fft-1* (second row) versus Col0 (top row). Top right: pollen production and dehiscence in complemented *fft-1*. (b) Variable success of silique production in *fft-1* versus Col0 and complemented *fft-1*.

mutant (*F*-test, $P < 0.005$), although mean seed size was not significantly different from that of the WT (Fig. 3b).

FFT *affects growth rates*

It was noticed that mutant seedlings appeared to grow and mature more quickly than the WT, so assays of root growth were carried out using plants on 1/2MS with and without sucrose (the former to promote anthocyanin production, in case of any effect). The mutant showed significantly faster root growth in all cases (Fig. 4a, b; for no sucrose: *t*-test, $P < 0.001$ day 8, 64 df). Although the different germination characteristics meant that fewer *fft-1* seeds were viable than WT seeds, the difference in mean rate of root growth developed after day 3–4 ps (when all viable seeds had successfully germinated) and continued to develop, suggesting that faster germination was not the primary cause of the root growth phenotype. The growth rate of *fft-1* up to 2 weeks was greater under all conditions tested, including cold (4 °C) and higher intensity light (250 µmol m^{-2} s^{-1}).

Fertility of fft-1 *is reduced*

When examined using cryo-SEM, a proportion of mutant pollen appeared shrunken and irregular, and/or had an aberrant surface structure (Supplementary Fig. S6 at *JXB* online). To examine this further, Alexander's stain was used to differentiate viable from non-viable pollen. Acid fuchsin stains the protoplasm of viable pollen purple, and malachite green (MG) is a background counterstain. This can give an overestimate of viability, since non-viable pollen can contain protoplasm (Pline *et al.*, 2002), but here a distinct difference between WT and *fft-1* flowers was shown (Fig. 5a). Series of flowers at identical stages and positions on the primary inflorescence were sampled and stained. In contrast to WT anthers, which were plump, full of purple-stained pollen, and showed many pollen grains released upon dehiscence, the mutant anthers appeared thinner and had a lower proportion of (but did contain some) round, purple pollen grains. Much of the pollen in mutant anthers stained only blue-green, the cell walls coloured only by MG (Fig. 5a).

Many *fft-1* anthers fail to dehisce, with no visible pollen outside each (Fig. 5a, Supplementary Fig. S6 at *JXB* online). This was also obvious in epifluorescence microscopy. DPBA was used to enhance cytoplasmic flavonol fluorescence: it gives high emission levels when bound to flavonols rather than other flavonoids, for example fluorescing at 520 nm and 543 nm when bound to the flavonols kaempferol and quercitin, respectively (Saslowsky and

Winkel-Shirley, 2001). WT flowers treated with DPBA showed bright yellow pollen: in contrast, only a few, non-fluorescent, pollen grains were present on mutant flowers (data not shown).

Arabidopsis siliques elongate as seeds develop following successful fertilization: reduced silique length is a visible demonstration of diminished fertility. In the *fft-1* mutant plants, silique length is very variable (Fig. 5b) and seed number per silique was lower than in the WT [WT mean, 51 (SE, 11); *fft-1* mean, 25 (SE, 5); *t*-test *P*=0.0001, 19 df].

Flavonoid metabolism is perturbed in mutant plants

In *fft-1* mutant seedlings up to 1 week of age, extracted anthocyanins were slightly reduced in level compared with the WT (not shown), but the pigment was visible even in young tissues and, in quantity, in mature *fft-1* and WT plants grown on high (5% w/v) sucrose, which were comparable, dark purple, colours.

Following epifluorescent imaging of the WT and *fft-1*, it was hypothesized that confocal microscopy would reveal differences in flavonoid content of the plants. Confocal laser scanning microscopy was therefore used essentially to perform fluorescence spectroscopy, and thus indicate any changes to flavonoid levels (a technique previously used by Hideg *et al.*, 2002, and discussed in Pfündel *et al.*, 2006). Since GUS staining had occurred strongly in floral guard cells, inflorescence tissues treated with DPBA were excited with UV light (364 nm) and an emission spectrum was

Fig. 6. (a) Col0 versus *fft-1* mean ratio of flavonoid (580 nm) and chlorophyll fluorescence (690 nm; Bolhar-Nordenkampf *et al.*, 1989). Inset: example of the anther guard cell fluorescence scanned for analysis as viewed with the confocal microscope. (b) Examples of replicate fluorescence emission spectra from guard cells in Col0 and *fft-1* anthers (background subtracted; normalized to 690 nm).

recorded (400–730 nm). Spectra were recorded from anther guard cells in WT or mutant flowers (inset, Fig. 6a) from the same position on an inflorescence (the first open flower below the unopened bud cluster), in which flavonoids should be comparable. A peak of emission from anther guard cells expected to result from flavonoid excitation could be visualized at 500–580 nm in WT tissues (Fig. 6b). Notably, a component of the fluorescence spectrum at ~520 nm was absent in *fft-1* (Fig. 6b), coinciding with the expected peak from flavonol–DPBA secondary fluorescence (Saslowsky and Winkel-Shirley, 2001). To quantify and test statistically the difference in replicate flowers, the well-defined flavonoid fluorescence peak at 580 nm and the peak from chlorophyll fluorescence were used to produce a ratio of flavonoid:chlorophyll emission. The ratios were significantly different between WT and *fft-1*, suggesting a lower flavonoid content in the mutant [WT, 1.60 (SE, 0.14) versus *fft-1*, 0.86 (SE, 0.05); *t*-test *P*=0.0016, 28 df; Fig. 6a]. Chlorophyll content was confirmed to be the same in WT and *fft-1* mutant plants to make certain that levels of that pigment were not responsible for the differences observed, nor for any other aspect of the mutant phenotype (other photosynthetic parameters were also unaffected; see Supplementary Note S7 at *JXB* online). The products of the reactions of DPBA with flavonoids have not been comprehensively identified, however, so this can only be an indication of a difference in the flavonoid content in *fft-1*.

To investigate specific changes in flavonoid composition resulting from loss of the *FFT*-encoded protein, LC-MS was carried out on *fft-1* mutant versus WT plants. A wide range of tissues was initially tested, guided by the position of GUS staining, to identify any differences between WT and *fft-1* samples at the same stages of development. More plants were then grown so that the tissues where *fft-1* apparently had different levels of particular metabolites could be sampled and analysed in replicate assays. Consistent differences were noted in plants from the separate sowings: in particular, mutant buds and siliques contained an altered spectrum of flavonol glycosides, with several peaks present at ~40–70% the level in the WT, namely quercitin–rhamnoside–glucoside, kaempferol–glucoside–glucoside (kGG), and kaempferol–rhamnoside–glucoside. In contrast, there were
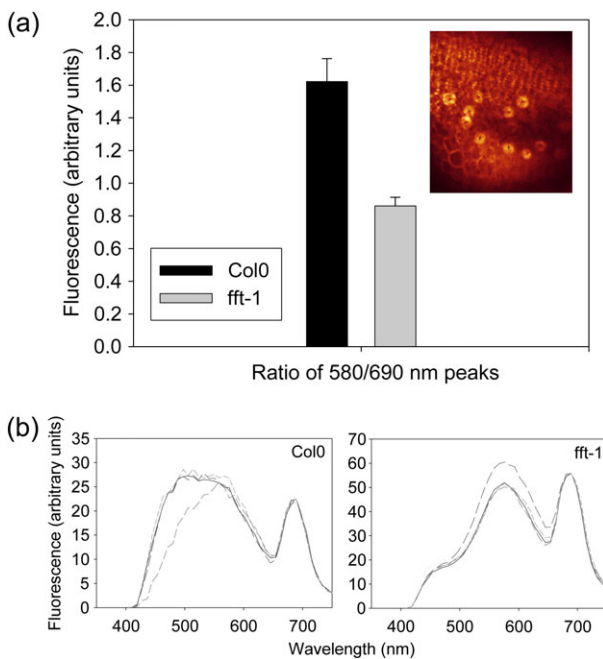
Table 1. Relative level of flavonol glycosides in mutant versus wild-type (WT) floral tissues (further data in Supplementary Fig. S8 at *JXB* online)

Peak and tissue type	Mean content in *fft-1* versus WT (%)
kGG bud*	40
kRG bud	70
kRG immature silique	73
qRGR bud	180
qRG bud	65
qRG immature silique	49

* *P*=0.018, 5 df.
q, quercitin; R, rhamnoside; G, glucoside; IS, immature silique; k, kaempferol.

almost doubled levels of quercitin–rhamnoside–glucoside–rhamnoside in buds (Table 1), and high levels of this flavonol were consistently found in mutant seedlings, suggesting that flavonoid synthesis and/or transport is perturbed in the *fft-1* mutant. The difference in the levels of kGG was significant (*t*-test *P*=0.018, 5 df; see also Supplementary Fig. S8 at *JXB* online).

Discussion

The lack of detail known about the transport of flavonoids and about some of their roles in plants contrasts with our knowledge of their biosynthetic pathways. Here, it is shown that a MATE protein in *Arabidopsis*, FFT, is necessary for correct accumulation of flavonols in the plant. The effects of removing this protein support a role for flavonols in reproduction in *A. thaliana* and suggest an effect on growth, in line with current literature documenting an influence of flavonoids on auxin transport.

Flavonoids are necessary for full fertility

A GUS–promoter construct showed that guard cells are the principal location of the *FFT* transcript in aerial tissues. This is not surprising in the context of flavonoid pathways: a large proportion of a plant's phenylpropanoid and flavonoid compounds are found in the vacuoles of guard cells, whose location and physiological importance places them at the forefront of UV protection and, indeed, other responses to the environment (Hutzler *et al.*, 1998; Gitz and Liu-Gitz, 2003). A possible role in flavonoid pathways makes the location of FFT in the vacuolar membrane likely and, by coincidence during the course of this work, it was one of the 30 most abundant proteins identified as likely to be located on the *A. thaliana* tonoplast membrane (Jaquinod *et al.*, 2007).

Of particular note is the presence of *FFT* transcript in specialized guard cells of the nectaries and those of anthers and hydathodes. There is sparse published literature describing the roles of guard cells in these three locations. The nectaries develop as a ring at the base of the stamens in the Brassicaceae, which is among the families of plants that exude nectar through modified stomata (Davis *et al.*, 1998). Flavonoids including quercitin and kaempferol and other phenolics are found in nectar, and CHS, the enzyme at the root of the flavonoid biosynthesis pathway (Fig. 1), is up-regulated during nectary development in tobacco (Nicolson *et al.*, 2007). The presence of *FFT* transcript in these specific guard cells suggests a direct role in transporting flavonols for the production of nectar. Nectar release and nectar fall coincide with the start and end of anthesis (Durkee, 1983; Davis *et al.*, 1998; Nicolson *et al.*, 2007), so it is interesting that *FFT* transcription is also directed to the anthers.

As a self-fertile plant, nectar composition is unlikely to affect reproductive success in *A. thaliana*, but male sterility can result from defects in anther or pollen development. *fft-1* mutant anthers contained a proportion of non-viable pollen, and dehiscence of anthers was perturbed. As there was no GUS staining in the pollen of *FFT*–promoter–GUS-transformed plants (in contrast to anther guard cells), the altered flavonoid profiles in anthers are the probable cause of the *fft-1* pollen and fertility phenotypes. This was seen to an even great degree in the *white anther* petunia mutant which lacks CHS and is functionally male sterile (Napoli *et al.*, 1999). Other mutants besides *fft-1* are defective in both anther and pollen development, e.g. *coi1* (*coronatine insensitive 1*) or *fad* (*fatty acid desaturase 6*) mutants, both related to jasmonic acid signalling in the regulation of anther dehiscence (Xie *et al.*, 1998; Sanders *et al.*, 1999). As was seen here, other mutations also reduce rather than abolish fertility. Altered exine development in the *ms2* (*male sterility 2*) mutant (Aarts *et al.*, 1997), for example, caused a range of effects, many similar to those seen in the *fft-1* mutant, including reduced seed set, shrivelled anthers, and the majority of the seeds being produced from apical flowers of the mature inflorescence.

An effect of altered flavonoid content could be perturbed lignification, as seen after disrupting the function of the first enzyme of the phenylpropanoid pathway, phenylalanine ammonia lyase (PAL; Fig. 1; Rohde *et al.*, 2004). Anther development and dehiscence follow defined pathways of maturation and cell death (Sanders *et al.*, 1999), lignified secondary walls in the anther being important in its dehiscence (and also of relevance here, in breaking open mature siliques; Mitsuda *et al.*, 2005). If anthers dehisce late, stigmatic papillae may no longer be receptive to pollination (in a self-fertile plant such as *Arabidopsis*). The guard cell location of the *FFT* transcript, however, suggests a connection in particular with appropriate dehydration of pollen and/or anther (Keijzer *et al.*, 1996; Taylor and Heppler, 1997; Scott *et al.*, 2004; Mitsuda *et al.*, 2005; Jung *et al.*, 2006), which would be expected to require proper function of the guard cells on the epidermis of the anther theca (Fig. 2b). Flavonoids in anther guard cells may also act within regulatory pathways that control co-ordinated development and maturation of pollen and anther (see comments on auxin, below). A role for FFT protein in dehiscence or dehydration of anthers and thus pollen coat formation and pollen viability seems most likely, but a direct chemical effect is also possible. Pollen's highly sculpted outer exine layer is mostly made up of sporopollenin, derived from long-chain fatty acids, but also contains some phenylpropanoids (Morant *et al.*, 2007). Preston *et al.* (2004) speculated that altered flux along phenylpropanoid pathways was the cause of male sterility in *myb4* and *myb32* pollen mutants. Likewise, altered flavonoid composition in *fft-1* could disturb the pollen coat and reduce pollination success, since exine is involved in recognition and adhesion.

The *fft-1* mutant phenotype certainly indicates that perturbing flavonoid metabolism in *Arabidopsis* affects this plant's fertility. Although this is known to be the case in other plants, e.g. petunia (Napoli *et al.*, 1999), the *Arabidopsis* CHS mutant, *tt4*, is fertile. The requirement for particular flavonoids for viable pollen and pollination in *Arabidopsis* has been discounted, therefore (Burbulis *et al.*, 1996), although these authors do point out that the high

levels of flavonols in pollen argue for an indirect role, at least, in fertility of the male gametophyte. In addition, a PAL double mutant (lesions in two of four *PAL* genes) did become sterile, with the siliques remaining small, and empty ovule sacs initially enlarging but then becoming shrivelled, suggesting male sterility (Rohde *et al.*, 2004).

As with *fft-1*, variability was seen in the fertility phenotype of the *mpk6* (*mitogen-activated protein kinase 6*) mutant (Bush and Krysan, 2007). Neither the present nor the latter authors could pinpoint specific environmental conditions that determined the severity of the effects. These mutations are clearly important but can (later in development) be overcome for reproduction in *Arabidopsis*, probably by developmental homeostasis. The variable success of fertilization in *fft-1* does suggest that there may be redundancy in the putative flavonoid transporters of *Arabidopsis*. If a threshold level of a, or some, flavonoids must be achieved for some fertilization, low levels of transport by other transporters might eventually compensate for the missing FFT. Interestingly, the TT12 MATE transporter was able to transport glycosylated anthocyanidins in *in vitro* transport assays although *in vivo* these do not appear to be the normal substrate (Marinova *et al.*, 2007).

FFT is necessary for correct seed development, germination, and growth

Numerous mutants with seedcoat phenotypes are defective in components of flavonoid biosynthesis or of its transcriptional regulation, e.g. *tt8*, *ttg1* and *ttg2* (Walker *et al.*, 1999; Broun, 2005; Ishida *et al.*, 2007). Changing the levels of flavonoids can have a range of effects on seed: in the *ttg1* mutant, seedcoat anthocyanin levels, dormancy, and seed mucilage are all affected (Walker *et al.*, 1999). In *fft-1*, accumulation of PAs and tannins is apparently not affected, since seeds are not paler than the WT. Fewer seed are set in the *fft-1* mutant, however, and varied seed sizes and heterogeneous mucilage release upon imbibition are seen amongst those that are produced. There is no evidence, from GUS staining, of *FFT* promoter activity in seeds, so it seems likely that the seeds' heterogeneity is a result of faulty flavonoid transport in the carpel and/or developing silique, in which RT-PCR did reveal high levels of transcript, and where there was strong GUS activity in *FFT*–promoter– GUS plants. Since, for example, oxidation of PAs contributes to seed dormancy (and protection) by strengthening the seedcoat (Debeaujon *et al.*, 2000), reduced dormancy in the *fft-1* mutant does also imply some change in seedcoat composition.

The GUS staining seen in *FFT*–promoter–GUS-transformed seedlings corresponds to the fluorescence from flavonoids seen previously in *Arabidopsis* roots using epifluorescent microscopy (Buer and Muday, 2004) and, notably, is consistent with possible accumulation of flavonoids in discrete locations of the root. *FFT*-directed GUS staining was notable in the cortex in the elongation zone and in the root tip, close to the locations of CHS and chalcone isomerase (Saslowsky and Winkel-Shirley, 2001).

The faster root growth of seedlings is intriguing, since flavonoid mutants can have altered auxin transport (Buer and Muday, 2004; Lazar and Goodman, 2005). After studies using several *tt* mutants, quite a body of literature (see Peer and Murphy, 2009) now documents a reduction or elevation in polar auxin transport because of increased or absent flavonols (respectively), and thus altered accumulation of auxin in apical tissues. Cecchetti and colleagues (2008) also recently discussed how auxin regulates anther dehiscence and pollen maturation: flavonol–auxin effects on fertility cannot be ruled out in *fft-1* and present an interesting avenue for future work.

MATE proteins transport-specific subsets of flavonoids

fft-1 has variable fertility, not complete infertility. Another flavonoid mutant's altered gravitational response was eventually corrected (Buer and Muday, 2004). These results suggest the existence of redundant mechanisms that allow roots or aerial tissues to recover. The existence of several *Arabidopsis* MATE proteins similar to FFT may explain redundancy of function, although some degree of specificity would correspond to the TT12 MATE transporter's limited substrates. Although it transports cyanidin-3-*O*-glucoside (acting as an H^+-antiporter) in yeast assays *in vitro*, work on the *tt12* mutant suggests that anthocyanins and glycosylated flavan-3-ols are its principal substrates *in vivo*, where it acts at the vacuolar membrane of PA-synthesizing cells of the seedcoat (Marinova *et al.*, 2007). This work has recently been extended, where complementation of the *tt12* mutant with a vacuolar *Medicago truncata* orthologue corroborates the identity of its substrate, epicatechin-3′-*O*-glucoside (Zhao and Dixon, 2009). In the present case, data from a tomato orthologue (Mathews *et al.*, 2003) led to speculation that *Arabidopsis* FFT is a transporter of anthocyanin in vegetative tissues. The present work does not support this suggestion. Spectroscopic measurement of anthocyanin extracts does show a small reduction in young seedlings, and RT-PCR reveals *FFT* transcript in senescent leaves (which have high levels of anthocyanin) but, conversely, anthocyanin visibly accumulates in the mutant. It is hypothezised that the small changes in anthocyanin levels are a result of other perturbations in the flavonoid biosynthetic pathways since *FFT*–promoter–GUS staining was most significant in non-anthocyanin-containing tissues (inflorescence guard cells and roots). In addition, LC-MS measurements did not show any significant change in peaks likely to be flavonoids arising from samples of vegetative tissues (data not shown).

Fluorescence spectroscopy with DPBA did suggest altered flavonoid content in mutant versus WT anther guard cells, and LC-MS data revealed reductions in the *fft-1* mutant in several flavonol glycosides in buds and immature siliques. Work to date suggests that a lack of FFT most affects levels of kaempferol (the more predominant of the two flavonols in whole *Arabidopsis* flowers; Shirley *et al.*, 1995), specifically a diglucoside and probably therefore kaempferol 3,7-*O*-diglucoside (see www.plantcyc.org:1555/

ARA/NEW-IMAGE?type=PATHWAY&object=PWY1F-FLAVSYN for detailed pathways and published contents of flavonoids and enzymes), but further work is required to confirm whether this is a transporter for that compound. Numerous novel dimers and oligomers of flavonols have been identified in *Arabidopsis* seeds (Routaboul *et al.*, 2006), totalling 26 different flavonoids, so perturbing this pathway in *fft-1* plants could clearly affect various aspects of reproductive development.

In summary, the characteristics of FFT support the case that MATE proteins are a component of flavonoid biosynthetic pathways. In addition, this protein appears to be necessary for proper reproductive development in *Arabidopsis*. Although *chs* mutants argue against an essential role in reproduction, transport of certain key flavonoids does seem to be essential for full male fertility.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. CLUSTALW alignment (N- and C-termini omitted to save space) and phylogram of FFT and the three most similar *Arabidopsis* MATE proteins with tomato putative anthocyanin permease (SlAnthP) and rice ripening protein (OsXP). The human MATE1 sequence (Q96FL8) is included to show an invariant glutamate (boxed) in the seventh transmembrane domain (Matsumoto *et al.*, 2008). Twelve putative transmembrane domains (TMPred) are underlined.

Figure S2. Tissue or stress-induced transcription of *FFT*. RT-PCR was conducted as described in the Materials and methods. Sil, silique (green, >1 cm); YS, young silique (green, <0.5 cm); IS, immature silique (green, <1 cm); ISP, IS with dehydrating petals attached; Pet, petals; Bud, unopened bud; BS, bolt stem; CL, cauline leaf; ML, mature rosette leaf; SL, senescent anthocyanin-pigmented leaf; d2/d5, day 2 or day 5 seedling; LS, rosette leaf of plant grown with 1% sucrose; Pt, petioles; Fl, plates flooded (25 ml of sterile water for 36 h); H2, 1 mM H_2O_2 added to root tips for 3 h; 4C, plates incubated at 4 °C with light for 24 h; *Pseudomonas syringae* was infiltrated into the leaf as follows: M4, *P.s.* maculicola M4 (pathogenic); RW, *P.s.* phaseolica RW60 (non-pathogenic); Hrp, *P.s.* DC300 HrpA⁻ (non-pathogenic).

Figure S3. Southern blot with a kanamycin probe showing one T-DNA band in genomic DNA from the *fft*-1 (N604224) mutant and the position of the T-DNA insertion.

Figure S4. RT-PCR in inflorescence tissues (top panel, *FFT* transcript; lower panel, *actin8*) from seven transformed mutants (lines A–G). Lines D, F, and G were investigated fully.

Figure S5. Cryo-SEM of inflorescence tissue from the WT (top panel, Col0-1), *fft-1* mutant (middle panel, Ab-1), and complemented mutant (lower panel, F-1). The mutant has non-dehisced anthers but receptive papillae on the stigma, whereas the WT and complemented mutant have dehisced anthers and pollen germinating on stigma papillae.

Figure S6. Cryo-SEM of WT (Col0) and *fft-1* mutant pollen and anthers, showing defective pollen and anthers in the mutant.

Figure S7. Photosynthetic parameters.

Figure S8. (a) Example of LC-MS runs for kGG in buds and immature siliques. Mutant, left (red); wild-type, right (green). (b) Mean values from which Table 1 proportions are derived. k, kaempferol; R, rhamnoside; G, glucoside; IS, immature silique; q, quercitin.

Acknowledgements

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