Plant Physiol. (1993) 102: 925–932

Chalcone Synthase and Flavonol Accumulation in Stigmas and Anthers of *Petunia hybrida*[1]

Peggy E. Pollak, Thomas Vogt, Yinyuan Mo, and Loverine P. Taylor*

Department of Horticulture and Program in Plant Physiology, Washington State University, Pullman, Washington 99164-6414

Flavonol aglycones are required for pollen germination in petunia (*Petunia hybrida* L.). Mutant plants lacking chalcone synthase (CHS), which catalyzes the first committed step in flavonoid synthesis, do not accumulate flavonols and are self-sterile. The mutant pollen can be induced to germinate by supplementing it with kaempferol, a flavonol aglycone, either at the time of pollination or by addition to an in vitro germination system. Biochemical complementation occurs naturally when the mutant, flavonol-deficient pollen is crossed to wild-type, flavonoid-producing stigmas. We found that successful pollination depends on stigma maturity, indicating that flavonol aglycone accumulation may be developmentally regulated. Quantitative immunoblotting, in vitro and in vivo pollen germination, and high-performance liquid chromatographic analyses of stigma and anther extracts were used to determine the relationship between CHS levels and flavonol aglycone accumulation in developing petunia flowers. Although substantial levels of CHS were measured, we detected no flavonol aglycones in wild-type stigma or anther extracts. Instead, the occurrence of a conjugated form (flavonol glycoside) suggests that a mechanism may operate to convert glycosides to the active aglycone form.

CHS catalyzes the initial step in flavonoid biosynthesis, and a lack of CHS activity has a pleiotropic effect: not only is flavonoid production inhibited, but the plants are self-sterile because of a failure of pollen germination and tube growth (Taylor and Jorgensen, 1992). An essential role for flavonoids in plant fertility was established using petunia (*Petunia hybrida* L.) and maize CHS mutants. Although the CHS-deficient pollen never functions in self-crosses, reciprocal crosses in petunia show that the mutant pollen can function on wild-type stigmas, a phenomenon termed CMF by Taylor and Jorgensen (1992). This observation led to the isolation of compounds from wild-type stigmas that, when added to an aqueous GM, are capable of stimulating CMF pollen to germinate (Mo et al., 1992). Using this as an in vitro rescue assay, Mo et al. (1992) showed that the germination-inducing activity resides in a single class of compounds, the flavonol aglycones. Moreover, the fertility defect is completely reversed by biochemical complementation; addition of nanomolar concentrations of kaempferol or other flavonol aglycones to the stigma at pollination or to the GM restores full seed set in vivo and pollen germination in vitro (Mo et al., 1992). Furthermore, HPLC analysis detects kaempferol in postdehiscent anther and stigma extracts from wild-type petunia, whereas stigma extracts from transgenic petunia lacking CHS fail to produce detectable amounts of kaempferol (Mo et al., 1992).

Recently, support for the concept that flavonoids are required for pollen function was provided by the observation that transgenic petunia containing an antisense CHS gene were male sterile because of an arrest in pollen development (van der Meer et al., 1992). Additionally, experiments with in vitro cultured tobacco pollen showed that development and germination were enhanced by addition of flavonols to the culture medium (Ylstra et al., 1992).

Our earlier observation (unpublished) that extracts from immature wild-type stigmas are unable to rescue CMF pollen, whereas extracts from mature stigmas can restore pollen germination and tube growth, suggested that flavonol aglycone accumulation is developmentally regulated. Although the factors specifically regulating flavonol aglycone accumulation are unknown, the first committed step in the pathway, catalyzed by CHS, is considered to be an important regulatory point in overall flavonoid biosynthesis (Niesbach-Klösgen et al., 1987; Ryder et al., 1987; Feinbaum and Ausubel, 1988; van der Meer et al., 1992). We undertook to test the hypothesis that the increasing competence of wild-type stigmas to rescue flavonol-deficient CMF pollen parallels increasing CHS and flavonol aglycone levels. If this is so, CHS and the flavonol aglycones required for pollen germination in petunia should accumulate in a coordinate manner during the development of wild-type stigmas and anthers.

In this report, we show that stigma maturity has a profound effect on pollen germination and tube growth in vivo and in vitro. The pattern of CHS and flavonol accumulation in maturing wild-type stigmas and anthers is described and correlated with the competence of stigmas to rescue the flavonol-deficient CMF pollen. Although mature wild-type stigmas can rescue CMF pollen in vivo, contrary to expectation, we detected no flavonol aglycones in extracts from either stigmas or anthers at any stage of development tested. Instead, we found evidence of an additional mechanism to regulate flavonol aglycone production in petunia reproductive tissues.

[1] This work was supported by U.S. Department of Agriculture-National Research Initiative Competitive Grants Program grant 9103184 to L.P.T.

* Corresponding author; fax 1-509-335-8690.

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

MATERIALS AND METHODS

Plant Material

The *Petunia hybrida* L. plants used in this study were described by Taylor and Jorgensen (1992) and Napoli et al. (1990); the inbred V26 (wild type) is competent to produce flavonoids and is self-fertile, and the transgenic derivative of V26 is suppressed for CHS activity and lacks flavonoids. This transgenic derivative exhibits the CMF phenotype, defined by Taylor and Jorgensen (1992) as the condition in which pollen is nonfunctional in self-crosses but functions in outcrosses. Pollen from CMF plants is unable to germinate in an in vitro system (Mo et al., 1992). All experiments were performed with V26 stigmas and CMF pollen unless otherwise noted. The 11 flower stages used in this study were defined by corolla length and specific morphological and anatomical events (Table I).

Expression of CHS Protein in *Escherichia coli* and Antibody Production

The maize *C2* gene encoding CHS (Wienand et al., 1986) was cloned into the T7 promoter-driven expression vector pP09 (Rottmann et al., 1991) as follows: an *Eco*RI fragment of the CHS cDNA clone (pC2-c46) was cloned into *Eco*RI-digested pBluescript II SK(+) (Stratagene) so that a unique *Nae*I site in *C2* was proximal to the *Sma*I site of the vector polylinker. This construct, designated pSK-c2.6, was digested with *Eco*RV and *Nae*I, and the fragment containing CHS sequences was ligated into *Eco*RV-digested pBluescript II SK(+). This yielded pSK-c2.16 with the *Nae*I/*Eco*RV junction adjacent to the *Eco*RI site in the pBluescript II SK(+) polylinker. *Eco*RI digestion of pSK-c2.16 produced a 1.4-kb fragment that was then cloned into the *Eco*RI site of pP09 in two orientations. Nucleotide sequencing determined that the resulting plasmid, pP09-c2.101, contains a complete C2 sequence fused to the T7 promoter in frame with four extra amino acids at the N terminus; pP09-c2.102 contains the same fragment in opposite orientation and was used as a negative control.

E. coli (strain BL21 DES) was transformed with the chimeric construct, and the CHS protein expressed after isopropyl β-D-thiogalactopyranoside induction was purified from inclusion bodies (Studier et al., 1985). After further purification by preparative SDS-PAGE (Laemmli, 1970), the band representing CHS was excised, lyophilized, ground to a fine powder, suspended in PBS, and injected into a sterile, whiffle-type golf ball that had been inserted subcutaneously into a virgin New Zealand rabbit (Reid et al., 1992). Granuloma fluid was used directly as the source of antibody. The CHS protein used as a standard in electrophoresis, western blots, and determination of antibody titer was obtained by electroelution of the *E. coli*-expressed protein from preparative acrylamide gels.

SDS-PAGE and Western Blots

Proteins were isolated from staged petunia flowers by grinding fresh tissue in ice-cold grinding medium (200 mM Tris [pH 7.8], 0.5 mM DTT, 10 mg/mL of polyvinylpolypyrrolidone, 10% [v/v] Dowex 1 × 2). Stigma proteins were extracted in a buffer supplemented with 10 mM *N*-ethylmaleimide (replacing DTT), 22 μM leupeptin, 14.3 μM pepstatin, and 200 μM PMSF. After the crude extract was centrifuged, an aliquot of the supernatant was loaded onto a 12% bisacrylamide gel in a Bio-Rad Mini-Protean II dual slab cell and run at a constant 200 V using the Laemmli buffer system (Laemmli, 1970). A standard curve was constructed using a range of stigma protein to establish that the amount of protein loaded (100 μg) bound quantitatively to the nitrocellulose

Table I. *Flower stages*

Length measured from base of receptacle to tip of corolla.

Stage	Bud Length	Floral State
	mm	
1	0–10	Bud totally green and enclosed in sepals; tapetum present; microspores in vacuolate stage
2	11–15	Corolla uncolored, beginning to emerge from sepals; tapetum beginning to disintegrate; uninucleate microspores with many small vacuoles
3	16–20	Corolla yellow
4	21–25	Corolla yellow; tapetum disintegrated; pollen binucleate
5	26–30	Corolla beginning to color in V26
6	31–35	Corolla deeply colored
7	36–40	Corolla fully colored; pollen grains binucleate with prominent exine and small vacuoles
8	41–50	Corolla fully colored, long and twisted; pollen wall thinning at pores
9	51–60	Corolla beginning to open; stigma dry; pollen wall with prominent pores
10	51–60	Corolla ¼ to ¾ open; stigma may be wet
11	51–60	Corolla fully open; stigma wet; anthers within 4 h of dehiscence
12	51–60	Anthers dehiscent

membrane during immunoblotting. Protein was determined according to the method of Bradford (1976).

Immunoblotting was carried out as described by Harlow and Lane (1988). The antibody-antigen complex was visualized with alkaline phosphatase conjugated to goat anti-rabbit immunoglobulin G (Bio-Rad). Serial dilutions of antibody indicated that 1:1000 (antibody:buffer [100 mM Tris (pH 8.0), 150 mM NaCl, 0.05% Tween 20] plus 1% BSA) clearly recognized 0.1 μg of CHS protein on western blots (Fig. 2); therefore, this dilution was used routinely. Band intensity was measured using an image analysis system consisting of a Pulnix TM-7CN video camera interfaced with a Macintosh Quadra 950 computer equipped with the Image 1.4 image analysis program. Total CHS levels were calculated based on comparison with a known amount of CHS and expressed as a percentage of total extractable protein.

In Vitro and in Vivo Pollen Rescue

Restoration of CMF pollen function in vitro was accomplished by the addition of purified flavonol aglycone or stigma extract to pollen suspended in GM as described by Mo et al. (1992) with the following modifications. Extracts were prepared by macerating five stigmas in 100 μL of either methanol or aqueous GM. In one set of experiments, GM extracts were further treated by incubation at room temperature for 15 to 30 min before extraction in either chloroform or ethyl acetate to dissolve flavonol aglycones (GM/organic solvent extract). Extracts in organic solvents were either analyzed by HPLC directly or dried and reconstituted in 20 μL of DMSO before use in in vitro assays. An amount equivalent to one-quarter of a stigma (1.0 μL) was used for each assay. The competence of stigmas to rescue in vivo was determined by counting seed production in hand-pollinated, staged flowers.

HPLC of Stigma and Anther Extracts

HPLC analysis of stigma and anther flavonoids was performed using a Waters 820 Maxima chromatography work station equipped with a dual-pump system (Waters 510) and photodiode array detector (Waters 994). Chromatographic conditions were as follows: gradient of solvent B, acetonitrile (5% acetic acid), to solvent A, water (5% acetic acid); 1 min at 5% B, to 65% B in 20 min, to 90% B in 1 min, 1 min at 90% B, to 5% B in 1 min, 5 min 5% B; flow rate, 1 mL min^{-1} on a C_{18} reversed-phase matrix (Waters Nova-pak, 150 mm × 3.9 mm i.d.; particle size, 4 μm). Peaks were detected at 365 and/or 290 nm. Detection was linear between 5 and 500 pmol. Kaempferol and quercetin were identified in plant extracts by retention time and spectral comparisons (photodiode array detection; wavelengths, 230–400 nm) with authentic standards. Additional standards included myricetin and glycosylated or methylated derivatives of these flavonols. All pure flavonoid standards were obtained from Spectrum Chemical (Gardena, CA).

Extracts for HPLC were prepared by soaking a defined number of stigmas or anthers in 100% methanol at 4°C overnight (10 stigmas or anthers 200 μL^{-1}). Before HPLC analysis, samples were crushed at room temperature, centrifuged, and supernatant (50 μL) was injected. To convert flavonol glycosides to flavonol aglycones, individual extracts were hydrolyzed by mixing 50 μL of sample with 50 μL of 2 N HCl, heating at 50°C for 30 min, and adding 50 μL of methanol to prevent precipitation of flavonol aglycones. The extracts were centrifuged, and 75 μL of the supernatant were injected.

Microscopy

Staged flowers were fixed in 2% paraformaldehyde and 1.25% glutaraldehyde in Pipes buffer (50 mM, pH 7.4) and infiltrated in LR White resin. Sections 1 μm thick were cut with a glass knife on a Reichert OM U2 ultramicrotome, collected on gelatin-coated slides, stained with toluidine blue, and examined with a Zeiss microscope.

RESULTS

In Vivo Rescue of CMF Pollen

To establish that the competence of stigmas to restore CMF pollen function is age dependent, we measured seed set from crosses of CMF pollen onto V26 stigmas of increasing maturity. Seed set is a measure of many factors, including the frequency of pollen germination and the production of functional pollen tubes. By two criteria, the rescue of CMF pollen increases with V26 stigma maturity: the number of seeds per capsule (Fig. 1) and the frequency of successful crosses (capsules with seed; Table II). The average number of seeds per capsule increases 3-fold and the number of successful crosses almost doubles (53–93%) between stigma stages 9 and 11 (Table II). Crosses of mature V26 stigmas by CMF pollen consistently produce about half the number of seeds as V26 self-crosses (Taylor and Jorgensen, 1992; Fig. 1, stage 11). The same ratio is maintained with CMF crosses made at earlier stigma stages (Fig. 1, stages 9 and 10).

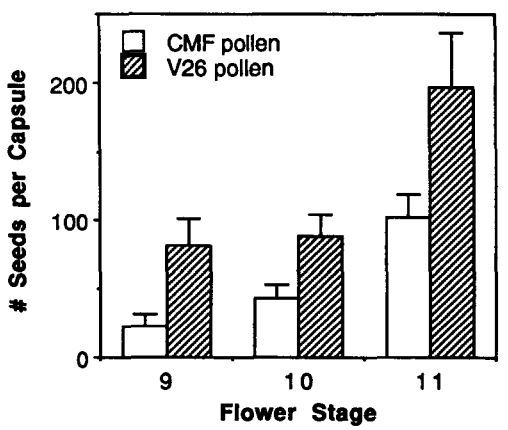

Figure 1. The competence of stigmas to rescue CMF pollen in vivo measured as seed set. V26 stigmas at different developmental stages were pollinated with either CMF or V26 pollen, and seed was collected 30 d later. The mean number of seeds per capsule is shown for the final three stages of flower development. Experiments were repeated twice with 10 flowers of each stage. Bars represent SE.

Table II. *Crosses of CMF pollen on wild-type stigma results in rescue of pollen function in vivo*

n, Number of crosses.

Stage	Seeds/Capsule				
	0	1–50	51–100	101–150	>150
	% of total capsules				
9 (n = 19)	47	32	16	5	0
10 (n = 20)	20	45	25	10	0
11 (n = 15)	7	20	27	20	27

CHS Antibody Production and Characterization

CHS levels during development were determined using an antibody raised against a maize CHS protein produced by overexpression of the cloned cDNA (see "Materials and Methods"). The antiserum recognizes maize CHS at 45 kD and petunia CHS at 43.3 kD (Fig. 2), a range of values exhibited by CHS subunits from many species (Mol et al., 1983; Kehrel and Wiermann, 1985; Ryder et al., 1987). The antiserum reacts with purified maize CHS produced in a bacterial expression system, with CHS in extracts from maize seedlings grown under high intensity white light, with corolla extracts from *Antirrhinum, Saintpaulia,* and *Nicotiana,* and with CHS from petals, stigmas (data not shown), and anthers of V26 petunia (Fig. 2). This cross-reactivity is consistent with the highly conserved amino acid sequence of CHS (Niesbach-Klösgen et al., 1987). The antiserum detects no CHS protein in CMF petunia tissue (our unpublished results).

The antiserum detects two closely spaced bands, in agreement with reports for CHS from carrot cell cultures (Gleitz and Seitz, 1989), pea leaves (Rommeswinkel et al., 1992), and petunia corollas (Mol et al., 1983). The antiserum does not recognize proteins from untransformed bacteria or proteins from bacteria transformed with the CHS gene in the antisense orientation. However, it cross-reacts with a 67-kD protein from both V26 and CMF petunia petals that is not related to CHS (our unpublished results).

Developmental Pattern of CHS and Flavonols in Stigmas and Anthers

The hypothesis that increasing competence of wild-type stigmas to rescue flavonol-deficient CMF pollen parallels increasing CHS and flavonol accumulation was tested by quantifying flavonol and CHS levels in developing stigmas and anthers. We selected CHS because it catalyzes the initial step and is a key regulator of flavonoid biosynthesis during development and in response to a variety of stimuli (Ryder et al., 1987, and refs. therein).

In anthers, CHS is not detected during the earliest two stages of floral development before microspore mitosis. However, it rapidly accumulates during pollen maturation, peaks during tapetum degeneration when the pollen is binucleate, and declines to as low as 0.02% of extractable protein thereafter (Fig. 3A). In contrast to anthers, stigmas at early stages contain low levels of CHS (0.003% of extractable protein), which gradually accumulates throughout development (Fig. 3B). Not only do CHS maxima occur at different flower stages in stigmas and anthers, but the peak amount of CHS protein in anthers is 4-fold higher than in stigmas (0.13% compared to 0.029% of extractable protein; Fig. 3). Flowers beyond stage 11 were not included in this study, therefore, we cannot preclude the possibility of changes in CHS levels with subsequent developmental events such as dehiscence or pollination.

Although flavonol aglycones are required for pollen germination (Mo et al., 1992), analysis of HPLC profiles of extracts from staged V26 anthers and stigmas established that no flavonol aglycones accumulate at any stage in either organ. However, when stigma and anther extracts were acid hydrolyzed, we did detect flavonol aglycones (Figs. 4 and 5). In many plant tissues, flavonoids occur predominately conjugated with sugars, and hydrolysis of flavonol *O*-glycosides, either enzymically or with acid, removes the sugar moieties to produce the aglycone form. A comparison of peak areas from the chromatograms of anther extracts before and after acid hydrolysis (Fig. 4) shows that peaks 1 and 2 (12.0 and 9.0 nmol 50 μL^{-1}, respectively) are quantitatively converted to peaks 4 and 5 (11.3 and 10.0 nmol 50 μL^{-1}, respectively) after correction for the slight difference in molar extinction coefficients. Isolation of peaks 1 and 2 followed by acid hydrolysis produces quercetin (peak 4) and kaempferol (peak 5), confirming the identity of the flavonol core of the flavonol-3-*O*-glycosides. Although we have yet to identify the sugar moieties, Zerback et al. (1989) established that the major flavonoids from petunia pollen are quercetin and kaempferol 3-*O*-(2′-*O*-β-D-glucopyranosyl)-β-D-galactopyranoside. Their attempts to hydrolyze this particular kaempferol glycoside with almond meal were unsuccessful. Likewise, we found that commercial β-glycosidase (almond meal) does not hydrolyze the flavonol glycosides represented by peaks 1 and 2. From the quantitative recovery

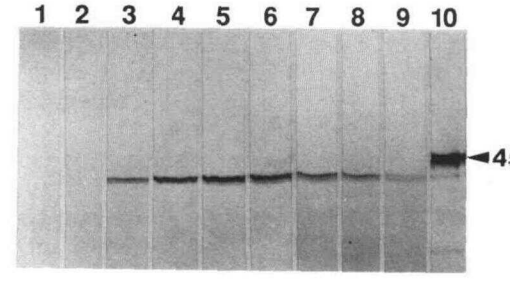

Figure 2. Immunoblot of CHS protein extracted from anthers of wild-type V26 flowers at nine developmental stages of increasing maturity (lanes 1–9). Stages are described in Table I. Lane 10 is the maize CHS protein purified from bacteria transformed with the maize *chs* gene. This fusion protein contains four extra amino acids, which may account for the higher molecular mass of the maize protein as compared to the petunia. Amino acid differences between maize and petunia CHS or the absence of posttranslational processing in the bacterial expression system may also contribute to the molecular mass difference. Crude extracts (100 μg of total protein) were separated with SDS-PAGE, blotted to nitrocellulose, and incubated with a polyclonal antibody to CHS. Visualization was achieved with goat anti-rabbit immunoglobulin G linked to alkaline phosphatase. Molecular mass in kD (arrowhead) was determined using protein molecular mass markers (Sigma Dalton Mark VIIL).

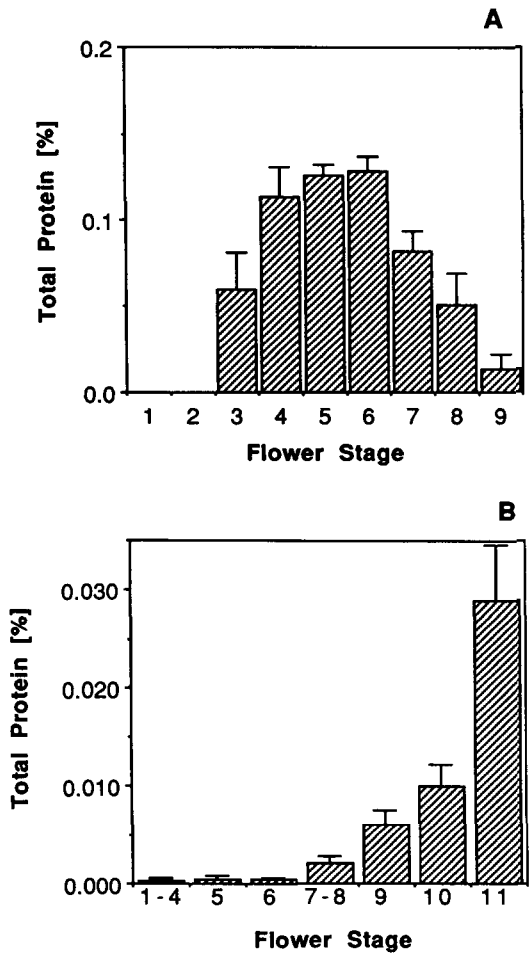

Figure 3. CHS levels as percentage of total soluble protein in anthers (A) and stigmas (B) of wild-type V26 flowers at successive developmental stages. Protein extracts were treated as described for Figure 2. A computer imaging system was used to quantify CHS by measuring the density of the immunoproduct formed after development of the antibody-alkaline phosphatase complex. Values are the means of four replicates. Error bars represent SE.

and UV spectra, we conclude that peaks 1 and 2 in the unhydrolyzed anther extract represent 3-*O*-glycosides of quercetin and kaempferol. In contrast to anthers, stigma extracts contain kaempferol but no quercetin after hydrolysis (Fig. 5B). Analysis of mature anthers verified that CMF pollen contains no detectable flavonol aglycones or flavonol glycosides (data not shown).

The pattern of CHS accumulation in V26 anthers correlates well with the appearance and peak levels of flavonol glycosides; CHS peaks at stage 6 and flavonols at stage 8, approximately 6 to 8 h later (compare Figs. 3A and 5A). This time lag agrees well with the accumulation kinetics determined for flavonoid biosynthetic enzyme transcripts (including CHS) and the anthocyanin product in light-induced maize seedlings (Taylor and Briggs, 1990). Although CHS levels decline in mature anthers, the amount of flavonol glycoside remains high, reflecting the stability of the compound. In stigmas, CHS levels begin to increase at stage 7 and continue to increase through stage 11 (Fig. 3B). On the other hand, flavonol glycosides are detected in the earliest stages tested and remain at a relatively constant, albeit low, concentration throughout development (Fig. 5B). In contrast to anthers, we find no correlation between flavonoid glycoside and CHS levels in the stages of V26 stigmas tested here (compare Figs. 3B and 5B). Based on the observation in anthers that peak protein and peak glycoside levels are separated by a 6- to 8-h time lag, the increase in stigma CHS at stage 11 may be followed by a commensurate increase in flavonol levels at a later stage.

In Vitro Rescue with Stigma Extracts

Stigmas extracted in aqueous GM (Table III, column 2) should contain the water-soluble flavonol glycosides detected by HPLC, and failure of these extracts to rescue CMF pollen

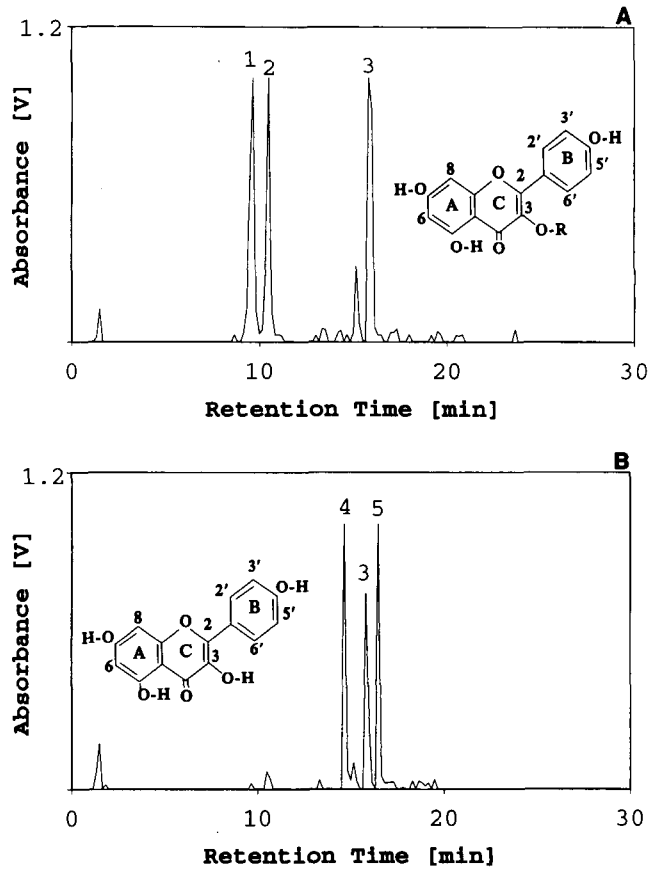

Figure 4. HPLC profiles of methanolic extracts from mature, wild-type V26 anthers. A, Extract before acid hydrolysis. Peaks 1 and 2 represent putative flavonol 3-*O*-glycosides of quercetin and kaempferol. Peak 3 is unidentified. No flavonol aglycones are detected. Inset depicts general molecular structure of flavonol glycosides (kaempferol glycoside), where R represents one or more sugar moieties. B, The same extract after acid hydrolysis. The appearance of quercetin (peak 4) and kaempferol (peak 5) is accompanied by the disappearance of peaks 1 and 2 of A. Inset depicts flavonol aglycone (kaempferol) produced after acid cleavage of sugar from the number 3 position in ring C.

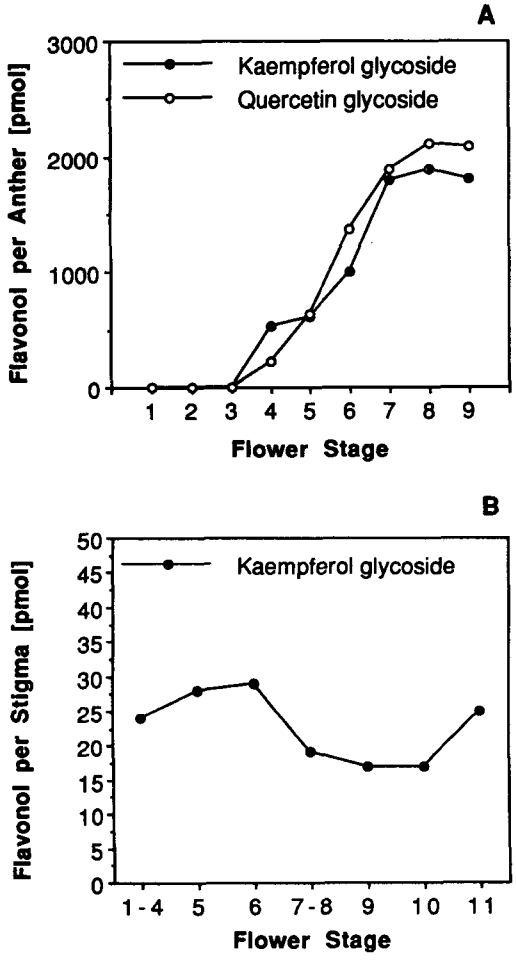

Figure 5. Accumulation of flavonol glycosides in anthers (A) and stigmas (B) during successive stages of floral development. Flavonols were detected by HPLC (λ = 365 nm) after acid hydrolysis of methanolic extracts. Values are the means of two replicates.

above control levels is consistent with previous demonstrations that flavonol glycosides cannot rescue CMF pollen (Mo et al., 1992). Methanolic extracts, in which flavonol aglycones are soluble, produced a low level of rescue (Table III, column 3), even though no flavonol aglycones were detected in these extracts by HPLC. This suggests that the sensitivity of the in vitro assay exceeds the detection limit of the HPLC analysis. To detect possible enzymic conversion of the flavonol glycosides to flavonol aglycones within the stigma extract, the tissue was macerated in GM, which allows recovery of water-soluble flavonol glycosides while retaining enzymic activity. After a short incubation in GM, the stigma suspension was extracted with an organic solvent to recover any flavonol aglycones, and an aliquot was tested in the in vitro germination assay. This procedure yielded the highest level of rescue of CMF pollen (Table III, column 4). If a mechanism operates to convert flavonol glycosides into the active aglycone form, then the glycosides should be present in sufficient quantities to produce an amount of aglycone that rescues pollen at the frequency we obtained (Table III). We measured

Table III. *Competence of different extracts from wild-type stigmas to rescue CMF pollen in vitro*

Extraction procedures are described in "Materials and Methods." For description of stages, see Table I. Extract from one-quarter stigma was used in each trial. Control, CMF pollen in GM with no added stigma extract. Values are mean percentages (±SE) of large pollen grains that developed tubes. The experiment was performed three times with five replicates per treatment.

Stage	GM Extract	Methanol Extract	GM/Organic Solvent Extract
9	0.36 ± 0.21	5.8 ± 0.35	8.2 ± 1.0
10	0.64 ± 0.31	6.0 ± 0.58	11.7 ± 2.6
11	2.3 ± 0.16	7.1 ± 1.6	16.1 ± 1.9
Control	1.5 ± 0.85	3.5 ± 0.1	5.7 ± 1.7

6.3 pmol of flavonol glycoside in one-quarter of the extract from a single stage 11 stigma (Fig. 5B), and this extract produced 16.1% rescue (Table III, column 4). A dose-response curve with purified kaempferol predicts that 6.3 pmol of pure kaempferol will produce 15% rescue (Fig. 6). Stigmas at stages 9 and 10 contain approximately 16 pmol of kaempferol glycoside (Fig. 5B) and produce 8.2 and 11.7% rescue (Table III, column 4) when 10% rescue is predicted. The near-perfect match between observed and expected levels of rescue suggests that an efficient mechanism exists to convert flavonol glycosides to flavonol aglycones.

Confirmation of the in Vitro Results by HPLC Analysis

The quantitative conversion of flavonol glycosides to aglycones was measured directly with HPLC to confirm the

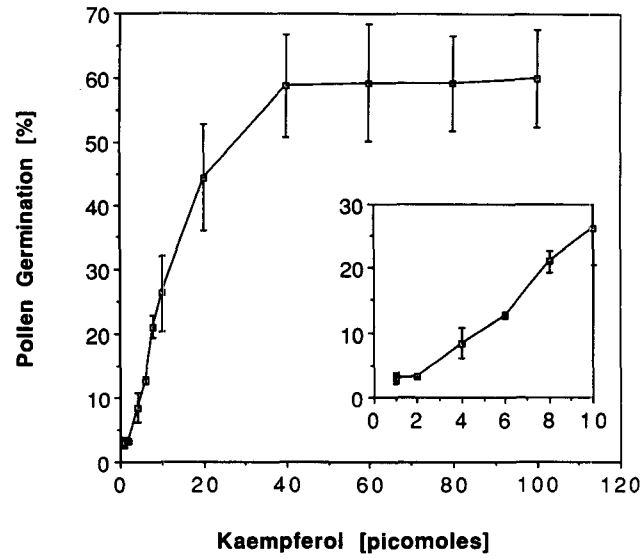

Figure 6. Frequency of CMF pollen germination in vitro as a function of increasing kaempferol concentration. Kaempferol (pmol) was added to a suspension of pollen in 100 μL of GM (Mo et al., 1992), and the number of germinated grains was counted after a 4-h incubation. Values presented are means of three replicates with at least 100 pollen grains counted per replicate. Error bars represent SE. Inset provides a magnified view of the left end of the abscissa, from 0 to 10 pmol of kaempferol.

enhanced in vitro rescue with incubated stigma extracts. Stage 11 stigmas were macerated and incubated in GM for 30 min before extraction with organic solvent and HPLC analysis. Stigmas treated in this manner have 30 times more kaempferol (29 pmol/stigma) compared to controls that were not incubated in GM before extraction (0.9 pmol/stigma). Further hydrolysis of these extracts with HCl did not produce an additional increase in kaempferol. These results indicate that (a) a factor endogenous to stigmas is able to convert precursor flavonol glycosides to flavonol aglycones, (b) all (100%) of the glycosides are converted to the corresponding flavonol aglycone, (c) the amount of kaempferol produced in this manner (29 pmol/stigma) closely matches the amount of flavonol glycosides measured in stage 11 stigmas (25 pmol/stigma; Fig. 5B), and (d) the in vitro bioassay is more sensitive than HPLC and is able to detect rescue in extracts with kaempferol concentrations (7.1% rescue in extracts with <1.0 pmol of kaempferol; Table III and Fig. 6) below the threshold for HPLC detection (5 pmol).

DISCUSSION

There are numerous reports of the regulation of flavonoid biosynthetic enzymes and products, especially anthocyanins and isoflavonoids (reviewed by Lamb et al., 1989; Dooner et al., 1991). Many of these studies focus on CHS as a key regulatory enzyme and show that product accumulation parallels the level of CHS gene transcription, protein, and enzyme activity (Lawton and Lamb, 1987; Ryder et al., 1987; Feinbaum and Ausubel, 1988; Welle and Grisebach, 1989; Weiss et al., 1990).

Little is known about the control of flavonol aglycone production and accumulation. This specific class of flavonoids gained biological significance when it was demonstrated that flavonol aglycones are necessary and sufficient for pollen germination and tube growth in maize and petunia (Mo et al., 1992). In the present study, we detected no flavonol aglycones in stigmas or anthers of wild-type petunia. However, significant amounts of flavonol glycosides were measured. Furthermore, in contrast to the studies described above, we found that flavonol aglycones and CHS are not coordinately regulated in petunia stigmas and anthers.

In view of the absolute requirement of CMF pollen germination for flavonol aglycones, we propose that a glycosidase cleaves sugar moieties from the inactive glycoside to create the active aglycone. This hypothesis is based on several observations. First, no flavonol aglycones were detected by HPLC or by the in vitro rescue of CMF pollen in either stigma or anther of wild-type petunia. However, a substantial pool of flavonol glycosides resides in anthers, as does a smaller but persistent pool in stigmas. Second, aqueous and methanolic extracts of wild-type stigmas lack detectable kaempferol and produce extremely low levels of rescue, yet the bioassay with extracts prepared by incubation in GM before organic solvent extraction repeatedly shows substantial rescue. Third, the level of rescue of CMF pollen by incubated stigma extracts corresponds closely to the amount of flavonol glycoside detected. Fourth, HPLC results confirm quantitative conversion of glycosides to aglycones in incubated stigma extracts. Finally, although glycosidase activity exhibiting a substrate specificity for flavonol glycosides has not been assessed in our system, we have measured nonspecific glycosidase activity, especially in germinating pollen (our unpublished data).

Data from several plant systems suggest that regulation of active signaling molecules is achieved by a mechanism that interconverts the conjugated, inactive form and the active, hydrolyzed form. In experiments with Douglas fir, Morris and Morris (1990) showed that hydrolysis of coniferin, a phenolic acid glycoside, may be required for induction of the *vir* genes of *Agrobacterium tumefaciens*. They found a strong correlation between the tumorigenicity and β-glucosidase activity of various *Agrobacterium* strains. Subsequently, Castle et al. (1992) cloned an *Agrobacterium* β-glucosidase gene for an enzyme that shows marked substrate specificity toward coniferin. Recently, Campos et al. (1992) isolated a protein with β-glucosidase activity from maize coleoptiles based on its ability to bind an active auxin analog. The enzyme shows high substrate specificity for auxin conjugates that generate corresponding aglycones with biological activity. Finally, evidence is accumulating that the balance between free and sugar-conjugated forms of salicylic acid may regulate the hypersensitive response in tobacco infected with tobacco mosaic virus (Malamy et al., 1992).

Glycosylation of flavonol aglycones is a final step in the biosynthetic pathway, and it is the glycosylated form of flavonols that have been detected in pollen grains and stigmatic exudates from a broad array of plant taxa (Martin, 1969; Wiermann and Vieth, 1983; Heller and Forkmann, 1988; Zerback et al., 1989). The production of flavonol glycosides may be driven by the necessity to eliminate the insoluble aglycones from the aqueous intracellular environment, which creates, within petunia reproductive tissue, a reservoir of flavonol glycosides that are available for conversion to aglycones as required. The speed and completeness of the apparent conversion of flavonol glycosides to flavonol aglycones in extracts of petunia stigmas suggest the existence of an efficient mechanism for this interchange.

ACKNOWLEDGMENTS

We thank Udo Wienand for the C2 cDNA clone used to produce the CHS protein; William Ferner for performing the implantation surgery; Molly Cadle for initial antibody characterization; Howard Grimes, W. Linn Montgomery, and Clarence Ryan for critical reading of the manuscript; and Gerald Irzyk for helpful discussions.

Received January 15, 1993; accepted April 8, 1993.
Copyright Clearance Center: 0032-0889/93/102/0925/08.

LITERATURE CITED

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Campos N, Bako L, Feldwisch J, Schell J, Palme K (1992) A protein from maize labeled with azido-IAA has novel β-glucosidase activity. Plant J **2:** 675–694

Castle LA, Smith KD, Morris RO (1992) Cloning and sequencing of an *Agrobacterium tumefaciens* β-glucosidase gene involved in modifying a *vir*-inducing plant signal molecule. J Bacteriol **174:** 1478–1486

Dooner HK, Robbins TP, Jorgensen RA (1991) Genetic and developmental control of anthocyanin biosynthesis. Annu Rev Genet **25:** 173–199

Feinbaum RL, Ausubel FM (1988) Transcriptional regulation of the *Arabidopsis thaliana* chalcone synthase gene. Mol Cell Biol **8:** 1985–1992

Gleitz J, Seitz HU (1989) Induction of chalcone synthase in cell suspension cultures of carrot (*Daucus carota* L. ssp. *sativus*) by ultraviolet light: evidence for two different forms of chalcone synthase. Planta **179:** 323–330

Harlow E, Lane D (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Heller W, Forkmann G (1988) Biosynthesis. *In* JB Harborne, ed, The Flavonoids. Chapman and Hall, London, pp 399–425

Kehrel B, Wiermann R (1985) Immunochemical localization of phenylalanine ammonia-lyase and chalcone synthase in anthers. Planta **163:** 183–190

Laemmli VK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lamb CJ, Lawton MA, Dron M, Dixon RA (1989) Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell **56:** 215–224

Lawton MA, Lamb CJ (1987) Transcriptional activation of plant defense genes by fungal elicitor, wounding, and infection. Mol Cell Biol **7:** 335–341

Malamy J, Hennig J, Klessig DF (1992) Temperature-dependent induction of salicylic acid and its conjugates during the resistance response to tobacco mosaic virus infection. Plant Cell **4:** 359–366

Martin FW (1969) Compounds from the stigmas of ten species. Am J Bot **56:** 1023–1027

Mo Y, Nagel C, Taylor LP (1992) Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. Proc Natl Acad Sci USA **89:** 7213–7217

Mol JNM, Schram AW, de Vlaming P, Gerats AGM, Kreuzaler F, Hahlbrock K, Reif HJ, Veltkamp E (1983) Regulation of flavonoid gene expression in *Petunia hybrida*: description and partial characterization of a conditional mutant in chalcone synthase gene expression. Mol Gen Genet **192:** 424–429

Morris JW, Morris RO (1990) Identification of an *Agrobacterium tumefaciens* virulence gene inducer from the pinaceous gymnosperm *Pseudotsuga menziesii*. Proc Natl Acad Sci USA **87:** 3614–3618

Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in *trans*. Plant Cell **2:** 279–289

Niesbach-Klösgen U, Barzen E, Bernhardt J, Rohde W, Schwarz-Sommer Z, Reif HJ, Wienand U, Saedler H (1987) Chalcone synthase genes in plants: a tool to study evolutionary relationships. J Mol Evol **26:** 213–225

Reid JL, Walker-Simmons MK, Everard JD, Diani J (1992) Production of polyclonal antibodies in rabbits is simplified using perforated plastic golf balls. Biotechniques **12:** 660–666

Rommeswinkel M, Karwatzki B, Beerhues L, Wiermann R (1992) Immunofluorescence localization of chalcone synthase in roots of *Pisum sativum* L. and *Phaseolus vulgaris* L. and comparable immunochemical analysis of chalcone synthase from pea leaves. Protoplasma **166:** 115–121

Rottmann WH, Peter GF, Oeller PW, Keller JA, Shen NF, Nagy BP, Taylor LP, Campbell AD, Theologis A (1991) 1-Aminocyclopropane-1-carboxylate synthase in tomato is encoded by a multigene family whose transcription is induced during fruit and floral senescence. J Mol Biol **222:** 937–961

Ryder TB, Hedrick SA, Bell JN, Liang X, Clouse SD, Lamb CJ (1987) Organization and differential activation of a gene family encoding the plant defense enzyme chalcone synthase in *Phaseolus vulgaris*. Mol Gen Genet **210:** 219–233

Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW (1985) Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol **185:** 60–89

Taylor LP, Briggs WR (1990) Genetic regulation and photocontrol of anthocyanin accumulation in maize seedlings. Plant Cell **2:** 115–127

Taylor LP, Jorgensen R (1992) Conditional male fertility in chalcone synthase-deficient petunia. J Hered **83:** 11–17

van der Meer IM, Stam ME, van Tunen AJ, Mol JNM, Stuitje AR (1992) Antisense inhibition of flavonoid biosynthesis in petunia anthers results in male sterility. Plant Cell **4:** 253–262

Weiss D, van Tunen AJ, Halevy AH, Mol JNM, Gerats AGM (1990) Stamens and gibberellic acid in the regulation of flavonoid gene expression in the corolla of *Petunia hybrida*. Plant Physiol **94:** 511–515

Welle R, Grisebach H (1989) Phytoalexin synthesis in soybean cells: elicitor induction of reductase involved in biosynthesis of 6′-deoxychalcone. Arch Biochem Biophys **272:** 97–102

Wienand U, Weydemann U, Niesbach-Klösgen U, Peterson PA, Saedler H (1986) Molecular cloning of the c2 locus of *Zea mays*, the gene coding for chalcone synthase. Mol Gen Genet **203:** 202–207

Wiermann R, Vieth K (1983) Outer pollen wall, an important accumulation site for flavonoids. Protoplasma **118:** 230–233

Ylstra B, Touraev A, Benito Moreno RM, Stöger E, van Tunen AJ, Vicente O, Mol JNM, Heberle-Bors E (1992) Flavonols stimulate development, germination, and tube growth of tobacco pollen. Plant Physiol **100:** 902–907

Zerback R, Bokell M, Geiger H, Hess D (1989) A kaempferol 3-glucosylgalactoside and further flavonoids from pollen of *Petunia hybrida*. Phytochemistry **28:** 897–899