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# Effects of Flavonoids on Pollen Tube Growth in *Arabidopsis thaliana*

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***Arabidopsis* mutants deficient in flavonoid and sinapate ester (tt4 and fah1-7, respectively) were evaluated *in vivo* and *in vitro* to study the possible role of flavonoid compounds in pollen tube growth. *In vivo*, we investigated pollen tube growth in the pistils of the mutants and wild type (Ler). The growth of pollen tubes was significantly different among the three genotypes. In the fah1-7 pistils, the tubes grew to a greater length relative to those of the wild type or tt4. To examine *in vitro* pollen tube growth, a solid medium was devised for pollen germination and subsequent growth. *In vitro*, the identical result was obtained; fah1-7 pollens developed the longest tubes and elongated most rapidly. Therefore, the growth response of pollen tubes to phenolic compounds was examined by adding quercetin or sinapate ester in various concentrations to the media. Quercetin enhanced both germination rate and tube growth in the pollens of the mutants and the wild type, especially in tt4. In contrast, sinapate ester inhibits pollen germination and pollen tube growth in three genotypes. These results suggest that flavonoids and related phenolic compounds have a physiological role in the plant reproductive system.**

*Keywords*: flavonoids, sinapate ester, quercetin, pollen tube growth, pollen germination

Flavonoids function in plants as signal molecules and as protective agents against herbivores and UV light (Herrman, 1995). Recent work has uncovered a requirement for flavonoids in petunia and maize pollen development (Mo *et al.*, 1992; van der Meer *et al.*, 1992). The flavonoids accumulated on the stigmatic surface enhance the growth of pollen tubes and protect it from bacterial or fungal attack. Genes in the flavonoid-synthetic pathway are also expressed in anther tissues from the early stages of flower development (van Tunen *et al.*, 1988).

Since chalcone synthase (CHS) is a key enzyme in the flavonoid biosynthesis pathway, antisense inhibition of CHS gene expression results in male sterility in petunias (van der Meer *et al.*, 1992). In that case, fertility can be restored by exogenously supplying a flavonol such as quercetin to the stigmatic surface. Restoration of germination and tube growth in the CHS mutant petunia was limited to a specific class of flavonoids, flavonol (Mo *et al.*, 1992), that share the structural features of an unsaturated bond between the carbons at positions two and three in the C ring as well as an unsubstituted hydroxyl group in position three (ring C) of the flavonoid skeleton. The addition of quercetin, kaempferol, or myricetin in micromolar concentrations to a pollen germination medium stimulated the growth of tobacco pollen, but the addition of naringenin chalcone, naringin, or naringenin had no effect (Ylstra *et al.*, 1992).

Numerous flavonoid-mutants have been identified for *Arabidopsis*, including several mutant alleles of the CHS locus (Shirlly *et al.*, 1995). Surprisingly, all of these plants appear to be fully self-fertile in this species (Burbulis *et al.*, 1996). The fact that these CHS *Arabidopsis* mutants supported pollen tube growth of flavonoid deficient pollen leaves us to question the essentiality of flavonoids for pollen tube growth in *Arabidopsis thaliana*.

In this report, we describe pollination events that lead to the pollen germination in the pistils of *Ara-*

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*bidopsis thaliana*. The length of pollen tubes was estimated in the pistils of tt4 and fah1-7 mutants and the wild type which had been self- or cross-pollinated. Then, an artificial medium was developed to evaluate *in vitro* pollen germination. Concurrently, pollen tube growth was compared in media containing quercetin or sinapate ester in various concentrations. Our results show that flavonoids, even if not essential, have a role in pollen tube growth.

## MATERIALS AND METHODS

### Plants and growth conditions

The EMS-induced mutants (tt4 and fah1-7) used in this study were derived from the *Arabidopsis thaliana* cv. *Lendsberg erecta*. Mutation resulting in transparent seed coats represent a locus which is named TT. A mutant (tt4) at the TT4 locus are characterized by lack of flavonoids (Koorneef *et al.*, 1990). When a mutant that lacks sinapate esters was published, it was named sin1-7, but fah1-7 is now used in its place. The fah1-7 mutant can be identified by shining a UV light on green leaves to fluoresce red (Chapple *et al.*, 1992).

Until flowering, plants were grown in flats in a growth room with a photoperiod of 16 hrs light at 22°C for 4 weeks. To examine pollen tube growth *in vivo*, the anthers were emasculated by removing them one day prior to pollination and then cross pollinated by applying fresh pollens from one anther locule to each flower stigma. The experiment was performed on the main inflorescence axis of plants that had produced at least 3 fully mature flowers.

### Callose staining to monitor pollen tube growth

Callose staining was performed according to Vogts method (1994) with minor modifications. Crossed pistils were harvested 6 hrs after pollination, fixed in ethanol/acetic acid (3 : 1, v/v) for 16 hrs, rinsed with 1 M phosphate buffer, pH 7, and incubated in 8 N NaOH for 3 hrs to clear the tissues. Pistils were stained with decolorized 0.1% aniline blue in 0.1 M K-phosphate buffer, pH 9, for 2 hrs, infiltrated with 100% glycerol for 1 hr, and mounted on glass slides. At least 50 pistils were examined to determine pollen tube growth per genotype. Pollen tubes in the pistil were observed with a fluorescence microscope (Olympus, Japan), emitted at 405 nm and photographed with Kodak Tmax 400 ASA. The experiment was carried out in triplicate.

**Fig 1.** Photograph of pollen tubes growing in a pistil of *A. thaliana*, 16 hrs after pollination.

### *In vitro* pollen tube growth

The pollens were harvested from the first flower in each fully mature flower bud. Pollen tubes were grown on a pollen germination medium consisting of 15% (w/v) sucrose, 4.4 mM $CaCl_2$, 2 mM $H_3BO_4$, 10 mg/l myo-inositol, 100 mg/l MES, pH 7.0, solidified with 1% (w/v) agar, and incubated for 4 hours. Germination rates and pollen tube lengths were checked in 300 pollens. The experiment was repeated three times and the values were averaged.

## RESULTS

To determine the effect of flavonoids on pollen germination and pollen tube growth, we placed pollens on the stigma of a flavonoid-deficient chalcone synthase mutant (tt4), a sinapate ester deficient mutant (fah1-7), and a wild type flower. Flowers were used when the first flower had fully matured from primary inflorescence but just before they opened. 6 hrs after pollination, the number and length of growing pollen tubes were estimated under a fluorescence microscope (Fig. 1). Most pollens germinated and penetrated into pistils after pollination. But the length of the pollen tubes significantly differed in the

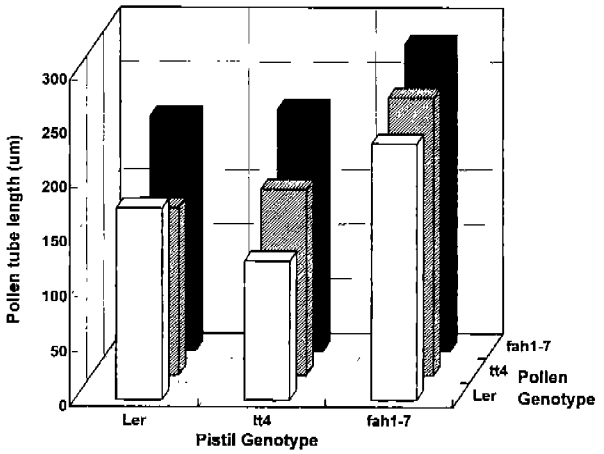


**Fig. 2.** Length of pollen tubes in the pistil of each genotype pollinated with the pollens of Ler, tt4 or fah1-7.

three genotypes (Fig. 2). In the pistils of fah1-7, tube growth occurred more rapidly than in tt4 or the wild type. Pollens from fah1-7 also developed longer tubes than those of other types.

To measure the pollen germination ratio, a medium for pollen germination was devised. Major components, including boric acid (2 mM) and calcium chloride (4.4 mM), were adjusted to provide a medium that supported pollen germination. In the medium, nutrients were omitted but an unusually high concentration of sucrose (15%) was provided.

Pollens of tt4, fah1-7 and wild type were germinated on the media and their germination rates were estimated along with tube lengths (Fig. 3). While germination was more frequent in the tt4 pollens, the tube length of Fah1-7 at about 240 μm was greater than that of tt4 and wild type tubes at 170 μm and 190 μm, respectively. Such genotype dependency in pollen tube growth forced us to examine the effects of flavonol and sinapate ester which are deficient in tt4 and fah1-7, respectively. So, quercetin and sinapinic acid were added in various concentrations to the media. An addition of 10 to 50 μM of quercetin doubled the pollen germination rate in tt4 (Fig. 4A). In the wild type and fah1-7, the pollens also show an enhancement of more than 30% in germination ratio. Tube length under the same conditions showed similar results but less strongly (Fig. 4B). However, the growth of pollen tubes in tt4 was affected the most significantly by exogenous quercetin treatment. Addition of sinapinic acid to the medium causes a decrease in the pollen germination ratio and tube length in all genotypes (Fig. 4C, 4D). While the pollen of the wild type was significantly inactivated at 2.5 μM of sinapinic acid, the pollens of fah1-7 revealed some resistance to the treatment of sinapinic acid so that they were still capable of germinating at a relatively high ratio. However, the addition of 25 μM of sinapinic acid completely inhibited pollen elongation in all genotypes.

## DISCUSSION

Thousands of flavonoid structures lead us to search for the functions that are carried by such diverse compounds. The mutant tt4, which contains neither flavonols nor anthocyanins, was blocked at a very early step of the biosynthetic pathway (Chapple *et al*, 1992). The mutant fah1-7 fails to accumulate any kind of sinapate esters in either leaf or seed tissue (Chapple *et al*, 1992) and is thought to be defective in ferulate-5-hydroxylase activity (Grand 1984).

The requirement for flavonols during germination has been well evaluated in petunias (Vogt *et al*, 1994). However, flavonoid deficient *Arabidopsis* do not show male sterility even though the mutants failed to produce flavonol (Li *et al*., 1993). Thus, it is suggested that there may be other mechanisms than the flavonoids involved in pollen germination and pollen tube growth in *Arabidopsis*.

To clarify this discrepancy, we have investigated pollen germination in the pistils of a flavonoid deficient chalcone synthase mutant (tt4) of *Arabidopsis* and found little evidence for flavonoid essentiality. This means that pollen germination still occurs in the absence of flavonols regardless of the germination efficiency in *Arabidopsis*. Therefore, the elongation of pollen tubes was carefully examined in tt4 pistils and fah1-7 because its flavonoid biosynthetic pathway is interrelated with the sinapate ester pathway. It was reported that increased amounts of some intermediate in the sinapate ester pathways may accumulate in tt4 (Li 1993).

While tube elongation occurs most rapidly in fah1-7 pistils, wild type pollens in tt4 pistils show the slowest growth of the tubes relative to any other combinations. These results show that the accumulation of flavonoids in the pistils rather than in pollens is more important for tube growth. In addition, the fact that fah1-7 had the longest pollen tubes suggests the presence of a positive or negative effector for tube elongation.

*In vitro* results showed significant differences in pollen tube growth among the three genotypes. The addition of quercetin to the medium results in dra-

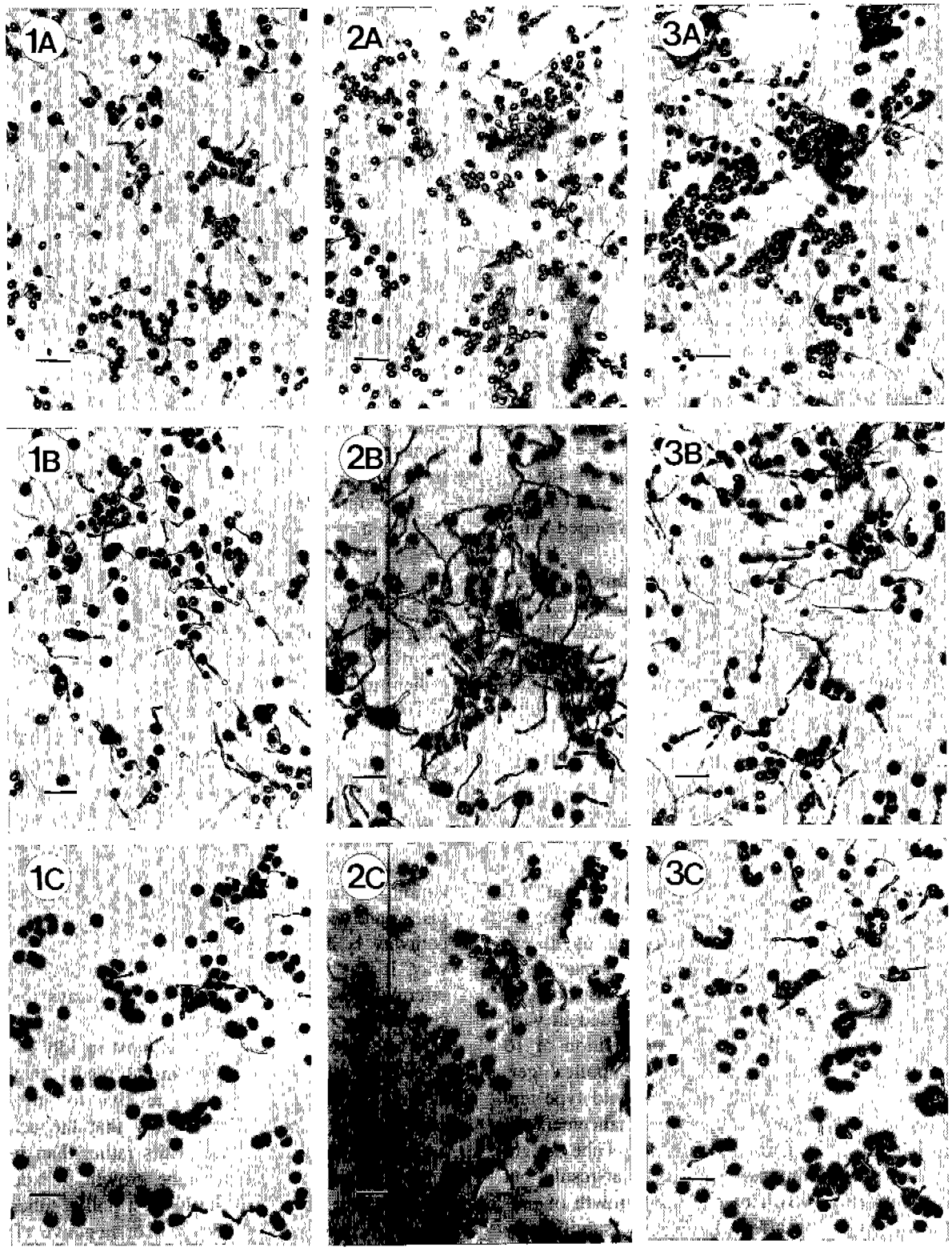


**Fig. 3.** Photographs of germinated pollen in Ler, tt4 and fah1-7 after 4 hrs on the germination medium.
1A, Ler pollens as control; 1B, tt4 pollens as control; 1C, fah1-7 pollens as control; 2A, Ler pollens on medium containing 10 μM quercetin; 2B, tt4 pollens on 10 μM quercetin; 2C, fah1-7 pollens on 10 μM quercetin; 3A, Ler pollens on medium containing 10 μM sinapinic acid; 3B, tt4 pollens on 10 μM sinapinic acid; 3C, fah1-7 pollens on 10 μM sinapinic acid.

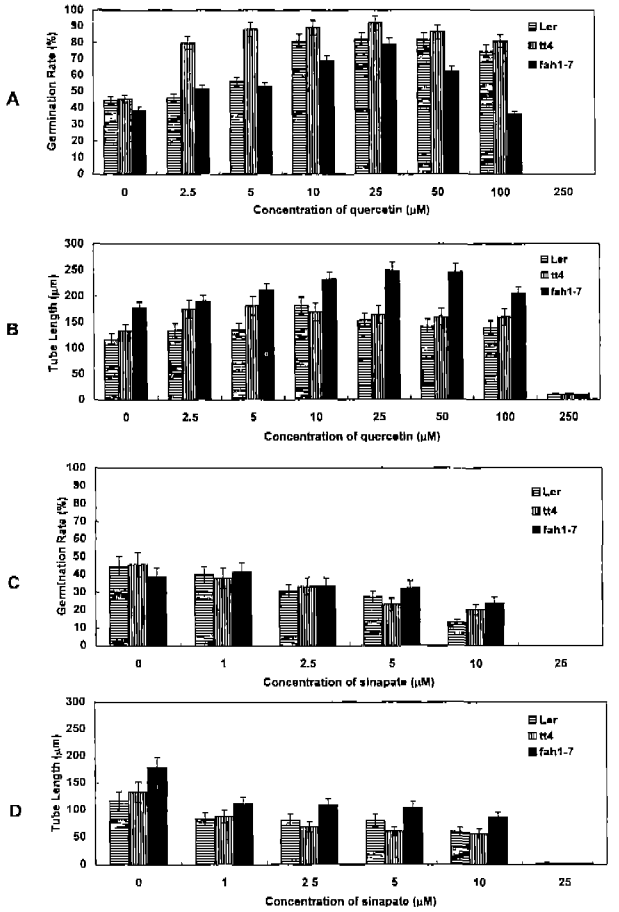


**Fig. 4.** Germination of pollens and pollen tube growth in wild type, tt4 and fah1-7 on the germination medium containing various concentration of quercetin and sinapinic acid. A, Germination of pollens on the medium containing quercetin; B, Pollen tube length on the medium containing quercetin; C. Germination of pollens on the medium containing sinapinic acid ; D. Pollen tube length on the medium containing sinapinic acid. * The data represent the averages from nine hundred pollens with standard deviation. * Pollens were incubated for 4 hrs on the medium.

matic increase of germination rate, most significantly in tt4. It shows that the presence of flavonol is important when the pollens are germinating. In contrast, the addition of synapinic acid significantly inhibited pollen tube growth in three genotypes. However, the germination rate of fah1-7 pollens, in which endogenous sinapate esters are not accumulated, did not seem to be seriously affected. This suggests that a main product of the sinapate ester pathway is an inhibitor for the elongation of pollen tubes.

Conclusively, the role of flavonol such as quercetin is important in pistils for pollen tube elongation. Also, it seems that some intermediate in the sinapate ester pathway may be involved in pollen tube elongation. The addition of caffeic acid to the media results in a dramatic increase of pollen tube growth (Song, unpublished data). In our next study, we will examine the effects of several intermediates in the flavonoid and sinapate ester pathways on pollen tube growth.

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