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Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen

(pollen germination/pollen tube/maize/petunia)

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ABSTRACT **Chalcone synthase catalyzes the initial step of that branch of the phenylpropanoid pathway that leads to flavonoids. A lack of chalcone synthase activity has a pleiotropic effect in maize and petunia mutants: pollen fertility as well as flavonoid synthesis is disrupted. Both maize and petunia mutants are self-sterile due to a failure to produce a functional pollen tube. The finding that the mutant pollen is partially functional on wild-type stigmas led to the isolation and identification of kaempferol as a pollen germination-inducing constituent in wild-type petunia stigma extracts. We show that adding micromolar quantities of kaempferol to the germination medium or to the stigma at pollination is sufficient to restore normal pollen germination and tube growth *in vitro* and full seed set *in vivo*. Further we show that the rescue ability resides in particular structural features of a single class of compounds, the flavonol aglycones. This finding identifies another constituent of plant reproduction and suggests that addition or removal of the flavonol signal during pollen germination and tube growth provides a feasible way to control plant fertility.**

In the postdispersal phase of male gametophyte development, pollen germinates on the stigma and extrudes a tube through a germination pore in the pollen wall. In flowering plants the pollen tube is a conduit for the migration of the two sperm cells through the stylar tissue to the embryo sac where they fuse with the egg and central cell nuclei forming the zygote and endosperm, respectively (1). Pollen-tube growth is confined to the extreme tip and is characterized by intense metabolic activity, including the rapid synthesis of wall components and plasmalemma precursors (2).

When released from the anther, pollen is a two- or three-celled spore containing the stored products of sporophytic gene expression, some of which arise from the inner layer of the anther wall (tapetum), and the products of haploid gene expression from the vegetative and/or generative cell within each grain (3, 4). The factors that regulate male gametophyte development between pollination and fertilization may originate from the pollen grain or from interactions between the pollen and pistil (stigma, style, and ovary) (2–4). Pollen–pistil interactions not only promote growth, but they also provide a recognition system to regulate fertilization. Self-incompatibility is a genetically controlled system, exhibited by about half the angiosperm families, which functions to prevent self-fertilization via arrest of pollen germination or tube growth (5, 6).

Flavonoids are an abundant class of small-molecular-weight (≈300) plant-specific metabolites that share a common skeletal structure of 15 carbon atoms (7). Pollen flavonoids have been detected in several species, where they impart a distinctive yellow color to pollen and can account for a large percentage (2–4%) of the dry weight (8–10). Analyses of petunia-pollen extracts have identified the major flavonoids as 3-*O*-glycosides of kaempferol and quercetin, 4,2′,4′,6′-tetrahydroxychalcone, and a dihydroflavonol, taxifolin (10–12). Maize pollen contains at least 10 glycosides of kaempferol, quercetin, and isorhamnetin (13).

Chalcone synthase (CHS) catalyzes the first step in flavonoid biosynthesis, the condensation of *p*-coumaroyl-CoA with three molecules of malonyl-CoA to form naringenin chalcone (14). The observation that maize and petunia mutants lacking CHS activity were not only deficient in flavonoids but were also male sterile suggested that flavonoids might be involved in pollen fertility. Recessive mutations in both CHS genes of maize, *C2* and *Whp*, produce the white pollen phenotype (15). Instead of functional yellow pollen, the mutant plants produce viable but nonpigmented white pollen that is male sterile in self-crosses (ref. 15; L.P.T., unpublished data). A similar phenotype (16) is exhibited by petunia plants down-regulated for CHS expression after the *Agrobacterium*-mediated introduction of an extra CHS gene (17). Microscopic examination of self-pollinated pistils of both the naturally occurring maize mutant and the genetically engineered petunia shows that the self-sterility results from a similar reproductive defect: the failure to produce a functional pollen tube [ref. 16; S. A. Modena, personal communication in (1982) *Maize Newsletter* **56**, 48].

Only pollen is affected; pistils of CHS-deficient plants are fully fertile when crossed by wild-type pollen. Most provocatively, the reciprocal cross in petunia showed that the CHS-deficient white pollen, which never functions on self-stigmas, could function on wild-type stigmas (16). When white pollen grains were placed on wild-type (inbred V26) stigmas, we observed white pollen tubes growing in the V26 styles 48 hr after pollination (L.P.T., unpublished data) and 30% of normal seed-set (16). This reciprocal effect is also seen in maize, but the frequency of seed set is highly dependent on the genotype of the female parent [E. H. Coe, Jr., personal communication in (1983) *Maize Newsletter* **57**, 37; L.P.T., unpublished data]. We use the term conditional male fertility (CMF) to describe the state whereby white CHS-deficient pollen is functional on wild-type stigmas but is not functional on CHS-deficient stigmas.

These results suggested that (*i*) CHS-deficient white pollen lacks one or more factors required for pollen-tube growth, and (*ii*) wild-type stigmas contain these diffusible factors that can biochemically complement pollen function at pollination. We developed an *in vitro* pollen-germination rescue assay based on this observation and used it to isolate and identify flavonols, a specific class of flavonoids, as the active compounds necessary and sufficient to produce functional pollen tubes in CHS-deficient petunia. Furthermore we show that it is feasible to control fertilization in maize and petunia by adding flavonols at pollination.

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Abbreviations: CMF, conditional male fertility; CHS, chalcone synthase; GM, germination medium; DMSO, dimethyl sulfoxide.

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MATERIALS AND METHODS

Plant Material and Genetic Crosses. All transgenic petunia plants displayed the CMF phenotype: no visible pigmentation, self-sterile, and partially fertile when crossed to wild-type stigmas (inbred V26) (16). Plant and pollen phenotypes, growth conditions, and pollen collection procedures for the V26 and CMF petunia are described in ref. 16. Biochemically complemented self-crosses were done by emasculating flowers 24 hr before application of ≈50 μg of flavonoid to the stigma, followed 12–24 hr later by pollination with CMF pollen. Flowers were bagged to prevent contamination.

The maize white pollen plants have stable recessive mutations at *C2* and *Whp* introgressed into a W23 inbred background. The plants are male sterile in self and sibling crosses and produce no visible flavonoid pigments in any tissues, including pollen and seeds (ref. 15; L.P.T., unpublished data). Standard genetic field practices were used to ensure that no contaminating pollen reached the silks of the white pollen plants. Mutant white pollen from 50–100 plants was collected, pooled, and divided into two portions. One portion was used "as is," and the other was mixed in an approximate 20:1 ratio with flavonoids (either quercetin, kaempferol, or a 50:50 mixture). The white pollen silks were bagged immediately after pollinating with either the untreated or the flavonoid-supplemented pollen. Mature ears were harvested 45 days after pollination.

Pollen-Rescue Assay. Petunia pollen grains were suspended in PEG 4000 germination medium (GM) (18) at a density of 1–2 × 10^4 grains per ml, and 100-μl samples were incubated at 25°C with shaking (150 rpm) in wells of a 96-place microtiter plate. Supplements in dimethyl sulfoxide (DMSO) were added to the GM before pollen addition. The concentration of DMSO was held constant in each assay at 1%. Pollen was scored as germinated when the tube was >1 pollen-grain diameter long. After 4-hr incubation a minimum of 1000 pollen grains were scored in each assay. Chemicals were obtained from Sigma, Extrasynthese (Genay, France), Spectrum Chemical (Gardena, CA), and Aldrich, except naphthylphthalmic acid, which was from Timothy Short (Carnegie Institution of Washington), and 4,2′,4′,6′-tetrahydroxychalcone, which was synthesized from naringenin according to ref. 19. Flavonoid purity was ascertained by HPLC analysis and repurified by collection of the appropriate peak where necessary.

Plant Extract Preparation. Aqueous extracts of V26 and CMF stigmas and pollen were made by mincing the stigmas in GM (100 per ml) or by mixing a pollen suspension in GM (50 mg/ml), centrifuging 5 min in a microcentrifuge, and applying samples of the supernatant to a CMF pollen suspension in GM. Similarly prepared methanol extracts were dried under vacuum and resuspended in GM before addition to the pollen suspension. To characterize the active component, the aqueous V26 stigma extract was heated (100°C, 5 min) or treated with 0.025 unit of papain for 30 min at 37°C in a 100-μl reaction volume before adding to the *in vitro* germination assay. The molecular mass of the active compound in V26 stigmas was estimated by passing the extract through a cutoff filter with a 3000-Da discrimination (Centricon-30, Amicon) and assaying the pollen-rescue activity of the filtrate.

Isolation and Identification of Kaempferol in Petunia Extracts by HPLC Analysis. Stigmas (300), anthers (500), or pollen (100 mg) was mascerated in a mortar and pestle with 3 ml of 50% methanol and then with 3 ml of 100% methanol; the extracts were pooled and concentrated to 200 μl. Aglycones were produced by acid hydrolysis (7). Replicate samples were analyzed at room temperature on a reverse-phase C_{18} column (Phenomenex Spherisorb 5 ODS 250 × 4.6 mm) with a solvent flow rate of 0.5 ml/min. Solvent A was 5% acetic acid, and solvent B was 5% acetic acid/80% acetonitrile. The gradient was 20% B (6 min), linearly increased to 95% B (20 min) and 95% B (14 min). Detection was at 360 nm with a Hewlett–Packard model 1040A photodiode-array detector. Kaempferol was identified in the plant extracts by retention time and UV/visible spectral comparisons with authentic kaempferol. Peaks of interest were collected and tested for rescue ability; identity was confirmed by rechromatography.

RESULTS

Biochemical Complementation of Pollen Function. Initial rescue experiments demonstrated that a diffusible factor in wild-type petunia pistils could restore pollen germination and tube growth. A one-fifth volume sample of an aqueous extract prepared from 10 V26 stigmas elicited a 33% germination rate when added to a suspension of CMF pollen in GM (data not shown). V26 pollen and anther extracts were also able to restore germination and tube growth to the CMF pollen, but no activity was associated with extracts from the CMF stigma, anthers, or pollen. The active component in wild-type stigma extracts was heat stable and behaved as a nonproteinaceous molecule with a molecular mass <3000 Da. Collectively, these results suggested that (*i*) one or more of the flavonoids normally present in wild-type pollen are required for germination and tube growth; (*ii*) the CMF pollen is deficient in these compounds; and (*iii*) the compounds can be supplied at pollination to restore pollen function.

Biochemical complementation of the CHS-deficient petunia pollen was achieved by adding a low concentration (1 μM) of kaempferol, a flavonol aglycone, to a suspension of CMF pollen in GM (Fig. 1). Side-by-side comparisons made throughout a 12-hr growth period confirmed that germination initiated simultaneously and that tube growth proceeded at

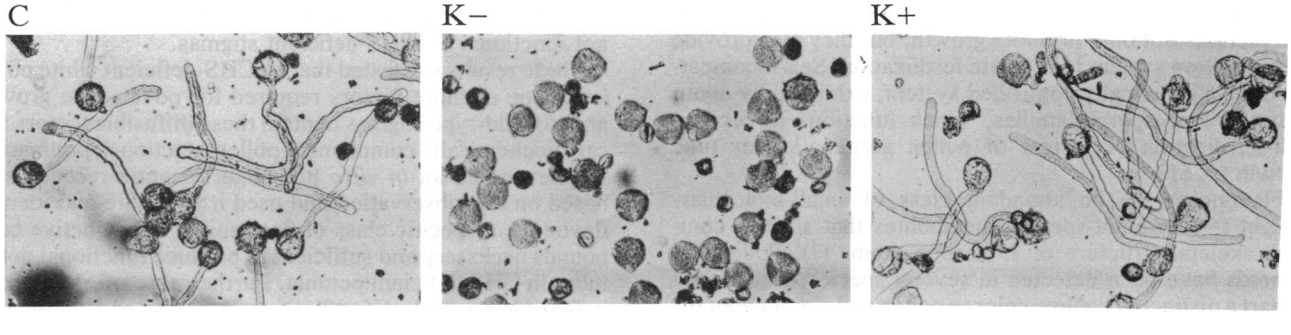


FIG. 1. Restoration of pollen germination and tube growth to petunia CHS-deficient pollen by kaempferol. Pollen was collected from CMF anthers and suspended in GM; kaempferol (K+) or DMSO (K−) was added to 1 μM final concentration. Representative fields are pictured after 4-hr incubation. The germination and tube growth seen in the kaempferol-rescued CMF pollen (K+) are indistinguishable from wild-type V26 control (C), which received only DMSO. Nonsupplemented CMF pollen (K−) shows swelling at the germination pore in some grains, but no pollen tubes are extruded. (×120.)

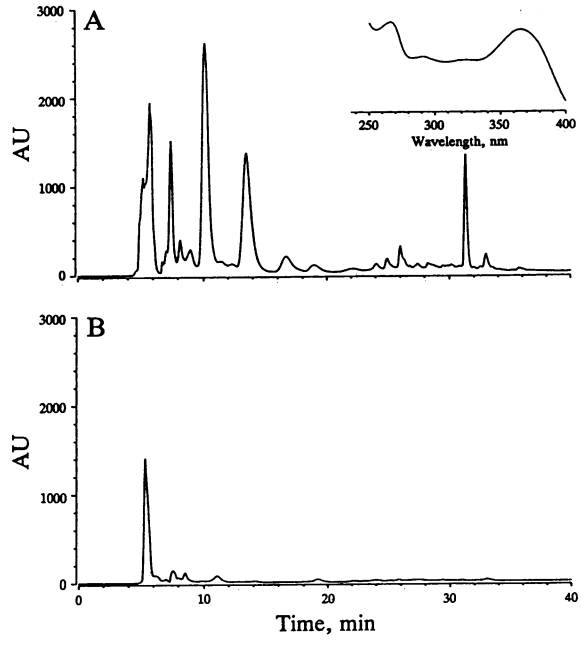
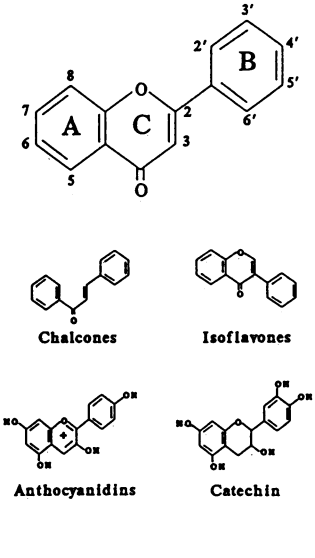


FIG. 2. HPLC profiles of methanolic extracts of wild-type V26 stigmas (*A*) and CMF stigmas (*B*). Absorption at 360 nm of 100-μl aliquots of extracts prepared from 150 stigmas and fractionated in a methanol/water gradient on a reverse-phase C_{18} column. (*A Inset*) UV/visible spectrum of the peak at 33.2 min; this spectrum is identical to that produced by an authentic kaempferol standard. An HPLC profile and UV/visible spectrum of an acid-hydrolyzed V26 stigma extract indicates that the major peaks at retention time 7.4, 10.1, 13.5, and 16.7 are glycosides of kaempferol and quercetin. The solvent-front peak occurs in both chromatograms at 5.6–5.8 min. AU, relative absorbance units.

the same rate and to the same extent in the rescued CMF pollen compared with wild-type V26 pollen that received no flavonol supplement. The flavonoid-supplemented pollen showed an 80% germination frequency relative to the V26 pollen. CMF pollen to which only the DMSO solvent was added showed no significant germination (1–2%), and the pollen tubes, if they germinated at all, never progressed >2 pollen-grain diameters.

To confirm that wild-type stigma extracts that can rescue pollen germination and tube growth contain kaempferol, an unhydrolyzed extract was fractionated by HPLC and analyzed by UV/visible absorption spectroscopy. A peak with a retention time and typical flavonol spectrum (absorption maxima ≈260 and 360 nm) was detected in the V26 stigma extract (Fig. 2*A* and *Inset*). This putative kaempferol peak was collected, evaporated to dryness, resuspended in DMSO, and added to the *in vitro* GM, where it elicited a full germination and tube-growth response from CMF pollen. Re-chromatography of this active fraction with an authentic kaempferol standard confirmed its purity and identity. From this analysis, we calculate the amount of kaempferol in V26 stigmas at 60 ng per stigma. By assuming a stigma volume of 34 μl (volume displacement), we estimate flavonol concentration in a V26 stigma at ≈6 μM, a level capable of eliciting a strong germination response. An identical analysis on extracts from 150 CMF stigmas or from 500 CMF anthers yielded no peaks, having a typical flavonoid spectrum (Fig. 2*B*). Extracts from V26 pollen and anthers produced a chromatogram similar to that shown in Fig. 2*A*, and the peak, with a retention time and UV/visible spectrum indicative of kaempferol, fully stimulated CMF pollen germination. This analysis confirms that kaempferol is also present in wild-type pollen and anthers (data not shown).

Structural Features Required for Pollen-Rescue Activity. Wild-type pollen and stigma extracts from petunia contain other compounds, in addition to kaempferol, which may also stimulate pollen germination and tube growth (Fig. 2*A*; refs. 10–12). Therefore, representatives from all the major classes of flavonoids—flavones, flavanones, flavonols, isoflavonoids, chalcones, anthocyanins, and catechins—were assayed for pollen-rescue activity. Flavonols successfully restored maximal germination frequency and tube-growth capacity to the CMF pollen, but among the other classes of flavonoids only the



Compound	Substitution 3	5	7	2'	3'	4'	5'	Concentration for response (μM)
Flavonols C_2=C_3								
Galangin	OH	OH	OH					1
Kaempferol	OH	OH	OH			OH		1
Iso-rhamnetin	OH	OH	OH		OCH_3	OH		1
Quercetin	OH	OH	OH		OH	OH		10
Morin	OH	OH	OH	OH		OH		10
Myricetin	OH	OH	OH		OH	OH	OH	100
Fisetin	OH		OH		OH	OH		100
3-Hydroxyflavone	OH							>100
Dihydroflavonol C_2-C_3								
Taxifolin	OH	OH	OH		OH	OH		>100
Flavones C_2=C_3								
Flavone								NR
7-Hydroxyflavone			OH					NR
Apigenin		OH	OH			OH		NR
Luteolin		OH	OH		OH	OH		NR
Flavanones C_2-C_3								
Flavanone								NR
Naringenin		OH	OH			OH		NR
Eriodictyol		OH	OH		OH	OH		NR

FIG. 3. Structural features and the pollen-rescue capability of specific flavonoid compounds. At left is the basic flavonoid structure with rings and substitution positions labeled. Response categories are based on the lowest concentration of compound that produces a full germination response. Compounds that caused <20% germination at 100 μM are indicated as >100 μM, and inactive compounds are designated NR. Also pictured is the basic structure of four additional classes of nonresponsive flavonoids analyzed, including catechin, 4,2',4'6'-tetrahydroxychalcone (chalcone), genestein (isoflavone), pelargonidin, and delphinidin (anthocyanidins).

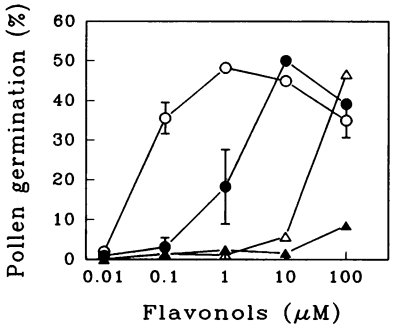


FIG. 4. Pollen-germination frequency as function of increased flavonol aglycone concentration. Kaempferol (○), morin (●), myricetin (△), and 3-hydroxyflavone (▲) were added to the GM at the indicated final concentrations, and germination was scored after 4-hr incubation. Mean germination frequency measured in three separate experiments is plotted with the SEM. SEM values <1.4 are not visible.

closely related dihydroflavonol, taxifolin, produced a modest (≈18%) response at 100 μM (Fig. 3). Additionally, several classes of nonflavonoid compounds were tested including *p*-coumaric, salicylic, chlorogenic, dihydroascorbic, naphthylphthalmic, 1-naphthaleneacetic, indol-3-acetic, gibberellic acids, and hydroquinone. None produced a germination response. Hence, the ability to rescue pollen function at physiologically relevant concentrations appears to reside in the flavonols.

From the range of flavonoids tested, the following general structural characteristics appear to be necessary for maximal pollen germination and tube growth. There are absolute requirements for a keto group at position 4 and an unglycosylated hydroxyl group at position 3 in the C ring [concentrations of quercetin 3-*O*-glucoside and rutin (quercetin 3-*O*-rhamnoglucoside) up to 100 μM produced no response]. A maximal response depends on an unsaturated bond between carbons 2 and 3 in the C ring and the degree of hydroxylation in both the A and B rings.

The requirement for a keto group at position 4 in ring C is indicated by the fact that catechin, which has no keto group, lacks activity. A comparison of the relative efficiencies of taxifolin (≈18% at 100 μM) and quercetin (≈50% at 10 μM) shows that a double bond between carbons 2 and 3 in the C ring increases the response by ≈30-fold. A comparison of quercetin with fisetin or with 3-hydroxyflavone shows that each additional hydroxyl group at either position 5 or 7 on the A ring increases the response ≈10-fold. This increase may depend on the stabilizing effect of an interaction between the 5-hydroxyl group and the adjacent keto group in ring C. Finally, hydroxyl substitutions on the B ring are not necessary for full activity and, in fact, increasing them actually decreases the activity (compare kaempferol with quercetin and myricetin). This difference could be from poor uptake or an increase in nonspecific binding caused by the more polar nature of the flavonols with numerous hydroxyl groups.

A report that some nonactive flavonoids act to antagonize active flavonoid induction of nodulation genes in the *Rhizobium* legume system (20, 21) prompted us to test whether the compounds that were nonactive in rescuing pollen function could antagonize the action of the flavonol aglycones. CMF pollen in GM was exposed to inactive compounds at concentrations of 1 and 10 μM for 30 min before adding kaempferol to 1 μM. The experiment was also done by simultaneously adding both the inactive compound and kaempferol, at 1:1 or 10:1 ratios, to the pollen suspension. No antagonizing action was detected from any of the following inactive compounds: apigenin, chalcone, eriodictyol, flavone, flavanone, luteolin, naringenin, catechin, chlorogenic acid, *p*-coumaric acid, hydroquinone, and salicylic acid.

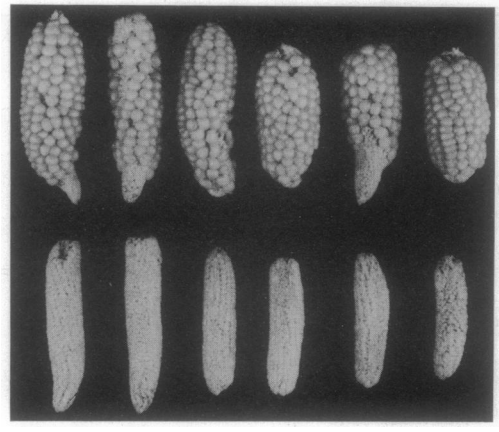


FIG. 5. White pollen self-crosses 45 days after pollinations done with (top row) or without (bottom row) flavonols. Kaempferol, quercetin, or a mixture of the two was added to the pollen at pollination. Fecundity of the biochemically complemented self-crosses is the same as white pollen backcrosses.

A comparison of the pollen-rescue ability of four flavonol aglycones showed that some flavonols are more potent than others (Fig. 4). The minimum concentration of flavonol required to produce the maximum germination frequency ranged over three orders of magnitude from a low of 1 μM for kaempferol to morin at 10 μM and to myricetin at 100 μM. One flavonol, 3-hydroxyflavone, never achieved a maximal germination frequency, even at 100 μM. Concentrations >100 μM were tested but appeared to have deleterious effects on the pollen and were not examined further.

The flavonol exposure required for complete germination and maximal tube growth was determined by scoring percent germination and tube length in aliquots of kaempferol-supplemented CMF pollen collected at timed intervals. Although a measurable increase in germination was detected in 10 min, between 1- to 2-hr exposure was required for a maximum response (data not shown).

***In Vivo* Complementation Leads to Successful Fertilization.** The restoration of *in vitro* germination to the flavonoid-deficient pollen by flavonol aglycone implies that these compounds function critically during pollen germination and tube growth. If they perform similarly *in planta*, self-fertility should be restored to the CMF mutants by supplying the flavonol aglycone at pollination. This hypothesis was tested by scoring the successful fertilizations that resulted from self-crosses of the CMF petunia done with added flavonols. We performed 47 self-crosses with added kaempferol or quercetin, and nearly 60% (27 of 47) produced seed capsules (data not shown). The number of seeds per capsule varied from 31 to 287, and in germination tests >90% of the seeds in any single pod were viable. All self-crosses done without added flavonols (>30 trials) yielded no seed set. We used the linked kanamycin-resistance marker (17) to test for segregation of the CMF character in the seeds produced from the flavonol-complemented crosses. A total of 221 seeds from three different capsules borne on three different plants were germinated with 100 μg of kanamycin, and they segregated in a 3:1 ratio of kanamycin resistance/sensitivity as expected for a dominant trait.

Even more dramatic results were obtained from a large-scale field trial involving 105 maize white pollen plants. A total of 58 self-crosses were performed with added flavonols, and all (100%) produced fully filled ears, whereas self-crosses (47 trials) done without added flavonols showed seed set <1% of normal (Fig. 5). These experiments show that in two widely divergent species, one a monocotyledon and the other

a dicotyledon, addition of flavonols alone at pollination can restore full function to flavonoid-deficient pollen.

DISCUSSION

Our results support a role for flavonols in functional pollen. Methanol and aqueous extracts of wild-type stigmas and pollen are capable of fully restoring germination and tube growth to flavonoid-deficient pollen. These extracts contain the same flavonols that are active in our bioassay. The ability to rescue pollen germination and restore full tube growth *in vitro* and full seed set *in vivo* is restricted to the flavonol aglycones. The concentrations that are active (<10 μM) are well within the concentrations of flavonol aglycones found in wild-type stigma.

Although the predominant form of plant flavonols, including petunia and maize pollen, is the 3-*O*-glycosylated species (Fig. 2*A*) (10, 13, 22), we found that only the aglycone can rescue pollen function. From our HPLC analysis of V26 stigmas (Fig. 2*A*) we estimate that the flavonol glycosides are 10-fold more abundant than the aglycone. Our *in vitro* analysis of the concentration and exposure time required to elicit a maximum response suggests that the amount of kaempferol in wild-type pollen is sufficient to support germination and tube growth for, at least, the first few hours after pollination. The bulk of the 3-*O*-glycosylated flavonols in the pollen may act as a reservoir, requiring only the action of a glycosidase to generate the aglycone. We have detected glycosidase activity in petunia stigmas, but the substrate specificity for glycosylated flavonoids has not been determined.

In most plant tissues, flavonol glycosides are localized in the vacuoles of epidermal cells (22), and the more nonpolar flavonol aglycones are principally found on external plant surfaces, often in association with other lipophilic secondary products (23). The likely source of pollen flavonols is the tapetal cell of the anther wall. Late in pollen development the tapetal layer disintegrates, and the cellular contents are expelled into the anther locule, coating the outer layer of the pollen grain (24). Our data showing that the hydrophobic flavonols are the only flavonoids capable of inducing pollen germination and that they can be externally applied to the grain suggest that they are either rapidly internalized or function externally. It will be necessary to determine their subcellular accumulation site in germinating pollen as a first step toward understanding how flavonols promote germination.

The precise structural requirements for rescue activity may be indicative of flavonol–protein binding constraints. The phenomenon described here is suggestive of a signaling system based on flavonols that operates to stimulate germination. This system shares features with the flavonoid induction of the nitrogen-fixing *nod* operon in *Rhizobium* (25) and steroid-hormone action (26). In the latter example the small nonpolar steroids pass through the lipid bilayer to accumulate within a cellular compartment, where they interact with a transcription-regulating protein. Formation of the steroid–protein complex alters gene expression, which leads to dramatic developmental changes. It is of note that we have measured an increase in pollen transcripts in direct response to germination-inducing flavonols but not in response to inactive flavonoids.

Flavonoids have been identified in pollen extracts from several plant families (8) suggesting that they may be universal pollen constituents. On the basis of the similar reproductive defects displayed by two such widely diverged species as maize and petunia, we would predict that the requirement for flavonoids during pollen germination is widespread. Support for this view will be provided by the isolation of flavonoid-deficient self-sterile mutants in other species. The loss of CHS expression resulting in CMF plants acts as a natural gametostat, not a gametocide, which can be reversed to restore full male function. In addition to identifying a factor involved in higher plant fertilization, a potential benefit of our finding may be the development of a reversible male sterile system for the production of hybrid seed.

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