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THE STRUCTURAL REQUIREMENTS OF FLAVONOLS THAT INDUCE POLLEN GERMINATION OF CONDITIONALLY MALE FERTILE *PETUNIA*

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Key Word Index—*Petunia hybrida*; Solanaceae; pollen germination; tube growth; flavonols.

Abstract—Flavonols are essential for pollen germination and sustained tube growth in *Petunia hybrida*. An *in vitro* bioassay, based on biochemical complementation of flavonol-deficient pollen, was used to compare how modifications at different sites on the basic flavonol molecule affect the efficiency of pollen germination. This structural–activity analysis using methylated or glycosylated derivatives showed that only flavonols with unsubstituted hydroxyl groups at positions 3 and 7 could induce rapid pollen germination. In addition, the enhanced germination frequency associated with a hydroxyl group at position 5 was abolished by substituting a methyl group. Increased hydroxylation of the flavonol B-ring had an inhibitory effect on germination, but methylation of the same hydroxyl groups promoted germination. Additional hydroxyl groups within the A-ring at carbon 6 had a mixed effect, but a methoxyl group at position 6 enhanced germination in all cases. Substitutions at position 8 were somewhat inhibitory and introduction of an isoprenyl group into ring A was toxic to both mutant and wild type pollen.

INTRODUCTION

Flavonoids are C15 compounds, formed by condensation of phenylpropanoid and acetate units, that are almost universally found in angiosperms. They accumulate in most plant tissues, including pollen. The major classes of flavonoids are distinguished by differences in their oxidation states and substitution patterns of the basic flavonoid skeleton. Several physiological roles have been attributed to flavonoids including protection against oxidative stress [1], signalling in plant–microbe interactions, pollinator attraction, UV protection, and serving as phytoalexins [2]. Recently, one specific class, the flavonols, were shown to have an essential role in plant reproduction and fecundity [3–5]. Conditionally male fertile (CMF) plants lacking flavonols in the pollen were self-sterile due to their failure to produce a functional pollen tube [3]. In angiosperms the pollen tube is the conduit for transporting the two sperm cells to the embryo sac where a double fertilization produces the embryo and endosperm. The fertility defect was biochemically complemented by adding low concentrations of flavonols to the CMF pollen; not only was pollen germination and tube growth (pollen rescue) restored to an *in vitro* suspension of pollen, but adding dry flavonols to the stigmas at pollination led to full seed set of CMF self-crosses [4].

A rescue assay, based on the addition of plant extracts or purified chemicals to an *in vitro* suspension of the CMF pollen, was used to determine the specificity of compounds that restore pollen germination. The number of grains that germinate and the extent of tube growth were measured 4 hr after supplementation and compared to an unsupplemented control. Of the numerous compounds tested for their ability to induce germination only flavonol aglycones could successfully restore pollen function. Among the bioactive flavonols, there were differences in the level of germination; the most potent inducers were kaempferol, isorhamnetin and galangin, producing full rescue at 0.5 μM [4]. Microscopic observation indicates that flavonol-supplemented CMF pollen shows tube outgrowth within 20–30 min of addition, a rate which is very similar to the speed with which wild type pollen germinates *in vitro*. In this report we describe the use of substituted flavonols in the bioassay to identify more precisely the structural requirements for flavonol-induced pollen germination.

RESULTS AND DISCUSSION

A- and C-ring substitutions

Previous results identified some of the structural features of the flavonoids that are required to induce pollen germination *in vitro* and *in vivo* [4]. They included a double bond between carbons two and three, a keto group at carbon four and a hydroxyl group at carbon

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three of the heterocycle. Only the flavonol class has all these features (the basic flavonoid structure and numbering scheme are provided in Fig. 1). In this report we have extended these observations by analysing a chemically diverse collection of naturally occurring flavonols for their ability to induce pollen germination. The substitution of one or more hydroxyl (OH) groups by methoxyl or glycosyl moieties not only removes hydroxyl groups but it can also alter the polarity and size of the molecule. The resulting change in chemical reactivity of the molecule will depend on the sites and extent of substitution.

To confirm the germination requirement for an unsubstituted hydroxyl group at carbon three, various 3-*O*-methylated (3-*O* Me) derivatives, as well as 3- and 7-*O*-glycosylated derivatives of commonly occurring flavonols were tested for their ability to induce pollen germination *in vitro*. As shown in Fig. 1, none of the 3-*O*-substituted derivatives of kaempferol (Kae; 3,5,7,4′-tetrahydroxyflavone), quercetin (Que; 3,5,7,3′,4′-pentahydroxyflavone) or myricetin (Myr: 3,5,7,3′,4′,5′-hexahydroxyflavone) could restore pollen tube growth at concentrations up to 100 μM (**3**, **8**, **16** and **18**).

The same type of analysis confirmed that a free hydroxyl group at the 7 position in ring A is required for pollen to germinate in a timely manner. For example, isorhamnetin (3′-*O*-Me-Que) induced complete pollen germination, while rhamnetin (7-*O*-Me-Que) and 7,3′*O*-dimethyl quercetin did not stimulate pollen to germinate during the 4 hr incubation at concentrations up to 100 μM (Fig. 1). Identical results were found with the isomers 4′-*O*-methyl kaempferol and 7-*O*-methyl kaempferol. Likewise, when a sugar residue was present at position 7 as in kaempferol 7-*O*-neohesperoside (**17**, Fig. 1) no rescue activity was detected. Earlier experiments comparing the germination-inducing ability of fisetin (3,7,3′,4′-tetrahydroxyflavone) and quercetin showed that the presence of a hydroxyl group at C-5 enhanced pollen germination 10-fold [4]. In the present study we found that methylation of the hydroxyl group at C-5 completely prevented pollen germination (compare to the response induced by isorhamnetin and 5,3′-dimethyl quercetin [Fig. 1]). We speculate that this may be due to hydrogen bonding between the hydroxyl group at position 5 and the adjacent keto group in the C ring; this interaction might provide a stabilized structure for interactions with other cellular components. The lack of a hydroxyl group at C-5 or methylation thereof would prevent the formation of this structure with the attendant loss of germination stimulating activity. Isolation and characterization of proteins that interact with the bioactive flavonols will be required to confirm this model.

B-ring substitutions

The original structure–activity analysis showed that flavonols with increasing numbers of hydroxyl groups in the B-ring were less effective at stimulating pollen germination. For example, galangin (3,5,7-trihydroxyflavone) with no hydroxyl groups in the B-ring, and kaempferol and isorhamnetin, each with one B-ring hydroxyl group substitution, stimulated full germination at less than 1 μM. Quercetin with two hydroxyl groups in the B-ring was required at 10 μM and myricetin with three B-ring-hydroxyls needed 100 μM to produce an equivalent level of pollen germination [4]. The replacement of one or more polar hydroxyl groups in quercetin and myricetin with the more apolar methoxyl groups significantly enhanced pollen germination (compare myricetin with the 3′,4′-*O*-dimethylether and 3′,5′-*O*-dimethylether and quercetin with isorhamnetin in Fig. 1). A plausible explanation for this enhancement is that the multiple hydroxyl groups in the B-ring of quercetin and myricetin produce a hydrophilic molecule which may participate in non-specific binding or interactions which reduce diffusion into the pollen grain. A hydrophobic B-ring may therefore be essential for amphophilic flavonoids to pass through the pollen wall and plasma membrane.

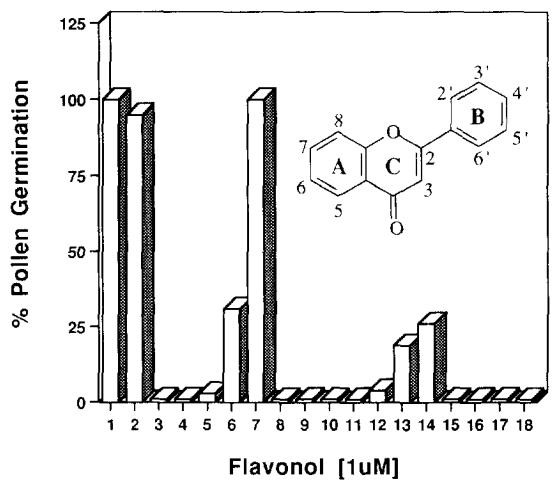

Fig. 1. CMF pollen germination frequency in response to supplementation with various flavonols. Inset: basic flavonoid structure.

A-ring substitutions at positions 6 *and* 8

Several flavonols with hydroxyl or methoxyl groups at carbons six and eight of the A-ring were tested and gave mixed results. Quercetagetin (6-hydroxy quercetin) and 6-hydroxy kaempferol induced pollen germination and supported normal tube growth at levels only slightly less than their non 6-hydroxy counterparts (compare **2** and **5** with **1** and **4** in Fig. 2). However, addition of a hydroxyl group at position 6 in galangin produces a compound that not only fails to stimulate significant germination, but has a detrimental effect on pollen tube growth of both CMF and wild type pollen at 1 μM. Microscopic analysis of a 6-hydroxy galangin-supplemented pollen suspension showed that germination was initiated and the tubes grew *ca* three to four pollen grain diameters before growth was arrested. Replacing a 6-hydroxyl group with a 6-methoxyl enhanced pollen tube growth producing levels comparable to unsubstituted kaempferol and quercetin. In the case of the galangin derivatives, the toxicity associated with 6-hydroxy galangin was completely abolished by methylation at this position. Prenylation of position 6 produces a molecule (platanetin) that appears to have a cytotoxic effect on both mutant and wild type pollen; concentrations of 3,5,7,8-tetrahydroxy-6-isoprenylflavone as low as 1 μM rapidly led to mis-shapen and bursting pollen grains (data not shown).

Addition of a methoxyl group at carbon 8 significantly reduced the percentage of germinated pollen grains *in vitro* (compare **7** with **10** and **4** with **12** in Fig. 2). The addition of a 8-hydroxyl group had a mixed effect on germination depending on the substitution at C-7. The combination of 7-hydroxy and 8-hydroxy groups decreased germination (8-hydroxy quercetin vs quercetin), while a 8-hydroxyl group adjacent to a 7-*O*-Me functionally stimulated a low level of germination (compare **11** in Fig. 2 with **5** in Fig. 1). The extremely diverse response to the same chemical modifications at positions 6 and 8 in the A-ring, coupled with the necessity for hydroxylation at carbons seven and five, indicate that this part of the flavonol molecule may be involved in precise structural recognition of an as yet unidentified target receptor.

A variety of phenylpropanoids, alkaloids, benzoic acid derivatives and other bioactive compounds were tested. Rescue assays were performed with jasmonic acid, methyl jasmonate, 17-β-estradiol, 24-epibrassinolide, an alkaloid (strictosidine), 5,7-dihydroxy-3-ethylchromone, phloroglucinol, glucose, galactose, glutathione, paraquat, NAD-/NADH, ATP, plus syringic, sinapic, ferulic and several hydroxybenzoic acid derivatives, *Helminthosporium carbonum* fungal toxin, and a series of compounds which stimulate or inhibit *Striga* ssp. seed and haustoria germination [6]. None induced germination at concentrations up to 100 μM. This further confirmed the absolute requirement of petunia pollen germination for flavonol aglycones.

How do flavonols promote pollen germination? Kaempferol may act as a first or second messenger in a signal transduction pathway, or as a structural component of pollen tube growth. Flavonols with an unsubstituted 3-hydroxyl group, are substrates for degradation by peroxidase–H_2O_2 [7]. Thus, we cannot exclude the involvement of kaempferol breakdown products in stimulating germination. We recently observed that germination can be induced *in vitro* by 7-*O*-methyl ethers after long incubation (> 8 hr). This induction has no physiological significance, since pollen normally germinates and grows into the stigma within 30–45 min. However, this slow induction and growth (tubes never reach kaempferol-rescued lengths) may indicate that different levels of recognition and interaction exist which can be exploited for mechanistic studies. A recent analysis of the flavonol profile of petunia pollen detected no flavonol aglycones, but did measure high amounts of disaccharides of kaempferol and quercetin [8]. Although flavonol-3-*O*-glycosides did not rescue germination *in vitro* (Fig. 1 and ref. [4]) this may be due to a failure of the bulky, polar sugar-conjugated flavonols to be internalized, rather than their inherent inability to effect germination.

EXPERIMENTAL

Plant material and pollen germination assay. Petunia hybrida L. plants used in this study were described previously [3, 4]. Pollen from 2 anthers of wild type (line V26) or transgenic flavonol-deficient mutant (CMF) plants was dispersed in 1 ml of germination medium [4].

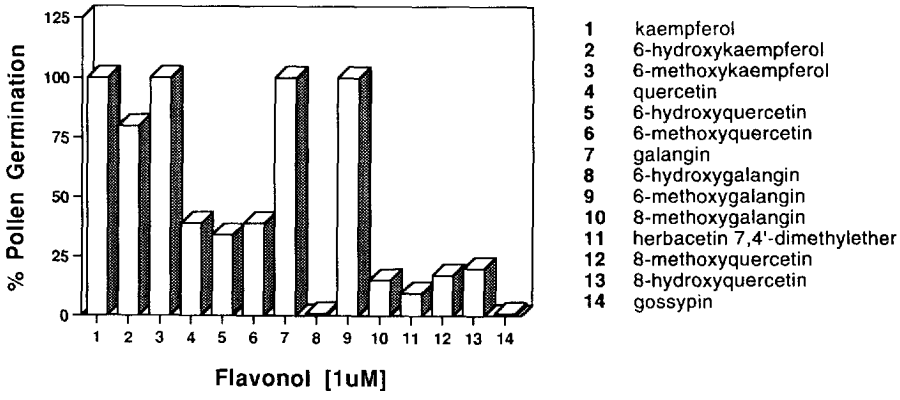

Fig. 2. CMF pollen germination in response to flavonols with modifications in the A-ring.

Flavonols, dissolved in DMSO, were added to the pollen suspension (1 μl 100 μl^{-1}) at concns ranging from 1 to 100 μM, and gently rotated for 4 hr at room temp. Successful germination was scored as the percentage of pollen grains which germinate and produce a tube greater than 2 pollen grain diameters long within 4 hr. DMSO controls without added flavonols were treated in a similar manner. Each experiment was performed in triplicate using both CMF and wild type pollen, to detect any toxic effects of the individual compounds. In each trial 200–500 pollen grains were scored.

Flavonoids were either obtained commercially (Spectrum Chemicals, Gardena CA, U.S.A. and Indofine Chemical Company, Somerville NJ, U.S.A.) or were isolated from various plant sources [9]. Purity was determined by HPLC and flavonoids were further purified where necessary. Gossypetin (8-hydroxy quercetin) was produced by acid hydrolysis of gossypin (3,5,7,3′,4′-pentahydroxy-8-*O*-glucosyloxy flavone).

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