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Short communication

Flavonols are *not* essential for fertilization in *Arabidopsis thaliana*

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Abstract

Flavonols are plant metabolites suggested to serve a vital role in fertilization of higher plants. Petunia and maize plants mutated in their flavonol biosynthesis are not able to set seed after self-pollination. We have investigated the role of these compounds in *Arabidopsis thaliana*. Like in all other plant species, high levels of flavonols could be detected in pollen of wild-type *A. thaliana*. No flavonols were detected in reproductive organs of the *A. thaliana* tt4 mutant in which the *chs* gene is mutated. Surprisingly, this mutant did set seed after self-fertilization and no pollen tube growth aberrations were observed *in vivo*. The role of flavonols during fertilization of *Arabidopsis* is discussed.

Abbreviations: CHS, chalcone synthase; TLC, thin-layer chromatography.

Chalcone synthase (CHS), the key enzyme in flavonoid biosynthesis, catalyses the condensation of 3 malonyl-CoA molecules with one coumaroyl-CoA molecule to chalcone. Chalcone is a central intermediate in the synthesis of flavonoids, a group of plant metabolites comprising anthocyanins, flavones, flavonols and isoflavonoids. Flavonols are detected in pollen of all higher-plant species tested so far, and promote tube growth in a wide range of species, including Cruciferae [9, 11]. Sedgley [7] has reported that the flavonol quercetin enhances tube growth of *Brassica oleracea* pollen. For maize and petunia it was demonstrated that mutants with blocked *chs* gene activity are self-sterile [4, 6, 11]. This sterility is due to an aberrant pollen tube growth, which could be restored by externally applied flavonols.

For *Arabidopsis thaliana* a *chs* mutant, designated *tt4-1*, was previously obtained by ethylmethane sulfonate (EMS) mutagenesis. RFLP mapping revealed that the *TT4* locus on chromosome 5 coincides with the position of the *chs* locus [2, 8]. It has been reported that *Arabidopsis* contains only one *chs* gene and that *tt4* would lack flavonols in vegetative tissues [2, 3]. However, the presence and role of flavonols in reproductive organs of *Arabidopsis thaliana* has not been studied.

To monitor the presence of flavonols in mature *Arabidopsis thaliana* and in the *tt4-1* mutant cv. Landsberg erecta pollen, a thin-layer chromatography (TLC) analysis was conducted. The TLC procedure was performed according to van der Meer *et al.* [4] and compromises a hydrolysation step in order to concentrate the different glycones into one aglycone spot thereby enhancing sensitivity. The chromatography was performed in a HAc/37%HCl/H_2O solution (30:3:10) after which the plates were sprayed with 0.05% NH_3 and diphenyl boric acid 2-amino-ethyl ester, to further enhance the detection level. High levels of the flavonols kaempferol and quercetin were detected in pollen of *Brassica napus* and wild type *Arabidopsis thaliana*, but not of mutant *tt4* (Fig. 1A).

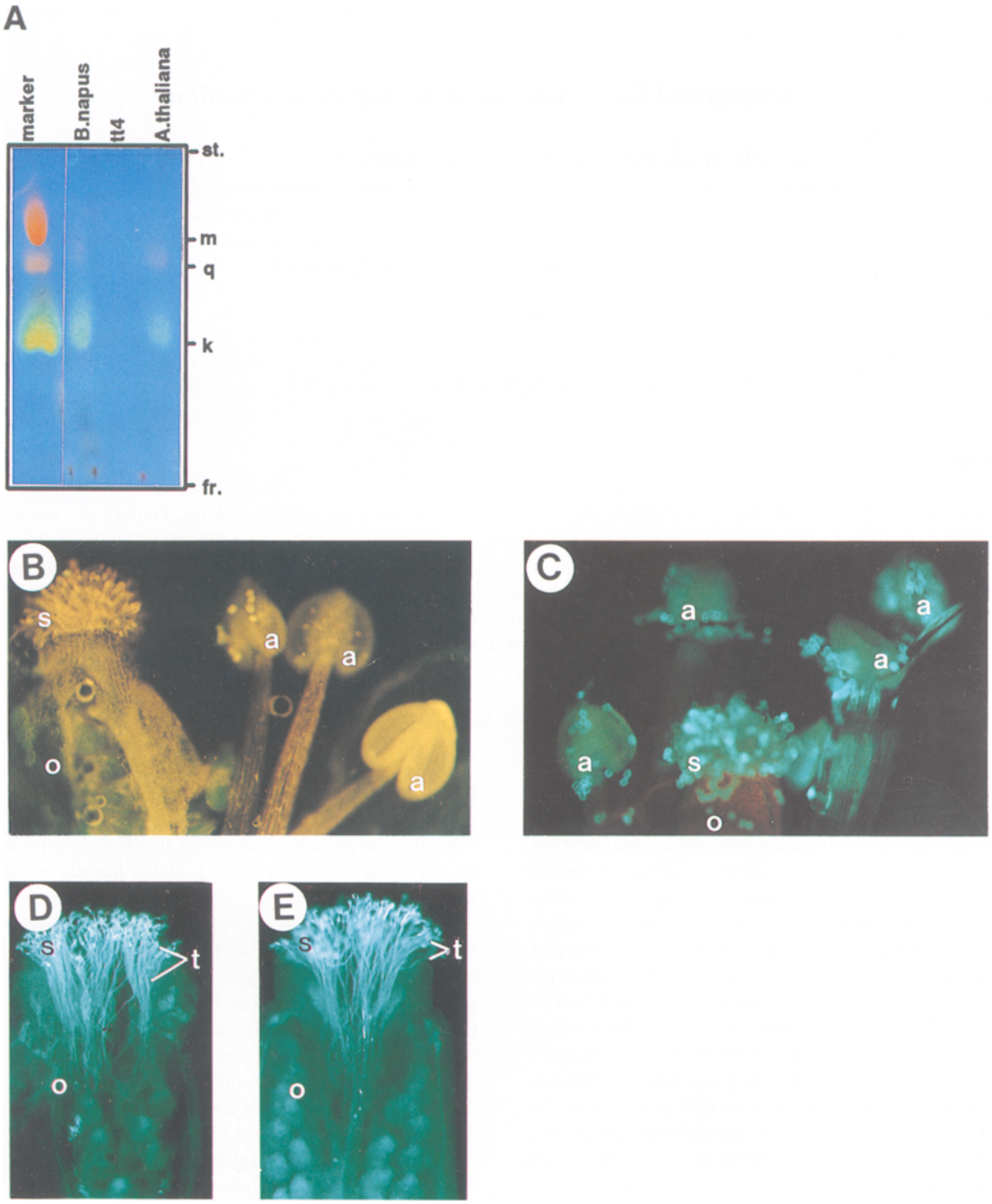

Figure 1. Analysis of flavonols and pollen tube growth in wild-type and mutant *tt4 A. thaliana* plants. To support an optimal fertility, plants were grown under controlled conditions, with 16 h of continuous light at 20 °C and 70% relative (RH) humidity decreasing in the night to 18 °C and 47% humidity. A. TLC analysis of *A. thaliana*, *tt4* mutant and pollen extracts. A flavonoid extract from *Brassica napus* pollen was loaded for comparison. From left to right: marker, *Brassica napus* (B. napus), *tt4* mutant, and wild-type *A. thaliana*. In addition to the RF values, the colours of the spots are indicative. B. Localization of flavonols in the reproductive organs of wild-type *A. thaliana*. C. Localization of flavonols in the reproductive organs of *A. thaliana tt4* mutant. D. Pistil and pollen with tubes of wild type after 4 h of growth. E. Pistil and pollen with tubes of *tt4* mutant after 4 h of growth. Abbreviations used: m, myricetin; q, quercetin; k, kaempferol; st, start; fr, front; a, anthers; o, ovary; s, stigma; t, pollen with tubes.

To localize flavonols in reproductive organs, diphenyl boric acid 2-amino-ethyl ester was used as a histochemical stain. Therefore, complete flowers of wild-type and mutant *tt4* were incubated for ca. 5 min in a saturated solution of the ester with 0.01% triton [11]. Presence of flavonols was analysed under an UV microscope and appeared as orange fluorescence in petals, anthers, pollen and pistils of wild-type *A. thaliana* flowers. In contrast, no flavonols could be detected in any of reproductive organs of *tt4* (cf. Fig. 1B and 1C). It furthermore indicates that the *tt4* mutation blocks the synthesis of flavonols and that no other (iso-)enzymes are active in reproductive organs.

Surprisingly, despite the absence of flavonols, the fertility of the *tt4* mutant did not seem to be severely affected. The amount of seeds in 10 siliques per plant from 30 wild-type or 30 *tt4* mutant plants was determined. The average for wild-type *Arabidopsis* was 607 seeds per 10 siliques (standard deviation (SD) 86.8) compared with 518 seeds for the *tt4* 30 mutant plants (SD 109.1). This difference (15%) is significant but the reduction is not so drastic as observed for the petunia or maize flavonol mutants [4, 6, 11].

To investigate the fertility of the *tt4* mutant in further detail, pollen tube growth was precisely monitored *in vivo*. Therefore, mature closed flowers were emasculated and self-pollinated the following morning. After different time intervals of 0.5 h, 1 h, 2 h, 4 h, 10 h and 24 h pistils were harvested, transferred to 1 M KOH and incubated overnight at 60 °C. After rinsing pistils with water, they were placed on a microscopic slide glass in a 50% glycine, 0.05% aniline solution to stain callose plugs in pollen tubes that were visualized by UV light. The amount of pollen germinating and the velocity of tube growth in the *tt4* mutant was indistinguishable from wild type (Fig. 1D and 1E).

From the experiments described here we conclude that, in contrast to the petunia and maize flavonol mutants, *A. thaliana* does not require *chs* expression or the presence of flavonols for fertilization. It appears quite contradictory that both petunia and maize require flavonols for proper seed-set and *A. thaliana* does not. This discrepancy may be explained by differences in the physical distance pollen tubes have to bridge. In the sterile petunia *chs* mutant, T17.02, pollen tubes grew a few millimeters in absence of flavonols [11]. In line with our results Burbolis *et al.* [1] found recently that fertility in their *Arabidopsis thaliana tt4* mutant was unaffected. Styles of *Petunia hybrida* cv. W115 are 4 cm in length and therefore fertilization might not occur. Style length of *A. thaliana* is only a few millimeters which might enable pollen tubes from a flavonol mutant to reach the ovules. An alternative explanation would be that in *A. thaliana* other phenylpropanoids or components like (brassino-)phytosteroids can compensate for a lack of flavonols [3, 5, 12]. In order to test this hypothesis we are currently studying the role of flavonols during fertilization in other Brassicaceae.

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