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RESEARCH ARTICLE

Pollination- or Wound-Induced Kaempferol Accumulation in Petunia Stigmas Enhances Seed Production

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Flavonols are essential for pollen germination and tube growth in petunia and can be supplied by either the pollen or stigma at pollination. HPLC analysis and a sensitive bioassay demonstrated that both pollination and wounding induce flavonol accumulation, especially kaempferol, in the outer cell layers and exudate of the stigma. Pollination and wounding induced nearly identical flavonol kinetics and patterns of accumulation in the same target tissue, suggesting that they share elements of a common signal transduction pathway. The wound response was systemic, because kaempferol accumulated in the stigma when distal tissues, such as the corolla, stamens, or sepals, were wounded. We have exploited the germination requirement for flavonols and the high level of kaempferol that accumulates after wounding to enhance plant fecundity. Seed set was significantly increased by mechanically wounding the corolla and stamens prior to the application of pollen to the stigma. A reproductive role for a plant secondary metabolite and the specific function of stigmatic kaempferol are discussed from an evolutionary perspective.

INTRODUCTION

In angiosperms, the stigma is the pollen-receptive surface of the female gametophyte-bearing pistil. In many species, the stigmatic surface is covered with a chemically complex exudate that provides the hospitable conditions and factors required for pollen adhesion, hydration, germination, and pollen tube penetration (Konar and Linskens, 1966; Knox, 1984; Heslop-Harrison, 1987). In addition, discriminatory events, such as the rejection of self-pollen in sporophytic incompatible reactions, occur at the stigmatic surface (Nasrallah et al., 1991). The stigma also appears to offer a hostile environment to bacteria and fungi because growth of these organisms on the stigma is rare (Jung, 1956).

Transcripts homologous to genes induced by wounding and stress have been detected in flowers from nonwounded plants (Gasser, 1991), and in some cases, defense-related compounds have been found to accumulate specifically in the stigma. For example, high levels of proteinase inhibitor (PI) proteins (Atkinson et al., 1993) and chitinase (Leung, 1992) have been measured in stigmas of solanaceous plants, and the promoters of an hydroxyproline-rich glycoprotein gene and a phenylpropanoid biosynthetic gene, phenylalanine ammonia-lyase, direct transcription of a reporter gene in transgenic tobacco stigmas (Liang et al., 1989; Wycoff et al., 1990). Although the defensive role of these compounds in wounded tissue is well known, a reproductive function has not been established.

Phenolic compounds function as stress indicators because they accumulate to high levels in many plant tissues in response to a wide range of biotic and abiotic signals, including wounding (Lawton and Lamb, 1987). Biosynthesis and accumulation of phenolics also occur as part of normal developmental programs in most higher plants (Wiermann, 1981). Chemical modifications to the basic aromatic structure lead to the proliferation of different classes of these so-called secondary products, representatives of which occur in most plant tissues. Although many roles in development have been suggested, their ubiquitous nature has made it difficult to identify critical functions. Those instances in which a specific role has been established, for example, as signal molecules between legumes and nitrogen-fixing *Rhizobium* spp or as UV protectants, have relied on the characterization of phenolic-deficient mutants (Burn et al., 1987; Li et al., 1993).

Chalcone synthase (CHS) catalyzes the initial step in flavonoid biosynthesis, and wild-type petunia and maize pollen contain large amounts of flavonoids. A lack of CHS activity in petunia and maize mutants has a pleiotropic effect: not only are the plants deficient in flavonoids, they also show abnormal pollen function and/or development (Coe et al., 1981; Taylor and Jorgensen, 1992; van der Meer et al., 1992). This correlation suggested a novel role for this specific class of phenolics. We found that although flavonoid-deficient pollen never

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functions in self-crosses, reciprocal crosses in petunia showed that pollen from mutant plants can function on wild-type stigmas, a phenotype defined as conditional male fertility (CMF) (Taylor and Jorgensen, 1992). The defect, a lack of pollen germination and tube growth, was biochemically complemented by adding nanomolar concentrations of kaempferol, a flavonol aglycone, to the nonfunctional CMF pollen. This treatment successfully restored pollen germination and tube growth and led to full seed set in both maize and petunia *CHS* mutants (Mo et al., 1992). The accumulation of flavonols and their effect on pollen germination in the different crosses of wild-type (V26) and CMF plants are summarized in Table 1.

Using an in vitro assay, we tested a large number of compounds for their ability to stimulate pollen germination. The capacity to restore germination and tube growth to the CMF pollen is limited to a specific class of flavonoids, the flavonol aglycones (Mo et al., 1992; T. Vogt and L.P. Taylor, manuscript in preparation), which 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, as shown in Figure 1. The specificity of flavonols for stimulating pollen tube growth in vitro has also been shown using cultured tobacco pollen (Ylstra et al., 1992). In this system, anther diffusates containing kaempferol, quercetin, and myricetin enhanced pollen tube growth.

In our initial study, we detected the flavonol aglycone kaempferol in stigmatic extracts from mature wild-type (inbred V26) petunia (Mo et al., 1992). Subsequent analyses of less mature flowers established that stigmas from V26 flowers with predehiscent anthers lack flavonol aglycones and contain only traces of a sugar conjugated form, kaempferol glycoside (Pollak et al., 1993). In this report, we describe the conditions that lead to the accumulation of high levels of kaempferol in petunia stigmas. Surprisingly, two seemingly different processes, a systemic wound response and normal pollination, produced virtually the same pattern of flavonol accumulation. Kaempferol is concentrated in the outer cell layers and exudate of the stigma where it is available to pollen grains. This accumulation of kaempferol in the stigma has a positive effect on reproduction, which we exploited to increase plant fecundity. By wounding the corolla 24 hr in advance of pollination and allowing the stigmas to accumulate kaempferol, seed set was increased in both wild-type and mutant crosses. The failure of kaempferol to accumulate in CMF stigmas pollinated with wild-type pollen confirmed that the stigma is the sole source of the flavonol.

Table 1. Flavonol Production and Pollen Germination in Crosses Described in This Study

Cross		Flavonols Produced[a]		Pollen Germination on Stigma[b]
Female	Male	Stigma	Pollen	
V26	× V26	+	+	+
V26	× CMF	+	–	+[c]
CMF	× V26	–	+	+
CMF	× CMF	–	–	–

[a] (+), competent to produce flavonols; (–), unable to produce flavonols.
[b] (+), pollen germinated within 30 min; (–), no pollen germination.
[c] Pollen germinated after 90 min.

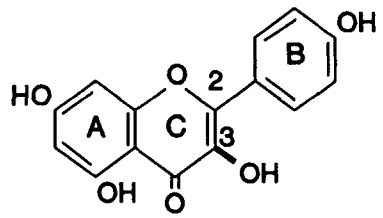

Figure 1. Structure of Kaempferol.

The structural features of the flavonol aglycone, kaempferol, required for pollen rescue are illustrated and include an unsubstituted hydroxyl group at carbon 3 and an unsaturated bond between carbons 2 and 3 of the C ring.

RESULTS

Kaempferol Accumulation in Stigmas after Pollination and Wounding

Flavonoids were separated and quantified by reverse phase HPLC, and the individual classes were identified by their characteristic UV spectra. We controlled whether the stigma, the pollen, or both provided the flavonols in a genetic cross by using flavonol-deficient plants as either male or female parents, as shown in Table 1. Large numbers of wild-type (inbred V26) and CMF flowers were synchronized to reach anthesis simultaneously by removing all open blossoms 24 hr prior to pollination. Wild-type stigmas from mature, stage 11 flowers (see Methods for a description of the stages) were pollinated with flavonol-deficient CMF pollen and harvested immediately (0 hr) or 24 hr after pollination; flavonoids were then extracted and separated by HPLC. Results of this analysis are displayed in the chromatograms in Figures 2A and 2B and show that pollination induced the production of a limited number of compounds in V26 stigmas. Based on spectral data showing absorption maxima at 260 and 350 nm, they were identified as flavonoids. The major new peak (peak 1) was kaempferol, a flavonol aglycone, which was identified by comparing retention time and spectral properties with a commercially available standard.

During earlier, preliminary experiments, we slit the corolla to gain easy access to the stigma. Surprisingly, we found that simply wounding the corolla in this way, without subsequent pollination, caused kaempferol to accumulate in the stigma.

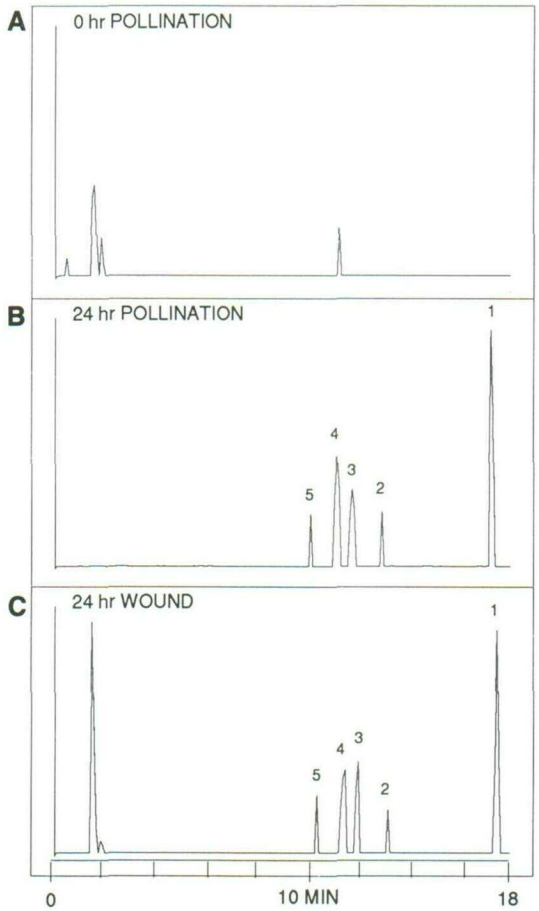

Figure 2. HPLC Analysis of V26 Petunia Stigma Extracts after Pollination (0 and 24 hr) with CMF Pollen or 24 Hr after Wounding.

Each injection is equivalent to 2.5 stigmas. Peak 1 is kaempferol and peaks 2 through 5 are flavonoid 3-*O*-glycosides. Peaks appearing before 5 min represent the solvent front. Chromatographic conditions are as described in Methods. Eluents were monitored at 365 nm.
(A) Extract from stigmas immediately (0 hr) after pollination.
(B) Extract from stigmas 24 hr after pollination.
(C) Extract from stigmas 24 hr after wounding.

Figure 2C shows that the HPLC pattern of flavonoid accumulation in extracts of unpollinated stigmas 24 hr after slitting the corolla (wounding) is virtually identical to that induced by pollination alone. HPLC analysis of extracts of corollas harvested at various times after wounding showed that no kaempferol or other flavonols accumulated in the wounded tissue after this treatment. This reaction resembles a systemic defense response because wounding the corolla, an adjacent nongerminal tissue, led to the accumulation of kaempferol in the stigma.

Restriction of Kaempferol Accumulation to the Outermost Cell Layers and Exudate of the Stigma

Whole stigmas were extracted for HPLC analysis; therefore, it was unclear in which of the various stigmatic tissues (papillae, epidermis, transmitting tract, vascular tissue, or parenchyma) the kaempferol accumulated. Localization was determined by examining longitudinal sections of unfixed V26 and CMF stigmas stained with the flavonoid-specific reagent Naturstoffreagenz A (diphenylboric acid 2-aminoethyl ester; Neu, 1957). In Figure 3A, the epidermal and subepidermal cell layers of a V26 stigma 24 hr postpollination display a vivid yellow–green fluorescence with a spectrum typical for flavonols

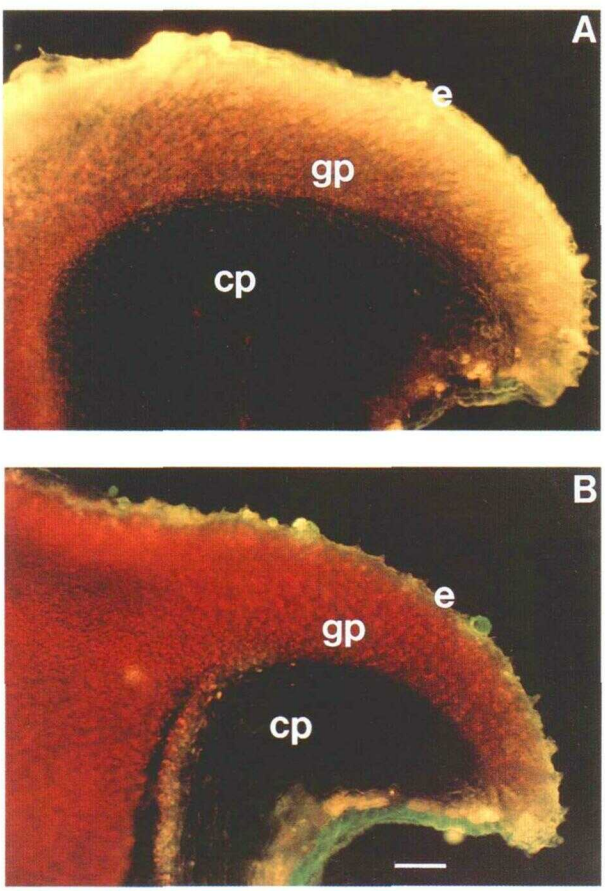

Figure 3. Flavonol Accumulation in Outer Cell Layers and Exudate of Stained Petunia Stigmas.

Longitudinal sections (100-μm thick) of unfixed tissue were incubated with diphenylboric acid 2-aminoethyl ester, a flavonoid-specific reagent, and examined by epifluorescence. e, epidermis; gp, glandular parenchyma; cp, cortical parenchyma.
(A) V26 stigma 24 hr after pollinating with CMF pollen.
(B) CMF stigma 24 hr after pollinating with CMF pollen.
Bar = 0.125 mm.

containing a single hydroxyl group in the B ring; kaempferol has this structure (Figure 1). The same localization occurred in V26 stigmas 24 hr after wounding the corolla (data not shown). On the other hand, control sections of CMF stigmas stained with diphenylboric acid 2-aminoethyl ester 24 hr after pollination synthesized no flavonols and showed only slight autofluorescence of cell walls (Figure 3B), as did V26 stigmas immediately after (0 hr) pollination or wounding (data not shown).

Kaempferol was also present in the exudate. This was demonstrated by briefly (20 sec) dipping tissues into chloroform and analyzing the wash solution by HPLC (Vogt et al., 1991). After this treatment, 70% of the kaempferol that accumulated in the V26 stigmas 24 hr after pollination was recovered in the chloroform wash. Kaempferol is amphiphilic and can permeate the plasma membrane of cells, which may explain how kaempferol from V26 stigmas gains access to CMF pollen grains. Upon contact with the stigma, the pollen grains are suspended in the kaempferol-rich exudate much as they were in the kaempferol-supplemented germination medium of the in vitro rescue assay. Due to its high concentration, kaempferol is readily available for uptake and induction of germination. The observation that kaempferol accumulated in the stigma agrees with previous reports that flavonoids preferentially accumulate in the outer cell layers of many tissues and that the less polar aglycone form of flavonoids are commonly found on the external surfaces of plants (Wollenweber and Dietz, 1981; Schmelzer et al., 1988; Hrazdina, 1992).

Time Course of Kaempferol Accumulation

Kaempferol induction in the stigma is developmentally controlled: pollinating or wounding immature flowers (stages 3 through 7) did not lead to kaempferol accumulation. Figure 4A shows that stigmas from flowers at stage 8 became competent to accumulate kaempferol when pollinated and wounded, although the levels were only ~50% of those measured at stages 10 and 11. The capacity of stage 8 stigmas to accumulate kaempferol when pollinated and wounded coincided with the ability of the pistil to sustain pollen tube growth; stage 7 stigmas did not support tube growth but stage 8 stigmas did. We have not determined whether seed set results from pollinating stage 8 stigmas, but we have measured limited seed set with stage 9 stigmas (Pollak et al., 1993).

The time course of kaempferol accumulation in V26 stigmas following pollination with CMF pollen and wounding by slitting the corolla is plotted in Figure 4B. These treatments did not produce detectable kaempferol at the time of pollination or wounding (0 hr) (see also Figure 2A). After a delay of ~4 hr, kaempferol levels began to rise in the pollinated stigma, and accumulation continued for at least 48 hr. At 24 hr after pollination, more than 200 pmol of kaempferol accumulated in each stigma, and levels of 450 pmol per stigma were measured at 48 hr after pollination. Wounding the corolla produced a similar pattern of kaempferol accumulation in stigmas, except

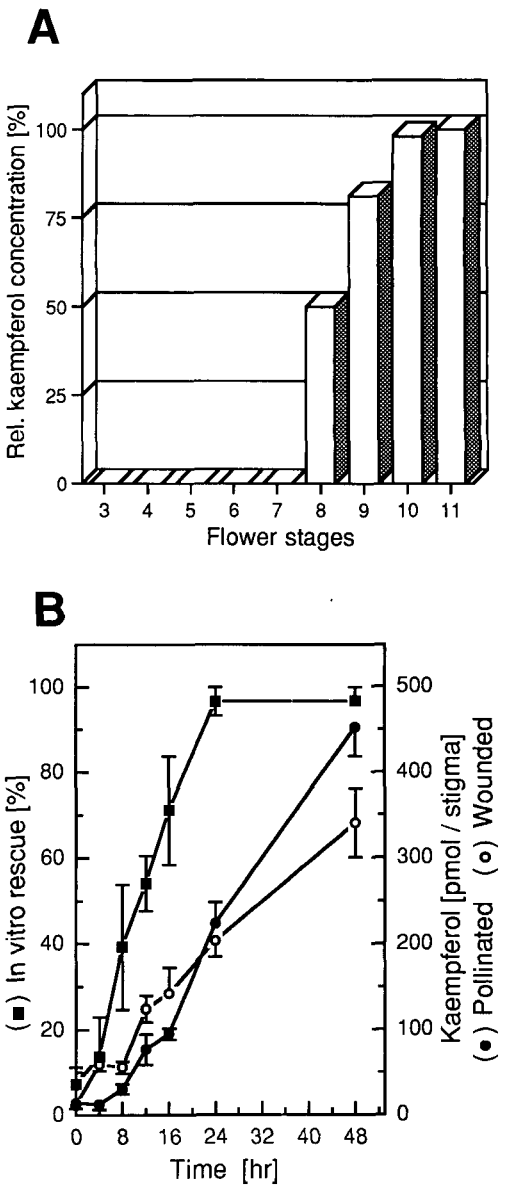

Figure 4. Kaempferol Accumulation in V26 Stigmas after Pollination and Wounding.

Kaempferol amounts were determined from the peak areas of HPLC separated methanol extracts of stigmas collected at various stages or times after pollinating with CMF pollen and wounding by slitting the corolla.

(A) Developmental profile of relative (Rel.) kaempferol accumulation in V26 stigmas. Stages are as described in Methods.

(B) Time course of kaempferol accumulation in V26 stigmas after pollinating (●) and wounding (○). An aliquot of the pollinated extract was dried and dissolved in DMSO (extract of 10 stigmas per 20 μL of DMSO); 1 μL was added to a 100-μL suspension of CMF pollen in germination medium (Mo et al., 1992). The degree of pollen rescue (■) was calculated by scoring the number of germinated pollen as a percentage of the total grains counted. Values equal the average of three trials (± SE).

that the 4-hr time lag was abolished, and 48-hr levels were ~25% lower than pollination amounts (Figure 4B). These data, together with the HPLC pattern presented in Figure 2, indicate that wounding and pollinating produced almost identical kaempferol profiles in stigmas.

The in vitro pollen germination assay confirmed the HPLC analysis. Because the number of CMF pollen grains that germinate in the in vitro system is directly related to kaempferol concentration (Mo et al., 1992; Pollak et al., 1993), it follows that stigma extracts isolated at successive times after pollination (or wounding) should stimulate CMF pollen germination at levels corresponding to the amount of extractable kaempferol. This was confirmed by correlating the kaempferol concentration in pollinated stigma extracts, measured by HPLC, with the number of CMF pollen grains induced to germinate by an aliquot of the stigma extract (Figure 4B). Between 4 and 24 hr, there was a near linear correlation between the kaempferol content of a stigma and the ability of the stigma extract to stimulate pollen germination. At 24 hr after pollination, full rescue (>95% of the pollen grains germinate) was achieved with stigmas containing ~250 pmol of kaempferol. Calculated on the basis of stigma volume, this is approximately the same concentration (0.5 μM) that produced full rescue in the in vitro assay when using commercially available, HPLC-purified kaempferol (Pollak et al., 1993). When applied to wild-type pollen, the stigma extract did not inhibit pollen germination, indicating that the amount of kaempferol extracted from the stigma is solely responsible for the levels of CMF pollen germination in this assay.

Treatments Stimulating Kaempferol Accumulation in V26 Stigmas

Wounding and pollination involve touching the stigma and corolla. Plants are mechanosensory and can respond to mechanical stress by changes in gene expression, which translate into changes in developmental patterns (Braam and Davis, 1990). To determine if additional physical treatments or developmental events besides pollination or wounding of the corolla induce stigmatic flavonols, we compared the relative levels of kaempferol after various treatments and events. Treatments included emasculation by removing the anthers, touching the stigma or corolla with a blunt instrument, applying a quantity of glass beads equivalent to the amount of pollen received at pollination, pollinating with *Nicotiana alata* or heat-killed CMF petunia pollen, and wounding increasingly distal tissues. As graphically presented in Figure 5, the combination of slitting the corolla plus removing the anthers led to the largest accumulation of kaempferol in the stigma (170%). Emasculation alone and pseudopollination with glass beads, heat-killed pollen, or pollen from another solanaceous species stimulated almost as much kaempferol accumulation in V26 stigmas as pollination with live pollen or slitting the corolla (wounding). Touching the stigma produced a response ~50% of that produced by pollination or slitting the corolla. Touching the corolla had no effect. The wound effect became attenuated with increasing distance of the wound site from the stigma. Slitting a sepal or subtending leaf induced kaempferol in V26 stigmas to levels 61 and 39%, respectively, of the level produced by slitting the corolla.

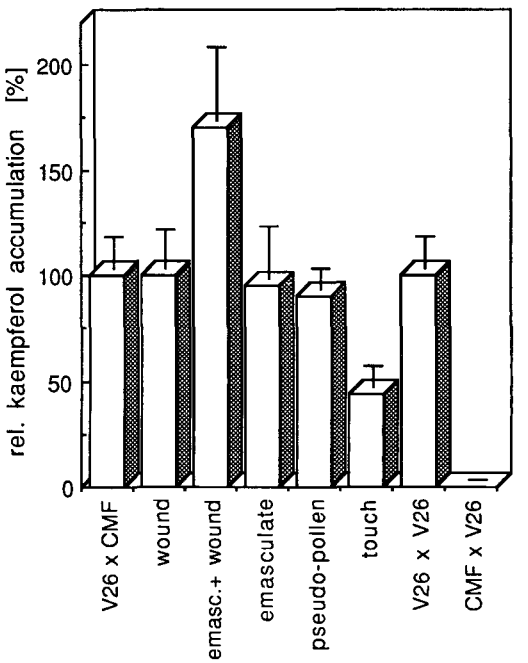

Figure 5. Kaempferol Accumulation in V26 Stigmas 24 Hr after Various Sensory Treatments and Developmental Events.

Kaempferol was quantitated from the peak areas of HPLC-separated stigma extracts and expressed relative (rel.) to the amount induced by pollination with CMF pollen. All extracts were from V26 stigmas, except for the control extract from CMF stigmas pollinated with V26 pollen. Treatments included the following: (1) pollination with CMF pollen, (2) slitting the corolla (wound), (3) emasculation (emasc.) plus slitting the corolla, (4) emasculation (removal of anthers), (5) pseudopollination (pseudo-pollen) with glass beads (0.2 mm), heat-killed CMF pollen, or tobacco pollen, (6) touching the stigma, (7) pollination with V26 pollen, and (8) pollinating CMF stigmas with V26 pollen.

Pollination of V26 stigmas with V26 pollen produced a response identical to pollination with CMF pollen, suggesting that accumulation of kaempferol in stigmas is a normal post-pollination event and not an atypical response to the flavonoid-deficient CMF pollen. Control pollinations confirmed that the V26 stigma is the sole source of the kaempferol that collects after pollination or wounding. In the reciprocal cross of CMF × V26, the stigma is unable to produce flavonoids because the plants lack CHS activity (Napoli, et al., 1990; Mo et al., 1992). HPLC analysis of stigma extracts from CMF pistils pollinated with V26 pollen detected no kaempferol or other flavonol (Figure 5). This analysis excludes the possibility that leachate from V26 pollen is the source of the kaempferol that accumulates in V26 stigmas following pollination. When anthers and/or pollen are mechanically disrupted (Mo et al., 1992),

kaempferol can be generated from kaempferol glycosides (T. Vogt, P. Pollak, and L.P. Taylor, unpublished results). However, using a nondisruptive extraction procedure and HPLC analysis (detection limit <2 pmol per anther), we could not detect the aglycone form in intact anthers and pollen even though each mature V26 anther contained more than 2000 pmol of kaempferol glycosides (Pollak et al., 1993; T. Vogt and L.P. Taylor, unpublished results). As expected, stigma extracts from CMF self-crosses accumulated no flavonols.

Dehiscence precedes pollination and involves rupture of the anther along the stomium to liberate the pollen grains. To determine whether dehiscence contributes to flavonol induction in the stigma, we compared kaempferol levels in unpollinated stigmas from flowers with dehiscent and nondehiscent anthers. We found that kaempferol does not accumulate in the stigma in response to dehiscence.

Germination and Tube Growth of CMF and V26 Pollen on V26 Stigmas

In a V26 × V26 cross, both the pollen and stigma could supply flavonols, but the stigma was the only source of flavonols when V26 stigmas were pollinated with CMF pollen. The frequency of CMF pollen germination was dependent on kaempferol concentration; at very low concentrations (<0.5 μM), fewer grains germinate (Mo et al., 1992; Pollak et al., 1993). Therefore, the time and extent of CMF pollen germination on V26 stigmas should reflect the kinetics of kaempferol accumulation in the pollinated stigma. In addition, if the extent of pollen germination and rate of tube growth in a V26 × V26 cross are increased relative to that of CMF pollen on a V26 stigma, this would suggest that the flavonols present in wild-type pollen are mobilized for germination and tube growth. V26 flowers were emasculated 24 hr prior to pollination with either V26 or CMF pollen, and the stigmas were harvested at various intervals. Pollinated pistils were squashed in decolorized aniline blue, and callose in the growing tubes was visualized by epifluorescence. The results of three separate trials confirmed that pollen germination was retarded in V26 × CMF crosses relative to V26 × V26 crosses. In Figure 6, photomicrographs of selected regions of pollinated pistils show that 90 min after pollination most of the V26 pollen grains had germinated and the growing tips had penetrated well into the stigma. In contrast, CMF pollen did not reach this point until ~6 hr after germination. In fact, 90 min after placing the CMF pollen on the stigma, few if any grains had germinated.

Although there are exceptions, the majority of pollen tubes grew in a mass, the leading edge of which was monitored and scored for length. At 8 hr after pollination, the growing front of pollen tubes from a V26 × V26 cross had passed the stigma–style interface, whereas most of the CMF tubes were still in the stigma proper. Fertilization normally occurs in petunia 48 hr after pollination (Herrero and Dickinson, 1979), and we found V26 pollen tubes entering the ovary at this time. On the other hand, the majority of the CMF tubes reached this point ~54 hr after pollination. Entry into V26 ovules by CMF pollen tubes was therefore delayed relative to V26 self-crosses. This delay is presumably due to the time required for kaempferol to accumulate in V26 stigmas to levels sufficient to stimulate germination of CMF pollen. The 6-hr delay established at germination of CMF pollen appeared to be maintained throughout tube growth in the pistil, indicating that once germination has occurred, V26 and CMF pollen tubes grow at the same rate.

Seed Set Increased by Wounding before Pollinating

Although seed set is affected by many factors, one of the most important is pollen germination frequency and the production of functional pollen tubes. The high levels of stigmatic kaempferol induced by pollination or wounding combined with our earlier finding that CMF pollen germination rates depend on kaempferol concentration (Mo et al., 1992; Pollak et al., 1993) predict that pollen germination will be enhanced by wounding corollas before pollen is applied to stigmas. This hypothesis was confirmed by a comparison of seed set in flowers wounded 24 hr before pollination versus nonwounded control flowers; these data are shown in Table 2. We found that slitting V26 corollas (wounding) and removing anthers (emasculation) 24 hr prior to pollinating with CMF pollen caused a 14% relative increase in seed set (Table 2; crosses V26 × CMF). The increase in the average number of seeds per capsule, from 180 to 206, was significant (Student's *t*-test, $\alpha = 0.0467$). In addition to increased seed set, microscopic examination of pollinated V26 pistils from wounded flowers showed that slitting the corolla 24 hr before pollination eliminated the lag seen in CMF pollen germination: within 90 min, most of the CMF pollen had germinated and the majority of tubes had reached the ovary by 48 hr after pollination. In fact, V26 pistils from wounded flowers pollinated with CMF pollen looked virtually identical to the unwounded V26 × V26 cross shown in Figure 6.

The V26 inbred strain sets fewer seeds per capsule than many other petunia varieties (Taylor and Jorgensen, 1992). To determine whether we could exploit the accumulation of kaempferol in the stigma following wounding to increase wild-type plant fecundity, we slit corollas 24 hr before pollinating with V26 pollen. The comparison of V26 × V26 crosses in Table 2 shows that the average number of seeds per capsule increased by more than 100% (125 versus 252), a difference that is highly significant (Student's *t*-test, $\alpha = 0.0046$). In addition, the proportion of capsules with seeds in excess of 250 increased from 13 to 49% in wounded versus nonwounded flowers. This result is surprising because we measured levels of kaempferol 3-*O*-glycosides up to 2000 pmol per mature V26 anther (Pollak et al., 1993). The speed with which V26 pollen germinated both in vivo on V26 or CMF stigmas and in vitro would suggest that flavonols are not limiting for pollen germination. Our current results provide no explanation for this paradox. The flavonol content of individual pollen grains may

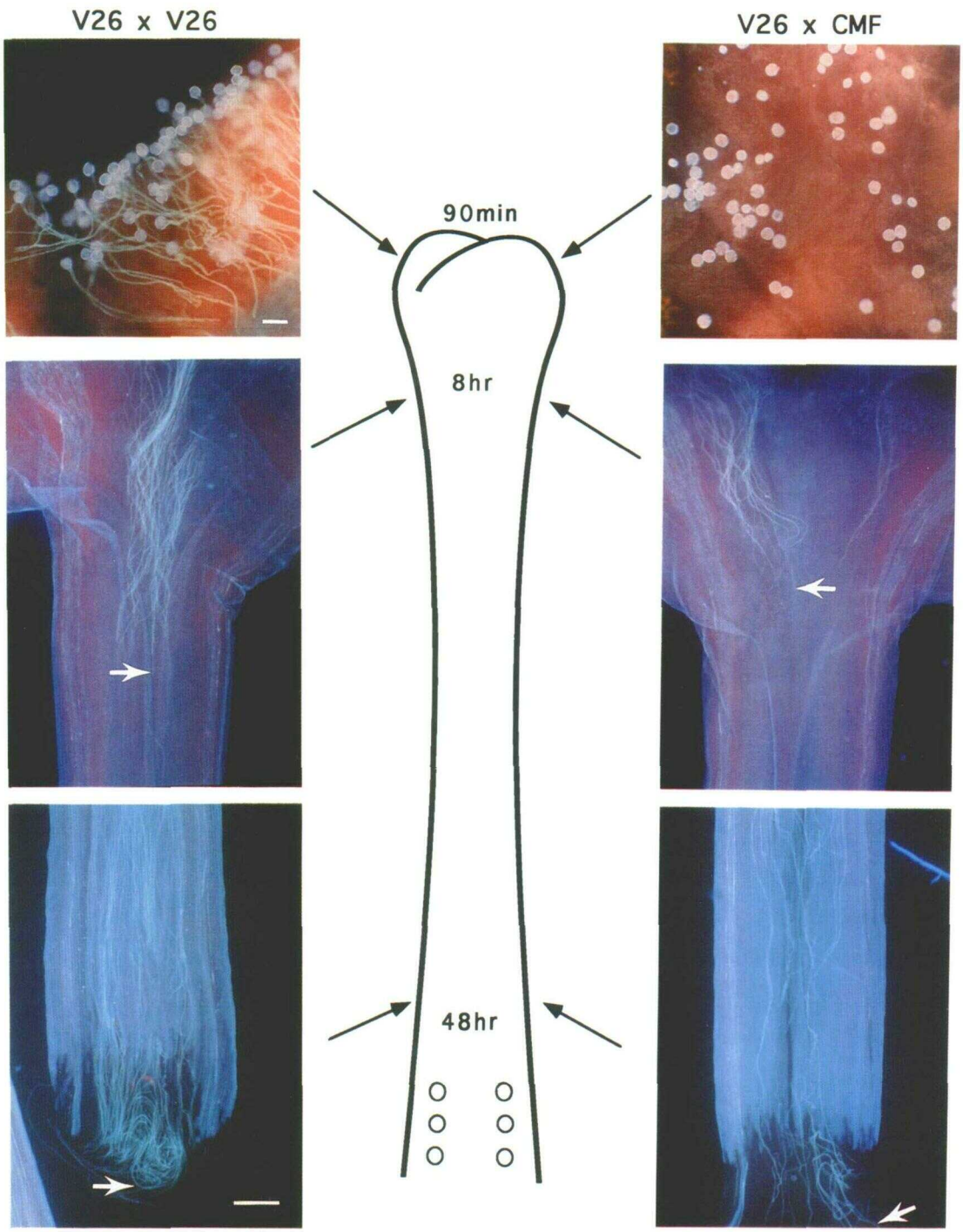

Figure 6. Fluorescence Micrographs Showing the Extent of Pollen Germination and Tube Growth in V26 Pistils at Three Intervals after Pollination.

V26 pistils, pollinated with either wild-type pollen (left) or flavonol-deficient CMF pollen (right), were harvested at 90 min, 8 hr, and 48 hr, fixed, cleared in NaOH, and stained with decolorized aniline blue. Callose in the growing tubes was visualized by epifluorescence. Arrows indicate the leading edge of the growing pollen tubes. Pollen tubes in the lower left panel (V26 × V26) have grown beyond the end of the style and appear in a coiled mass. The bar in the lower left panel = 0.35 mm and applies to the lower four micrographs. Micrographs of the stigma head (top two micrographs) are at twice the magnification of the four panels shown below (Bar = 0.175 mm).

Table 2. Wounding before Pollination Increases Seed Set

Cross	Treatment	Pollinations with Seeds per Capsule					Average per Capsule	Total Seeds	No. Crosses
		0	1–150	151–250	251–350	>350			
V26 × CMF	Wounded	5	21	32	26	6	206[a]	18,500	90
V26 × CMF	Nonwounded	4	25	35	17	5	180[a]	15,500	86
CMF × V26	Nonwounded	15	34	8	17	6	153	14,600	95
V26 × V26	Wounded	1	23	42	28	18	252[b]	23,700	94
V26 × V26	Nonwounded	9	50	20	8	4	125[b]	11,300	91

[a] Student's *t*-test, $\alpha = 0.0467$.
[b] Student's *t*-test, $\alpha = 0.0046$.

vary widely and only those with the highest levels reach the embryo sac, or alternatively, unidentified environmental effects may influence seed set.

CMF stigmas cannot accumulate flavonols even after wounding (Mo et al., 1992; Pollak et al., 1993) so that, in a cross of CMF pistils by V26 pollen, all of the flavonols required for pollen germination come from the pollen itself. A similar situation exists during the first few (up to 4 hr) hours of a nonwounded V26 self-cross. The concentration of kaempferol in immature flowers is 20 pmol per stigma (Pollak et al., 1993), which is all in the sugar-conjugated form that is unable to stimulate pollen germination (Mo et al., 1992). Therefore, CMF × V26 crosses and nonwounded V26 × V26 crosses have little or no stigmatic kaempferol during the first few hours after pollination. These two crosses showed similarity in the average seed set per capsule (153 for CMF × V26 versus 125 for V26 × V26) and in the distribution of seeds per capsule (well over half in the <150 category).

Increased CHS Transcript and Protein Levels Following Pollination

Production of phenylpropanoids and flavonoids is generally controlled and preceded by an increase in transcript and protein levels of the key biosynthetic enzymes, phenylalanine ammonia–lyase and CHS (Dangl, 1992). In petunia, *CHS* is represented by a family of eight genes, two of which are expressed in the corolla (Koes et al., 1987). No data exist on CHS RNA levels in petunia stigmas, but we recently detected CHS protein in developing stigmas using an anti-CHS antiserum (Pollak et al., 1993).

Accumulation of CHS transcripts in V26 stigmas following pollination or wounding was determined by RNA gel blot analysis, as presented in Figure 7A. Using the glyceraldehyde phosphate dehydrogenase hybridization signal to correct for loading differences, relative amounts of CHS transcript at different times after pollinating with CMF pollen or slitting the corolla were determined. Increased levels (1.8 times higher than at 0 hr) of CHS RNA were detected in V26 stigmas 4 hr after pollination and well before kaempferol was measurable (Figure 7A, lanes 1 and 2). A similar induction was measured 4 hr after wounding the corolla (data not shown). At 24 hr after pollinating with CMF pollen, wounding by slitting the corolla, or pollinating with V26 pollen, CHS RNA amounts were 3.5, 2.1, and 2.0 times higher than the 0 hr baseline level (Figure 7A, lanes 3, 4, and 5, respectively). CMF stigmas cosuppressed for CHS and showed no transcripts homologous to the CHS cDNA probe (Figure 7A, lane 6). Although a separate signal must be involved to transmit the wound stimulus from the corolla to the responding stigma cells, the pathways of pollination- and wound-induced CHS must converge at some point. It may be that the signals induce transcription of different *CHS* genes(s); this could be determined by hybridization with gene-specific probes.

Another candidate for the control of flavonol accumulation is flavanone 3-hydroxylase (F3H) that catalyzes the addition of an hydroxyl group at position 3 on the C ring of flavanones, producing dihydroflavonols. Using an F3H cDNA from Antirrhinum (85% similarity to petunia F3H at the amino acid level) as a hybridization probe in an RNA gel blot analysis, we found the gene to be constitutively expressed at moderate levels in all stigmas tested, including CMF stigmas (Figure 7A). The lack of correlation between F3H transcript levels and kaempferol levels in response to wounding and pollination (Figure 4) indicated that kaempferol accumulation is not controlled at the level of F3H transcription and that this step is not rate limiting in flavonol biosynthesis in petunia. CHS and F3H transcripts were not detected in anthers of mature flowers (data not shown); this is consistent with our previous result showing that CHS protein peaks and declines before flower maturity and anthesis (Pollak et al., 1993). The coordinate rise in kaempferol and CHS RNA in the stigma following pollination and wounding suggests that regulation of flavonol accumulation may be controlled at the level of CHS transcription.

In Figure 7B, immunoblot analysis using an anti-CHS antiserum shows that CHS protein accumulated in stigmas after the various treatments. Using the signal from purified CHS protein to quantify relative CHS protein amounts in stigma extracts, we found CHS levels increased from 0.04% of total soluble protein at 0 hr to 0.05 and 0.06% of total protein at 8 and 24 hr, respectively, after pollination with CMF pollen and 0.09% at 24 hr after pollinating with V26 pollen (Figure 7B, lanes 2 to 5). Wounding (0.10%) and pseudopollination (0.11%) with glass beads (Figure 7B, lanes 6 and 7, respectively) also showed increased stigmatic CHS protein levels relative to the

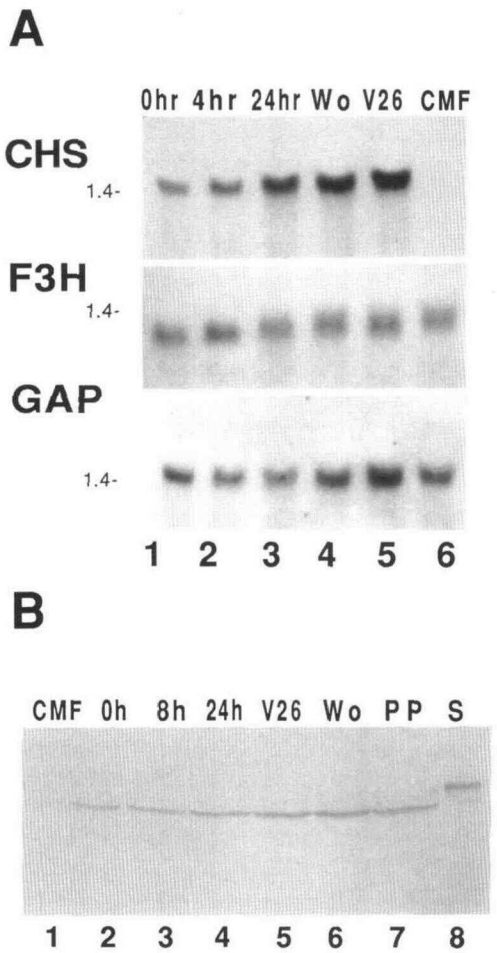

Figure 7. Effect of Pollination or Wounding on the Accumulation of Transcripts in the Flavonol Pathway and CHS Protein in V26 Stigmas.

(A) RNA gel blots hybridized with a CHS cDNA probe from petunia, an F3H cDNA probe from *Antirrhinum*, and, as a loading control, glyceraldehyde phosphate dehydrogenase (GAP). Each lane contains 15 μg of total RNA. Lane 1, V26 stigmas at 0 hr after pollinating with CMF pollen; lane 2, V26 stigmas 4 hr after pollinating with CMF pollen; lane 3, V26 stigmas 24 hr after pollinating with CMF pollen; lane 4, V26 stigmas 24 hr after wounding (Wo; slit corolla); lane 5, V26 stigmas 24 hr after pollinating with V26 pollen; and lane 6, CMF stigmas 24 hr after pollinating with CMF pollen. Length markers in kilobases are given at left.

(B) Immunodetection of CHS protein in stigmas after pollination or wounding. Total protein from petunia stigmas was separated by SDS-PAGE (100 μg per lane; 0.1 μg CHS standard), immobilized on nitrocellulose, reacted with a rabbit polyclonal antibody to CHS, and visualized with anti–rabbit IgG-conjugated alkaline phosphatase. Lane 1, self-pollinated CMF stigmas 0 hr after pollination; lane 2, CMF-pollinated V26 stigmas 0 hr after pollination; lane 3, CMF-pollinated V26 stigmas 8 hr after pollination; lane 4, CMF-pollinated V26 stigmas 24 hr after pollination; lane 5, self-pollinated V26 stigmas 24 hr after pollination; lane 6, V26 stigma 24 hr after wounding (Wo) the corolla; lane 7, V26 stigma 24 hr after pseudopollination (PP) with glass beads; lane 8, maize CHS protein standard (S).

amount measured at 0 hr. Flavonols are chemically stable and once synthesized, they tend to remain in plant tissues until senescence; therefore, modest increases in CHS RNA and protein can lead to high levels of stigmatic kaempferol 24 hr after pollination or wounding. This coordination between kaempferol accumulation (Figure 4B) and CHS protein and RNA levels contrasts with the regulation in immature stigmas in which CHS protein levels rose during development but kaempferol levels remained low (Pollak et al., 1993). This difference revealed that a change in the regulation of CHS is coincident with stigma maturity and receptivity.

DISCUSSION

Kaempferol Induction in the Stigma

In this report, we have characterized the accumulation of high levels of kaempferol in the outer cell layers and exudate of petunia stigmas in response to either pollination or wounding. The similar pattern of kaempferol accumulation suggests that the signals generated by these two processes may be transduced through a common pathway. In both, kaempferol levels were initially low but ultimately rose and remained elevated until well after fertilization had taken place. Our previous structure–activity analysis showed that only flavonol aglycones are able to stimulate pollen germination, and in this study we have shown that the aglycone form was not produced until the stigma was pollinated or the flower was wounded.

The biological reason for kaempferol accumulation in the stigma in response to pollination is not obvious. The timing differential between peak kaempferol levels and pollen germination is strong evidence that germination and early tube growth do not depend on kaempferol in the stigma. Within minutes of landing on the stigmatic surface, wild-type pollen germinates and tubes grow into the transmitting tract. By the time kaempferol has risen to detectable levels in the outer cell layers (4 to 6 hr after pollination), the growing tips of pollen tubes are far beyond this part of the stigma. In addition, the fact that wild-type pollen germinates and produces moderate seed set when used to pollinate flavonoid-deficient CMF stigmas (Table 2; Taylor and Jorgensen, 1992) supports the notion that stigmatic kaempferol is not required for successful pollen function. This raises the question of whether the accumulation of kaempferol in stigmas after pollination has any reproductive function.

Lipids and sugars are major constituents of the colorless exudate that accumulates on the head of the stigma late in petunia flower development (Konar and Linskens, 1966). This liquid presents an attractive environment for growth of fungi and bacteria. In addition, penetration of pollen tubes is accompanied by secretion of lytic enzymes and the breakdown of surface barriers; these are processes that could encourage pathogen invasion. Yet, infections of the stigma are rare (Jung, 1956). The antiseptic, phytoalexin properties of phenolics and flavonoids are well documented (Tal and Robeson, 1986;

Lawton and Lamb, 1987), and high levels of kaempferol are even reported to inhibit the growth of viruses (French and Towers, 1992). The effective concentration of kaempferol in the stigmatic exudate 24 hr after pollination or wounding is between 10 and 100 μM, which approaches a level that is inhibitory to pollen germination in vitro (Mo et al., 1992). It reaches this level long after the pollen has germinated and at a time when pollen tubes are at least two-thirds of the way down the style. Therefore, we propose that high levels of kaempferol in the stigma following pollination may have an allelopathic function. The mechanism of kaempferol toxicity is not known, but kaempferol can promote radical formation that might interfere with vital functions of pathogens (Ahmad, 1992). The slow induction of kaempferol may serve as a sort of rear guard to growing pollen tubes, preventing infections in the parts of the stigma and style wounded by "invading" pollen tubes. These regions are presumably more susceptible to pathogen colonization, and these pathogens might then overtake the growing pollen tubes and possibly infect the ovary.

Kaempferol accumulation in the stigma after various wound treatments fits the definition of a systemic defense response. However, unlike a classic systemic wound response, kaempferol does not accumulate in the wounded tissues. In addition, biochemical analysis revealed no flavonoids in stylar extracts, confirming that kaempferol is not transported from the wounded tissues to the head of the stigma (this study; Mo et al., 1992). At pollination, many tissues of the mature flower are approaching senescence, and we speculate that enzymes of the flavonoid pathway are not active in the corolla, sepal, and subtending leaf at this time. Analysis of CHS RNA in the corollas of transgenic petunias showed that transcripts from the endogenous CHS gene peaked in 40-mm-long flowers and were virtually undetectable in mature flowers (Napoli et al., 1990). Although wounding produces a flavonoid profile that is virtually identical to that induced by pollination, the wound response requires the movement of a signal from the wounded tissue to the responding stigma. Although the identity of the transducing molecule is unknown, ethylene is an attractive possibility because it is known to be produced by petunia stigmas following pollination and in response to wounding (Hoekstra and Weges, 1986).

Other defense-related molecules, including the PI proteins, are systemically induced upon wounding. Recently, PI II transcripts and proteins were detected in stigmas of healthy, nonwounded tobacco plants (Atkinson et al., 1993). In situ localization showed that PI II transcripts accumulated in the epidermal and subepidermal cell layers of the stigma, which is the same site in which kaempferol accumulates in petunia stigmas. However, PI proteins are present at high levels in stigmas throughout tobacco flower development from very young (1-cm-long buds) to mature flowers, whereas only mature stigmas of petunia accumulate large amounts of kaempferol and do so only in response to defined developmental events and wound signals. Also, in contrast to flavonols (Mo et al., 1992), no reproductive role for PI proteins is known. It is worth noting that we detected PI II transcripts in both wounded and pollinated petunia stigmas (T. Vogt and L.P. Taylor, unpublished results).

The findings described here bridge a gap between two earlier studies. In an initial biochemical complementation analysis of CMF pollen, we used a stigma extract from mature wild-type flowers, and kaempferol was detected in this extract (Mo et al., 1992). In a subsequent analysis of developing stigmas, we found no kaempferol; however, only stigmas from flowers with predehiscent anthers were analyzed (Pollak et al., 1993). It is now apparent that the kaempferol in the initial study came from pollinated stigmas. In retrospect, it is worth noting that, in anticipation of the flavonol burst upon pollination, CHS protein levels increased threefold in predehiscent stigmas at the stage immediately preceding anther dehiscence (Pollak et al., 1993). Although there are earlier reports of unidentified phenolics in stigma extracts from several species (Martin, 1969), in this study, we have identified specific environmental and developmental processes that lead to the accumulation of a particular flavonoid, kaempferol, in defined cell layers of the stigma.

Seed Set Increased by Kaempferol Accumulation in the Stigma

Regardless of the function of kaempferol in the stigma, its accumulation following wounding presents an opportunity to enhance plant fecundity. In an experiment designed to increase seed set, we combined three characteristics of flavonol-dependent pollen germination: (1) the frequency of pollen germination is directly related to flavonol concentration; (2) flavonols applied at the time of pollination stimulate germination; and (3) 24 hr after wounding the corolla or stamens, high levels of kaempferol accumulate in the stigma. On stigmas of the same genotype (V26), two different types of pollen, one flavonoid deficient and the other containing ample flavonol glycosides, each produced enhanced seed set in wounded flowers. In the cross of CMF pollen onto V26 stigmas, seed set was increased by 14%, and in a V26 self-pollination, the number of seeds was doubled in wounded flowers compared to nonwounded flowers. In addition to a net seed increase, crosses done in the presence of elevated levels of flavonol produced a higher proportion of seed capsules with more than 350 seeds. An inadequate amount of flavonol aglycone may limit germination of some V26 pollen grains, producing selective pressure for pollen grains with higher levels of flavonol aglycones. We are now testing other petunia varieties and other solanaceous species to determine if the seed set of these plants can also be increased by wounding before pollinating.

Evolution of a Role for Kaempferol in Pollen-Pistil Interactions

A reproductive function for flavonols may have evolved from a defensive role. Flavonols, including kaempferol, are present

in asiphonogamous plants, such as liverworts and ferns, in which pollination is not involved in reproduction (Markham, 1988; Stafford, 1991). In these plants, flavonols may act in plant defense. In siphonogamous plants, penetration of the pistil by pollen tubes may open a conduit for pathogens. Allelopathic flavonoids in the stigma or pollen may act to prevent introduction of pathogens into the pistil. Pollen that could tolerate and/or metabolize the potentially toxic kaempferol would have been selected, and those grains that could utilize kaempferol to enhance germination or tube growth would have a competitive advantage (Mulcahy, 1979), thus selecting for kaempferol as a growth promoter.

Angiosperm pollen is metabolically very active and successful fertilization is highly dependent on two factors: rapid germination and pollen tube growth (Hoeckstra, 1983). As our results demonstrated, increased kaempferol levels, which eliminate the germination lag of CMF pollen, led to increased seed set. Other organisms also may be dependent on flavonols for growth. Certain mycorrhizal fungi respond with enhanced hyphal growth to the same flavonol aglycones that are required for pollen tube growth in petunia (Gianinazzi-Pearson et al., 1989; Bécard et al., 1992; Chabot et al., 1992). More data showing the specificity of phenolics and flavonoids as interactive compounds among plants, fungi, and microbes are refocusing our perception of these universal secondary metabolites.

METHODS

Plant Material

The *Petunia hybrida* plants used in this study are described by Taylor and Jorgensen (1992) and Napoli et al. (1990); the inbred line V26 (wild type) produces flavonols and is self-fertile. Transgenic derivatives of V26, suppressed for chalcone synthase (CHS) activity, fail to accumulate flavonoids and are self-sterile. They exhibit a conditional male-fertile (CMF) phenotype, which was described by Taylor and Jorgensen (1992): these transgenic derivatives produce pollen that is nonfunctional in self-crosses but is able to function in outcrosses. Also, in contrast to wild-type V26 pollen, CMF pollen is unable to germinate in vitro (Mo et al., 1992). All experiments were performed with V26 and CMF plants at the developmental stages described by Pollak et al. (1993). Stages are defined by bud length and morphology: stage 1, buds up to 10 mm from the base of the receptacle to the tip of the corolla; stage 2, buds 11 to 15 mm; stage 3, buds 16 to 20 mm; stage 4, buds 21 to 25 mm; stage 5, buds 26 to 30 mm; stage 6, buds 31 to 35 mm; stage 7, buds 36 to 40 mm; stage 8, buds 41 to 50 mm; stage 9, buds over 50 mm with corolla beginning to open; stage 10, corolla up to three-quarters open; stage 11, corolla open, anthers predehiscent; stage 12, anthers dehiscent. Table 1 summarizes the different genetic crosses described in this study: the ability of stigmas and anthers to produce flavonols, and whether pollen will germinate on different stigmas.

Crosses of CMF × CMF, V26 × V26, CMF × V26, and V26 × CMF were performed by applying pollen from one locule to each stigma from nonwounded flowers at stage 11, just prior to anther dehiscence. Wounding was performed by slitting the corolla, sepal, or subtending leaf along its entire length with forceps. To prevent uncontrolled self-pollination during the dehiscence experiments, a straw (3-mm i.d.) was placed over the stigma. At harvest, stigmas were separated into two groups: the dehiscent population was from flowers that had at least three ruptured anthers; the nondehiscent controls had no open anthers.

Seed set experiments were performed as described above, except that wounded flowers were also emasculated by removing anthers 24 hr prior to pollination. To control for possible variation among plants, at least 10 different plants were used for each treatment with 10 crosses per plant. Wounded and nonwounded flowers were pollinated at the same time using pollen from a pooled source of several flowers. Seed capsules were allowed to develop for 6 weeks, harvested, and dried at room temperature; the number of seeds per capsule was then determined.

HPLC Analysis of Plant Extracts

Our current extraction procedure and HPLC analysis differ from those used previously (Mo et al., 1992). The earlier analysis required the use of highly concentrated extracts from pulverized tissue to compensate for a less sensitive detection system. We have substituted an extraction procedure that does not disrupt the tissues as a protection against enzymatic hydrolysis of the flavonol glycosides. Using a more sensitive detection system, HPLC analysis was performed on an extract equivalent to 2.5 anthers or stigmas, whereas in the study of Mo et al. (1992) an extract equivalent to 250 anthers was used.

Extracts for HPLC were prepared by soaking stigmas (five stigmas per 100 μL of 100% methanol) overnight at −20°C. Samples were centrifuged briefly, and 50 μL of the supernatant was injected. To convert flavonol glycosides to the aglycone, 50 μL of the methanol extract was mixed with 50 μL of 4 N HCl and hydrolyzed for 30 min at 60°C. Samples were reconstituted to 150 μL with 100% MeOH to ensure solubility of the aglycones and centrifuged; 75 μL of the supernatant was injected. Each time course was performed at least twice with sampling in triplicate. Wounded or nonwounded tissue was extracted into 100% methanol (200 mg/mL), as was described for stigmas, and analyzed for flavonoids by HPLC.

HPLC analysis of stigma extracts was performed as described by Pollak et al. (1993), using a linear gradient of increasing acetonitrile concentration in water (both 5% acetic acid) to elute compounds from a reverse phase analytical column (Nova-Pac C18) supported by a chromatography workstation (model 820 Maxima) equipped with a dual pump system (model 510) and a photodiode array detector (model 994 [all from Waters Chromatography System, Millipore Corp., Milford, MA]). Elution of individual compounds was monitored at 365 and/or 290 nm. Using kaempferol as an external standard, detection proved to be linear between 5 and 500 pmol. All standards, including kaempferol, quercetin, myricetin, and various glycosylated flavonols, were obtained from Spectrum Chemicals (Gardena, CA).

In Vitro Rescue Assay

Methanol extracts of stigmas were prepared as described for HPLC analysis, and 100 μL was evaporated to dryness and redissolved in 20 μL of DMSO. An aliquot (1 μL) was added to a 100-μL suspension of CMF pollen in germination medium as described by Mo et al. (1992). The percentage of pollen grains that germinated after a 4-hr incubation at room temperature was scored. As described by Taylor and Jorgensen (1992), the V26 inbred line produces a significant proportion of small, nonviable pollen grains, which are also present in the

CMF transgenic derivative. These grains never germinate and are excluded from the analysis. Percent germination values are calculated on the remaining proportion of large, viable grains.

Staining and Microscopic Imaging

Kaempferol Induction in Stigmas

Pollinated and/or wounded V26 and CMF stigmas were sectioned longitudinally with a vibrating microtome. Sections 100-μm thick were washed for 5 min in water, transferred to 0.1% Naturstoffreagenz A (diphenylboric acid 2-aminoethyl ester; Sigma) in 10 mM KPi, pH 6.0, 10% sucrose, 2% DMSO, and immediately photographed using a Leitz epifluorescence microscope (Wetvlar, Germany) (emission λ = 450 nm). Micrographs were taken using Kodak Ektachrome 800/1600 ASA.

Callose Staining to Monitor Pollen Tube Growth

V26 pistils were harvested 1.5, 8, and 48 hr after pollinating with V26 or CMF pollen, fixed in ethanol/acetic acid (3:1 [v/v]) for 24 hr, rinsed with 1 M phosphate buffer, pH 7, and incubated in 8 N NaOH for 3 to 24 hr to clear the tissue. Pistils were stained with decolorized aniline blue in 0.1 M KPi, pH 9.0, for 2 to 4 hr, infiltrated with 100% glycerol for 1 hr, and mounted on glass slides. At least 10 pistils were examined per time point, and the experiment was repeated three times on separate days using different plants each time. Pistils and pollen tubes were visualized with a fluorescence microscope (Jenalumar; Aus Jena, Jena, Germany) (emission λ = 410 nm) and photographed with Kodak Ektachrome 400 ASA.

RNA Gel Blot Analysis

Total RNA was isolated according to the method of Maes and Messens (1992) with minor modifications. Stigmas (350 to 500) yielded between 500 μg and 1 mg of total RNA. Total RNA was separated on a 1% agarose gel containing 2.2 M formaldehyde and transferred to a nylon membrane (Magna NT; Micro Separations Inc., Westboro, MA) by capillary blotting. Filters were hybridized at 42°C for 36 hr to gel-purified DNA fragments labeled with phosphorus-32 by the random priming method (Feinberg and Vogelstein, 1983). Hybridization signals were quantified using an image analysis system as described in Pollak et al. (1993).

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting

CHS protein levels in stigmas after various treatments were performed as described in Pollak et al. (1993).

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