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THE STIGMA SURFACE AND POLLEN-STIGMA INTERACTIONS IN *SENECIO SQUALIDUS* L. (ASTERACEAE) FOLLOWING CROSS (COMPATIBLE) AND SELF (INCOMPATIBLE) POLLINATIONS

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Senecio squalidus (Asteraceae) has been shown to possess a stigma with characteristics of both "dry" and "wet" types of stigma. The "semidry" stigma of *Senecio* is characterized by the presence of a surface cuticle overlaid by a proteinaceous pellicle and a small constitutive surface secretion consisting of lipid, carbohydrate, and protein. We anticipate that this semidry stigma may be a general feature of the Asteraceae. Secretion by the *Senecio* stigma is enhanced after both compatible and incompatible pollinations, when material secreted by the stigma combines with pollenkitt extruded from the alveolar exine of the pollen to form a heterogeneous "attachment foot" between pollen and stigmatic papillae. During this period, discrete inclusions, "wall bodies," can be seen within cell walls of papillae in contact with pollen grains, apparently exporting their contents across the cell wall and onto the surface of the stigma. Following compatible pollination, the emergent pollen tube grows through the attachment foot and between the tightly packed stigmatic papillae before penetrating the stigma at the base of the papilla cells, where the cuticle is absent. The pollen tube then grows intercellularly, within the middle lamella, through the stigma toward the style. Following incompatible pollinations, development of pollen is highly variable. Most incompatible pollen grains fail to germinate, but many do germinate to produce pollen tubes, some of which penetrate the stigma before they are inhibited. Such extensive development of incompatible pollen tubes is unusual for a species with homomorphic sporophytic self-incompatibility. These observations are discussed as a comparison with events at the dry stigma surface of *Brassica* following compatible and incompatible pollinations and in relation to current theories on the evolution of wet and dry stigmas.

Keywords: Asteraceae, pollen-stigma interactions, pollination responses, self-incompatibility, *Senecio*, stigma surface.

Introduction

The stigma surfaces of flowering plants have been classified as "wet" or "dry" based on the presence or absence, respectively, of a copious stigmatic secretion (Heslop-Harrison et al. 1975; Heslop-Harrison and Shivanna 1977). The secretions of wet stigmas, which can be primarily lipid rich, as in the Solanaceae, or primarily carbohydrate rich, as in the Liliaceae, are required for correct pollen hydration, germination, and penetration of the stigma by pollen tubes (Goldman et al. 1994). Recently, lipidic components of stigmatic secretions, particularly cis-unsaturated triacylglycerides, have been shown to be essential for pollen tube penetration of the stigma and probably for directional growth of the pollen tube on the stigma as well (Lush et al. 1998; Wolters-Arts et al. 1998). Indeed, in the presence of such lipids, pollen tubes will even penetrate leaves, albeit with the cuticle removed (Wolters-Arts et al. 1998). It has been proposed that the role of the lipids is to facilitate the establishment of a gradient of water within the stigmatic secretion that acts as a guidance cue for pollen tubes on the stigma (Lush et al. 1998, 2000). Upon germination, therefore, pollen tubes are presented with a path of increasing water concentration into the more aqueous environment of the conducting tissue of stigma and style.

Dry stigmas, which lack a copious surface secretion, are covered by a continuous cuticle that must be penetrated enzymatically by pollen tubes, using a cutinase, in order to effect successful fertilization (Heslop-Harrison et al. 1975; Maiti et al. 1979; Hiscock et al. 1994). Covering the cuticle is a thin proteinaceous surface layer, the pellicle, which can be detected, indirectly, by its strong nonspecific esterase activity (Mattson et al. 1974; Heslop-Harrison et al. 1975). The function of the pellicle is unknown, but it has been predicted to play an essential role in pollen-stigma recognition, because, in a variety of species, removal of the pellicle with dilute detergents prevents pollen tubes from penetrating the stigma (Mattson et al. 1974; Heslop-Harrison and Heslop-Harrison 1975; Heslop-Harrison et al. 1975; Hiscock et al. 1998). Interestingly, despite the dry nature of cuticularized stigmas, a lipidic surface environment is still essential for successful pollen hydration, ger-

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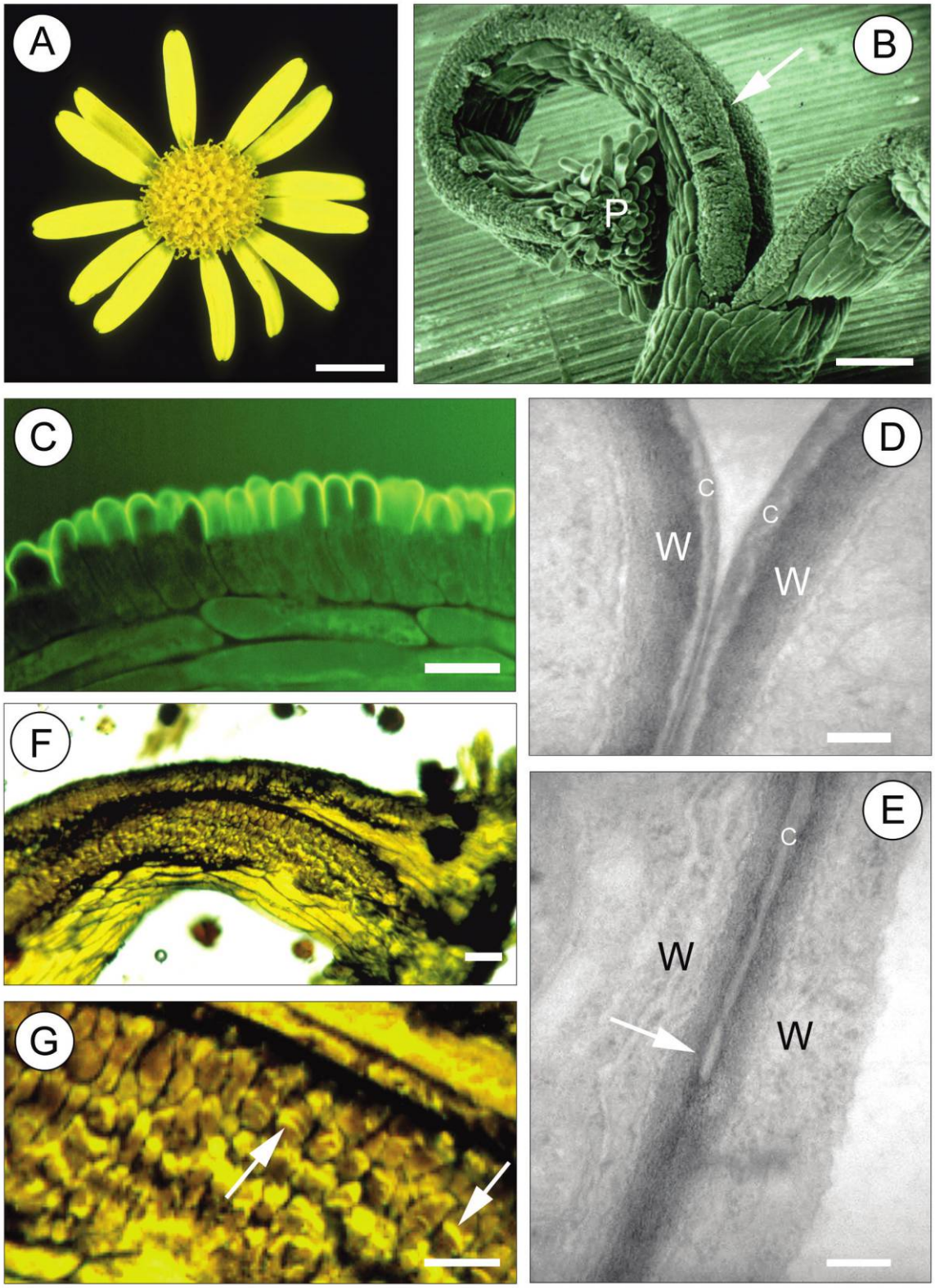

Fig. 1 Stigma surface of *Senecio squalidus*. *A*, Mature capitulum of *S. squalidus* showing outer zygomorphic carpellate ray florets and inner actinomorphic cosexual disk florets; bar = 5 mm. *B*, SEM of disk flower stigma showing receptive surface consisting of columnar papilla cells (arrow) and elongated pseudo-papillae (*P*) at the end of one of two reflexed stigma branches; bar = 0.1 mm. *C*, Cuticle of columnar papillae visualized by staining with auramine O. Note that the cuticle disappears as papillae become more tightly packed at their bases; bar = 10 μm. *D*, *E*, TEM of longitudinal section through cell wall region of two adjacent papillae showing the cuticle (*c*) near the surface (*D*) and as it disappears toward the basal region of the two papillae (*E*); *W* = papilla cell wall; bar = 0.2 μm. *F*, *G*, Localization of nonspecific esterase activity to stigmatic papillae. In *G*, the dark-staining esterase reaction layer can be seen flaking away from individual papillae (arrows). *F*, bar = 100 μm; *G*, bar = 10 μm.

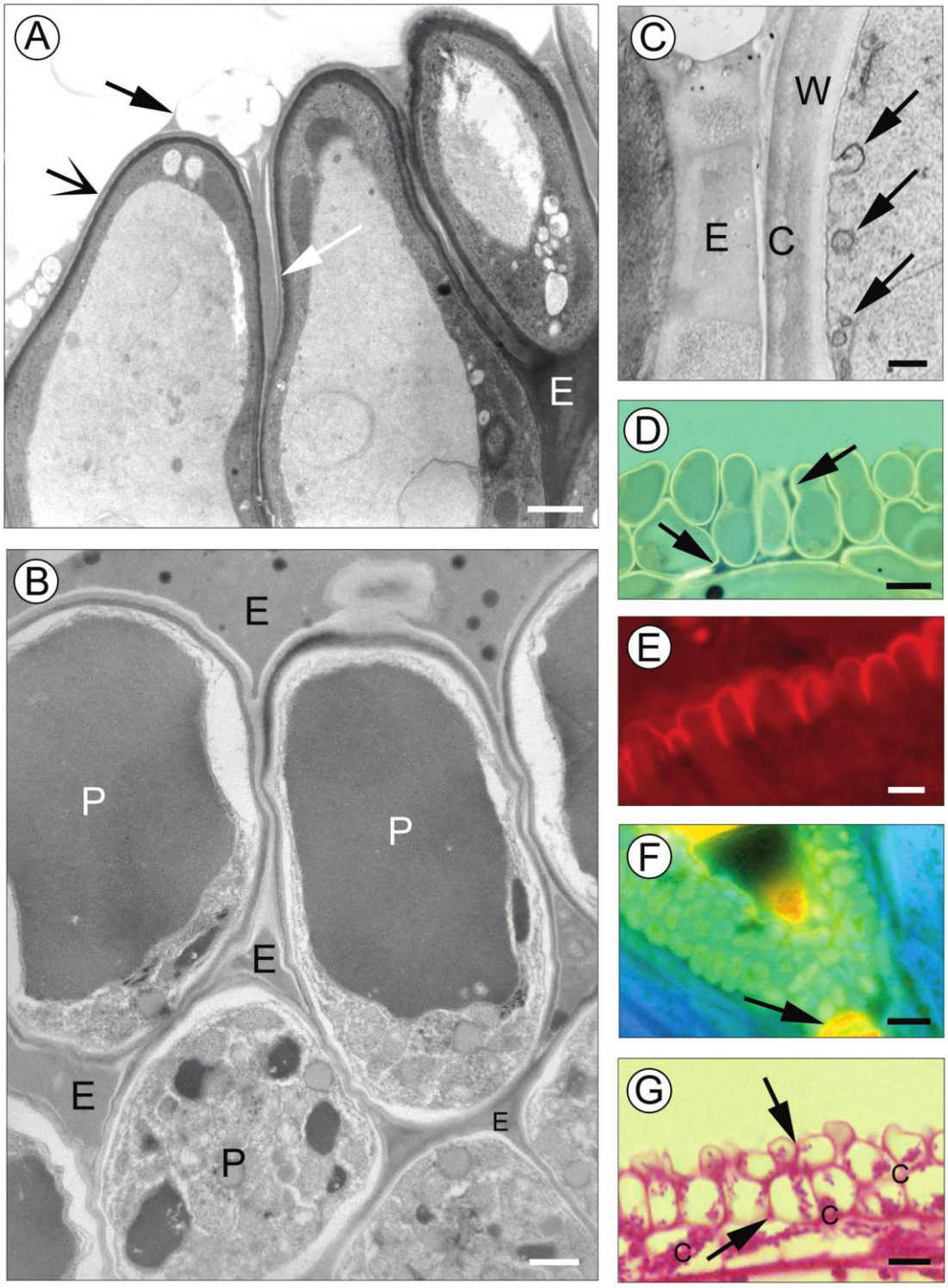

Fig. 2 Stigmatic secretion of *Senecio squalidus*. *A–C*, TEMs of stigma surface fixed in modified Karnovsky's fixative. *A*, Oblique section through three stigmatic papillae showing the presence of an extracellular secretion between the papillae (white arrow) and on their surface (filled black arrow) overlaying the cuticle (black arrow); bar = 1 μm. *B*, Transverse section through stigmatic papillae showing the copious extracellular secretion (*E*) between stigmatic papillae (*P*) near their bases; bar = 1 μm. *C*, Oblique section through the cell wall region of a stigmatic papilla showing vesicular activity (arrows) at the plasma membrane. *W* = cell wall; *C* = cuticle; bar = 0.2 μm. *D*, Oblique section through stigma stained with Sudan black B showing localization of lipids (staining black) within the extracellular secretion (arrows); bar = 10 μm. *E*, As in *D*, but stained with fluorescent lipophillic dye nile red; bar = 10 μm. *F*, Stigma surface probed with fluorescent lipophillic dye Rhodamine B hexyl ester. Surface lipids fluoresce pale green to yellow, whereas the lipidic pollenkitt of a nearby pollen grain (arrow) can be seen fluorescing bright yellow; bar = 20 μm. *G*, Oblique section of stigma stained with PAS to visualize carbohydrate. Positive (red) staining can be seen in and between the cell walls of papillae (arrows) and within chloroplasts (*c*); bar = 10 μm.

mination, and penetration of the stigma by the pollen tube—the lipids, together with additional proteinaceous recognition factors, being provided by complex pollen coatings (Dickinson 1993, 1994, 1995; Preuss et al. 1993; Hulskamp et al. 1995; Wolters-Arts et al. 1998). In the Brassicaceae, for instance, when a pollen grain alights on the stigma, lipid-rich pollen coating is released from the exine onto the stigma surface, where it establishes an "attachment foot" in the zone of contact between pollen and stigma (Elleman and Dickinson 1986; Elleman et al. 1992). During pollen hydration, water passes into the grain through the attachment foot, and upon germination, the pollen tube grows into the attachment foot, where it penetrates the stigma. Pollen from certain *Arabidopsis* male sterile mutants, which cannot produce a pollen coat or are defective in the synthesis of specific long-chain lipids, is unable to hydrate on stigmas (Preuss et al. 1993; Hulskamp et al. 1995), indicating that, as is the case with species with wet stigmas, lipids are essential for pollen development on the stigma (Dickinson 1993, 1994; Wolters-Arts et al. 1998). Interestingly, wet stigmas have been correlated with the possession of gametophytic self-incompatibility (SI) and dry stigmas with the possession of sporophytic SI (Heslop-Harrison 1975; Heslop-Harrison and Shivanna 1977).

The stigma surface of species in the Asteraceae has been described as dry based on observations of stigmas from 17 species from a variety of tribes within the family (Heslop-Harrison and Shivanna 1977). This observation correlates with the fact that members of the Asteraceae possess a sporophytic mode of SI (Gerstel 1950; Hughes and Babcock 1950; Hiscock 2000*a*). However, despite the importance of the Asteraceae as one of the largest families of flowering plants and a source of numerous agriculturally and horticulturally important species, there have been very few detailed studies of pollen-stigma interactions in this family.

In self-compatible (SC) *Ambrosia* sp. and SI *Cosmos bipinnatus*, Knox (1973) showed that compatible and incompatible pollinations were followed by a rapid release of pollen wall material (pollenkitt) onto the stigma surface within 10–15 min prior to and during germination of the grain. This pollen wall material (containing a diversity of enzymes, carbohydrates, and lipids) was subsequently shown to be released through the sexine pores and colpi and was proposed to play a key role in pollen-stigma recognition events leading to compatibility and/or incompatibility (Howlett et al. 1975). The self-incompatibility response in *C. bipinnatus* always occurred at the stigma surface, prior to or just after germination of the pollen tube, and was followed by deposition of callose in the nascent tube and surrounding stigmatic papillae (Knox 1973; Howlett et al. 1975). These observations were later confirmed in studies of *Helianthus* by Vithanage and Knox (1977), who noted that the stigma surface of *Helianthus* was dry.

As part of a small survey of pollen-stigma interactions in species with dry stigmas, Elleman et al. (1992) reexamined pollination events in *C. bipinnatus* and *Helianthus annuus* and showed that a secretory response by the stigma may accompany the release of pollen wall material directly after pollination. As a consequence, Elleman et al. (1992) questioned whether the stigma surface of species in the Asteraceae was indeed entirely dry or whether it was partially secretory. The aim of our study was to reexamine pollen-stigma interactions in a species from the Asteraceae, paying particular attention to the nature of the stigma surface and the response of the stigma to compatible and incompatible pollinations. The strongly SI species *Senecio squalidus* (Oxford Ragwort) was chosen for this study because it currently forms the basis of studies on the molecular genetic basis of sporophytic SI in the Asteraceae (Hiscock 2000*a*, 2000*b*).

Material and Methods

Plants

Senecio squalidus plants homozygous and heterozygous for self-incompatibility (*S*) alleles, S_1, S_2, S_3, and S_4 (Hiscock 2000*a*), were grown from seed and maintained in an insect-proof glasshouse at 15°–20°C under a 16 : 8-h light : dark regime.

Chemicals

Nile red, rhodamine B hexyl ester, and $DiIC_{18}$ (a dialkylcarbocyanine derivative) were obtained from Molecular Probes (Eugene, Oreg.; catalog nos. N-1142, R-648, and D-282, respectively). All other chemicals, unless otherwise stated, were obtained from Sigma-Aldridge (United Kingdom).

Pollinations

Flowering capitula of *S. squalidus* were removed from plants and maintained in tap water within the wells of microtitre dishes in the laboratory. Whole flowers (disk or ray) were carefully removed from capitula using forceps and were inserted into individual capillary wells within specially drilled Perspex blocks (20 mm × 15 mm × 10 mm) resting on wet filter paper in a petri dish. The capillary channels (ca. 50 per block) ensured a constant supply of water to the flowers through the pedicel and ovary. Prior to pollination, stigmas were checked for the presence of self pollen using a binocular microscope. Only stigmas uncontaminated with self pollen were used for manual pollinations; this was usually facilitated by using unisexual ray flowers as the female partner, although uncontaminated stigmas could also be found on some cosexual disk flowers. Cross (compatible) or self (incompatible) pollen was then applied to stigmas using a fine sable-hair paintbrush. Petri dishes containing pollinated flowers within capillary blocks were then placed in a large damp box (to prevent desiccation), where they were maintained for 6–12 h. After this period of time, flowers were removed and fixed (see below) in preparation for microscopy.

Light and Fluorescence Microscopy

Prior to fixation, petals were removed from flowers. For observations of stigmatic secretion, flowers were fixed in either 4.5% formaldehyde in 0.025 M phosphate buffer (pH 7.5) or 4% formaldehyde, 1% glutaraldehyde in 0.05 M phosphate buffer. These fixation methods were found to provide better preservation of the stigmatic secretion than did alcohol-based fixation methods. Flowers to be observed after staining with aniline blue and/or auramine O were fixed in absolute ethanol : acetic acid (3 : 1). Following overnight fixation, pistils were dissected out of flowers and dehydrated in an ethanol

series (10%, 25%, 50%, 75%, 90%, 95%, and 100%), with changes every 2 h; pistils fixed in ethanol : acetic acid (3 : 1) were dehydrated in a reduced ethanol series of 90%, 95%, and 100% ethanol. Pistils were then infiltrated and embedded in glycolmethacrylate (JB-4 embedding Kit; TAAB), according to the methods of Carmichael and Friedman (1995). Embedded pistils were sectioned using a Reichert-Jung 2040 microtome (Cambridge Instruments). Serial sections of pistils 3–5 μm thick were prepared, as described in Carmichael and Friedman (1995). Sections were then stained appropriately (see below). For aniline blue staining of pollen tubes, pollinated pistils were excised and squashed directly in stain (see below) between a microscope slide and a cover slip. Similar "squash" preparations were used for observing nonspecific esterase staining and with certain lipid stains (see below). Sections or squashes were observed and photographed under bright field or ultraviolet illumination using an Axiophot microscope (Zeiss).

Cytochemical Staining

Toluidine blue was used as a general stain for proteins and acidic polyanions; sections were immersed in 0.5% toluidine blue in 0.05 M phosphate buffer, pH 7.2, for 30–60 s prior to mounting in glycerol. Protein was also stained using 0.25% Coomassie brilliant blue B in water, methanol, and acetic acid (87 : 10 : 1 v/v) (Heslop-Harrison 1979). To visualize callose, sections were mounted in 0.1% decolorized aniline blue in 0.05 M phosphate buffer, pH 7.5, mixed 1 : 1 with glycerol. Cuticle was visualized by staining sections in 0.01% auramine O in 0.05 M phosphate buffer, pH 7.2 (Heslop-Harrison 1977). For dual observation of pollen tubes and cuticle, sections were mounted in a mixture of aniline blue and auramine O (1 : 3 in 0.05 M phosphate buffer, pH 7.2). Lipids were visualized using four lipophilic stains: (*a*) Sudan black B: sections were incubated in 70% ethanol for 2 min, stained in 0.3% Sudan black B weight/volume (w/v) in 70% ethanol (equilibrated for 3 h at 60°C and then filtered) for 1 h at 60°C, and destained in 70% ethanol for 1–2 min prior to mounting in glycerol; (*b*) nile red: sections were incubated in 10 mM nile red in phosphate-buffered saline (PBS) for 20 min, washed twice for 2 min in PBS, and mounted in glycerol; (*c*) Rhodamine B hexyl ester: whole stigmas were immersed in 30 mM rhodamine B hexyl ester in double-distilled water (ddH_2O) for 5–10 min, washed briefly in ddH_2O, and then squashed in 50% glycerol in ddH_2O between a slide and coverslip; and (*d*) $DiIC_{18}$: sections were incubated in 10 mM $DiIC_{18}$ in ethanol for 15 min and then mounted in glycerol. As controls for lipid staining, whole pistils or sections were incubated in lipase (0.2% in ddH_2O or 0.05 M Tris-HCl, pH 7.2, 8% sucrose) at 37°C for 1–12 h (Heslop-Harrison and Heslop-Harrison 1985). Carbohydrates were visualized with periodic-acid Schiff's reagent (PAS) (Pearce 1972); sections were first incubated in a saturated solution of dinitrophenylhydrazine for 30 min to block aldehydes, then incubated in 1% aqueous periodic acid for 10 min, and then immersed in Feulgen stain for 10 min. Sections were then rinsed in 0.5% sodium metabisulphite for 2 min followed by ddH_20 for 10 min prior to mounting in glycerol. Nonspecific esterase activity was detected using α-naphthyl acetate as substrate in a coupling reaction with fast blue RR salt (Pearce 1972); as controls, stigmas were incubated in pronase (0.1 mg mL^{-1} in 0.05 M Tris-HCl, pH 7.2, 8% sucrose) for 1 h (Heslop-Harrison 1977) or in a solution of fast blue RR lacking substrate.

Electron Microscopy

For scanning electron microscopy (SEM), flowers, with petals removed, were fixed and dehydrated in methanol according to the methods of Neinhuis and Edelmann (1996). Flowers were then placed in a pressure vessel and subjected to critical-

Fig. 3 (*Next page*.) Pollen-stigma interactions in *Senecio squalidus*. *A*, Oblique section through attachment foot region (*F*) of the contact zone between pollen and stigma 1 h after a compatible pollination, stained with toluidine blue (which stains proteins and acidic polyanions blue and sporopollenin, in pollen exine, green). The attachment foot consists of a mixture of lipidic pollenkitt, extruded from the exine cavities of the pollen wall, and material secreted by the stigma. A pollen tube (*pt*) can be seen growing from a pollen grain (detached from the attachment foot complex during preparation) and entering the foot region created by the two grains; *P* = papillae; bar = 10 μm. *B*, Oblique section through germinating pollen grain on stigma 1 h after compatible pollination stained with Sudan black B. Lipids, staining black, are clearly present within the remains of the attachment foot, through which the pollen tube is growing (arrows); bar = 10 μm. *C*, As in *B* but stained with PAS to visualize carbohydrate. Positive (red) staining is clearly visible within the attachment foot (*F*). The contracted nature of the pollen grain on the left indicates that it has germinated, whereas the grain on the right has just started to germinate and produce a pollen tube (filled arrow). PAS-positive chloroplasts are visible within papillae (arrow); bar = 10 μm. *D*, SEM of germinating pollen grains 15 min after compatible pollination. Pollen tube initials can be seen emerging from the colpi (white arrows); bar = 10 μm. *E*, As in *D* but 1 h after compatible pollination; only one of three pollen tube initials develops into a mature pollen tube (*pt*) that penetrates the stigma between papillae (white arrow); the other two tube initials abort (filled white arrow); bar = 5 μm. *F*, TEM of section through attachment foot (*F*) between pollen grain (*pg*) and stigmatic papillae 1 h after compatible pollination. The attachment foot is composed of highly heterogeneous material derived from pollen and stigma (black arrows) and contains a pollen tube (*pt*). Note the expansion of the papilla cell wall directly below the pollen grain (filled black arrow) and the presence within the wall of electron-opaque wall bodies. Fixation with modified Karnovsky's buffer; *pw* = pollen wall exine; *i* = pollen wall intine; bar = 2 μm. *G*, TEM of oblique section through attachment foot (*F*) containing a pollen tube (*pt*) 1 h after compatible pollination. The cell walls of papillae (*P*) in contact with the attachment foot are expanding and contain electron-dense and electron-translucent wall bodies (filled black arrows). Within the cytoplasm of papillae, numerous vesicle-like structures (black arrows) are apparent and give the appearance of an "active" cytosol. Stigmatic extracellular secretion is clearly seen to be part of the attachment foot (white arrow). Fixation with modified Karnovsky's buffer; *pw* = pollen wall; bar = 1 μm. *H*, As in *G*, but fixed in osmium vapor, and showing detail of wall bodies within the cell wall of a stigmatic papilla directly below a pollen grain 30 min after pollination. Here wall bodies appear moderately electron opaque. *W* = papilla cell wall; *pk* = pollenkitt; bar = 0.5 μm.

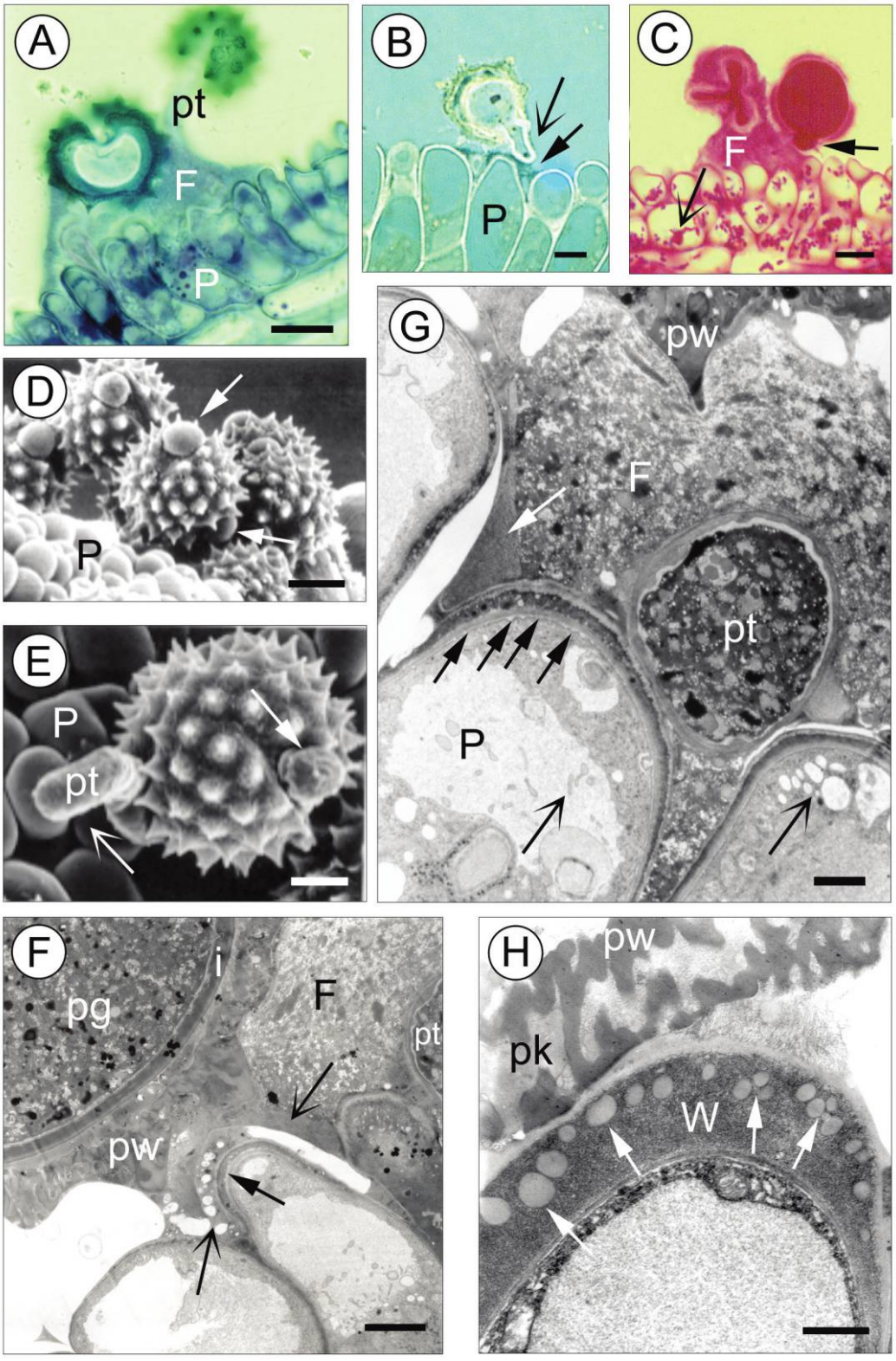

Fig. 3

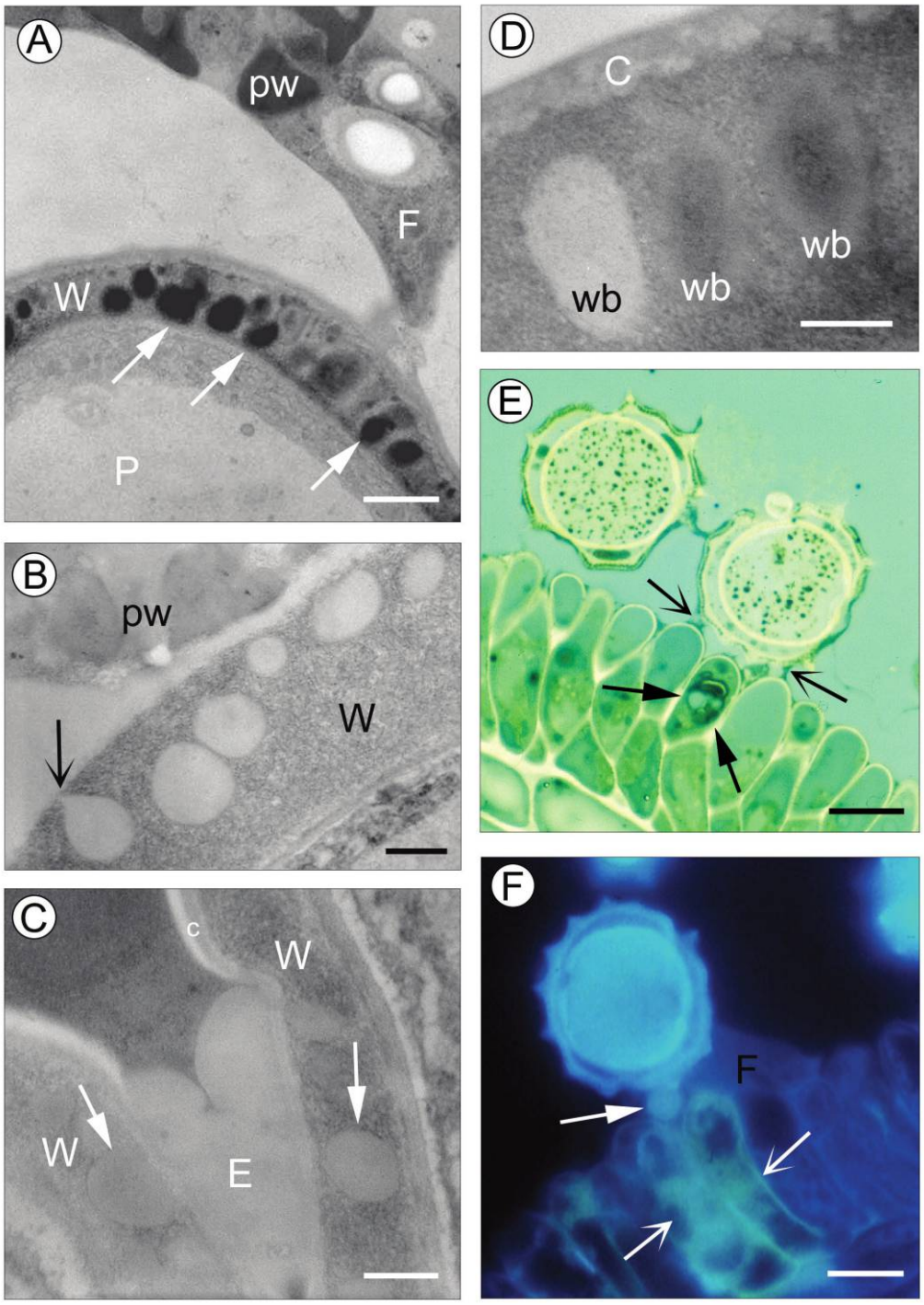

Fig. 4 Stigmatic responses to pollination in *Senecio squalidus*. *A*, TEM showing contact zone between pollen and papilla (*P*) 15 min after compatible pollination. Here wall bodies within the expanding papilla cell wall (*W*) appear highly electron opaque (arrows); osmium vapor fixed; *F* = attachment foot; *pw* = pollen wall; bar = 0.5 μm. *B*, As in *A*, but in this section wall bodies appear more electron translucent and of similar consistency to the stigmatic extracellular secretion (fig. 2). One wall body appears to be continuous with extracellular material in the attachment foot (arrow); bar = 0.2 μm. *C*, TEM of wall region between two papilla cells 15 min after incompatible pollination. Here the constitution of wall bodies (arrows) appears almost identical to extracellular material (*E*) within the attachment foot; osmium vapor fixed; *c* = cuticle; bar = 0.1 μm. *D*, As in *C*, but fixed in modified Karnovsky's fixative and showing detail of three wall bodies (*wb*). The wall body on the left appears electron translucent, whereas the two wall bodies on the right are more electron opaque; bar = 0.1 μm. *E*, Section through two pollen grains developing on a stigma 15 min after compatible pollination stained with Sudan black B to detect lipids. Positive (black) staining can be seen in the region of the attachment foot (arrows) and also within the cytosol of the papilla cell directly below and in contact with the right-hand pollen grain (filled arrows). Note that a significant proportion of the attachment foot has been washed away in preparation; bar = 10 μm. *F*, As in *E*, but 30 min after pollination and stained with fluorescent lipophillic dye $DiIC_{18}$ and aniline blue. Intense fluorescence attributable to lipid staining is clearly visible in the two papillae beneath the pollen grain (arrows). A pollen tube (filled arrow) can be seen growing through the region of the attachment foot (*F*); bar = 10 μm.

point drying with liquid CO_2 to remove methanol. Pistils were then mounted on SEM stubs using epoxy resin glue and were gold-coated in an argon chamber. Specimens were observed using a JEOL 35R microscope. For transmission electron microscopy (TEM), two fixation strategies were employed, one anhydrous and the other aqueous. The anhydrous method (Elleman and Dickinson 1986) was employed to visualize the stigma surface and pollen-stigma interactions in a "natural" dry state and as an attempt to better preserve any lipidic material associated with the stigma surface and pollen. For anhydrous fixation, flowers were fixed in osmium tetroxide vapor for 2 h, after which time pistils were dissected from the flowers and encapsulated in warm agar (2% w/v in ddH_2O) and postfixed in glutaraldehyde (2.5% v/v in ddH_2O), according to the methods of Elleman and Dickinson (1986). For aqueous fixation, pistils were removed from flowers and encapsulated in warm agar (2% w/v in ddH_2O) before fixation in modified Karnovsky's fixative (1.5% v/v glutaraldehyde and 2% depolymerized paraformaldehyde in 0.05 M phosphate buffer, pH 7.2) and postfixation in 2% w/v aqueous osmium tetroxide before dehydration in acetone series and embedding in epoxy resin (Elleman et al. 1992). Thin sections were cut using a Reichert ultramicrotome, stained in lead citrate and uranyl acetate, and examined in a JEOL 2000EX transmission electron microscope at 80 kV.

Results

The Stigma Surface of Senecio squalidus

The flowering capitulum of *Senecio squalidus* consists of an outer whorl of carpellate zygomorphic "ray" flowers and inner whorls of cosexual actinomorphic "disk" flowers (fig. 1*A*). In an immature state, the receptive papillate stigma surface is hidden between the two tightly appressed stigmatic lobes. In the disk flowers this prevents the receptive surface of the stigma from receiving self pollen as the maturing pistil grows through the tube of five united anthers. Sterile "pseudo-papillae" at the tips of the stigmatic lobes gather and force pollen from the anthers of disk flowers as they mature, thereby presenting pollen to the pollinating agents (usually hoverflies). At maturity, the lobes of the stigma reflex to expose two layers of receptive papilla cells (fig. 1*B*). The receptive stigma surface appears identical in both disk and ray flowers, but in ray flowers, the sterile pseudo-papillae are much reduced in length and number.

As was expected for a species from a family reported to have dry stigmas, the stigmatic papillae of *Senecio* were found to possess a prominent cuticle (fig. 1*C*). However, the cuticle did not extend fully to the base of the papilla cells; auramine O staining and TEM revealed the cuticle to disappear in regions in which papillae became more closely appressed, toward their bases (fig. 1*C*–1*E*). Strong nonspecific esterase activity was detected associated with the surface of the papillae (fig. 1*F*, 1*G*), indicating indirectly the presence of a proteinaceous pellicle overlaying the cuticle. In control treatments, in which the pellicle was digested with pronase prior to staining or in which substrate was omitted from the reaction buffer, no surface staining of the papillae was observed (data not shown).

Despite the characteristic features associated with a dry stigma, TEM observations revealed the presence of small amounts of an extracellular secretion between stigmatic papilla cells, frequently on the surface of the cuticle and presumably above the pellicle (fig. 2*A*). However, the extracellular secretion was most abundant in the basal regions of the papillate epidermis, where the secretion formed a more or less continuous homogeneous matrix between the papillae (fig. 2*B*). Closer examination of the plasma membrane toward the bases of papillae revealed the presence of vesicles subjacent to the cell wall, some of which appeared to fuse with the plasma membrane as though engaged in active secretion of material into the cell wall (fig. 2*C*). High-magnification observations of the stigma surface could not resolve the pellicle, so the relationship between the surface secretion and the pellicle was unclear.

The Nature of the Stigmatic Secretion

In order to elucidate the chemical nature of the extracellular surface secretion, a cytochemical analysis was carried out using standard methodologies (see "Discussion" for references to previous studies). The stigmatic secretion stained positively with Sudan black B, nile red, rhodamine B hexyl ester, and $DiIC_{18}$ (fig. 2*D*–2*F*), indicating the presence of lipids. In lipase-treated controls, staining/fluorescence at the stigma surface was greatly reduced or absent (data not shown). The stigma surface also stained positively with PAS (fig. 2*G*) and Coomassie brilliant blue B (data not shown), indicating that carbohydrate and protein, respectively, are also components of the surface secretion.

The Stigma Surface after Pollination

Within 15 min of pollination, a dramatic release of pollen wall–held material (pollenkitt) onto the stigma surface was observed leading to the formation of a distinct "attachment foot" beneath the pollen grain (fig. 3*A*). Pollenkitt was released through pores in the alveolar exine and also through the colpi, and its reaction with Sudan black B, PAS, and Coomassie brilliant blue B indicated that it contained lipids, carbohydrate, and protein, respectively (fig. 3*A*–3*C*). This response to stigmatic contact by the pollen was the same following both compatible and incompatible pollinations and always resulted in the establishment of an attachment foot at the pollen-stigma interface. Only in SEM observations of pollinated stigmas was the attachment foot absent (fig. 3*D*, 3*E*), presumably as a consequence of its removal during methanol fixation. TEM observations of pollinated stigmas highlighted a structural complexity to the attachment foot (fig. 3*F*, 3*G*). Highly granular material, containing spherical electron-opaque and electron-translucent bodies together with larger, more amorphous aggregates, appeared to be derived from the pollen wall, whereas more homogeneous material situated close to the stigmatic papillar cells was presumed to be the lipoidal stigma surface matrix. Electron-opaque fibrillar material was also observed within the foot and resembled neither pollen wall material nor stigma surface material. Frequently, the cytoplasm of papillar cells directly below pollen grains contained large numbers of small vesicles and larger vesicle-like bodies, giving the appearance of an active secretory reaction by the cytoplasm (fig. 3*F*, 3*G*).

In many sections of both compatible and incompatible pol-

linations, the cell walls of papillae in direct contact with pollen grains appeared to have swollen and expanded (figs. 3*F*–3*H*, 5*B*). TEM observations at higher magnification revealed the presence of discrete spherical bodies within the papilla cell walls (figs. 3*H*, 4*A*–4*D*). These "wall bodies" contained a homogeneous material that was quite variable in electron transparency; some wall bodies appeared highly electron opaque (fig. 4*A*), whereas others appeared almost electron translucent (fig. 4*B*). Wall bodies were consistently visible after both compatible and incompatible pollinations and in each case appeared to be releasing their contents onto the stigma surface within the vicinity of the attachment foot (fig. 3*H*; fig. 4*B*, 4*C*). On no occasion were wall bodies observed away from the region of contact between pollen and papillae, nor were they observed in the walls of papillae of unpollinated stigmas. The appearance of the material within the wall bodies was very similar to that of the extracellular surface secretion, suggesting that it may be lipidic. Interestingly, in certain sections stained for lipids using Sudan black B or $DiIC_{18}$, intense staining/fluorescence was visible within discrete regions of the cytosol of individual papillar cells beneath the point of contact with the pollen grain (fig. 4*E*, 4*F*).

Events at the Stigma Surface following Compatible Pollinations

Formation of the attachment foot appeared to occur simultaneously with hydration of the pollen grain, and within 15–30 min, a nascent pollen tube emerged from each of the three colpi of the pollen grain, but only one of these protuberances developed into an elongating pollen tube (fig. 3*D*, 3*E*). Elongating pollen tubes grew through the matrix of the attachment foot and between adjacent papillae directly below (fig. 5). Pollen tubes were frequently observed growing between papilla cells, the walls of which had swollen and expanded considerably (fig. 5*B*), but on no occasion were pollen tubes observed penetrating the expanded wall region. Indeed, no direct penetration of the stigmatic cuticle by a pollen tube was ever observed. In every instance, pollen tubes grew between papilla cells and continued growing toward the basal region, where the lipoidal stigma surface matrix was most abundant and where the cuticle disappeared. The path taken by a compatible pollen tube during its initial growth into the stigma was tracked using serial sections of a pollinated stigma double-stained with auramine O and aniline blue to visualize stigmatic cuticle and pollen tubes, respectively (fig. 5*C*–5*I*). The pollen tube, fluorescing blue, could be seen growing between adjacent stigmatic papillae, which fluoresced green because of the presence of an intact cuticle (fig. 5*C*). In the early sections (fig. 5*D*–5*G*), the cuticle of the papillae was clearly visible, but in the later sections, farther into the stigma epidermis (fig. 5*H*, 5*I*), the cuticle of papillae adjacent to the tube was no longer visible. At this point, pollen tubes penetrated the stigma and grew intercellularly between the cells of the stigmatic cortex (fig. 5*J*) before turning through 90° (fig. 5*K*) and growing parallel with the transmitting cells of the stigmatic lobe toward the style before growing downward within the style toward the single ovule.

Events at the Stigma Surface following Incompatible Pollinations

Incompatible pollinations in *Senecio* appeared very variable in terms of the stage at which pollen development was arrested (fig. 6). In most instances, incompatible grains hydrated and were then arrested prior to germination or following the appearance of short pollen tube initials (fig. 6*A*). In such cases, deposits of callose could frequently be observed within the papilla cells beneath the aborted pollen grains. Nevertheless, in many incompatible pollinations, pollen grains produced tubes that were arrested on the stigma surface as they grew between the papillae. Cessation of incompatible pollen tube development was accompanied by deposition of callose within the pollen tube and within the surrounding papillar cells (fig. 6*B*, 6*C*). Ultrastructural observations of incompatible pollinations revealed some dramatic responses by the stigma to incompatible pollen. A regular feature of pollinations in which pollen arrest occurred before or just after pollen tube germination was the presence of pronounced swellings in the cell walls of papillae in direct contact with arrested pollen grains (fig. 6*D*, 6*E*). These "wall swellings," which were visible within 30 min of incompatible pollinations, were never observed after compatible pollinations. The wall swellings contained a homogeneous moderately electron-opaque material of similar appearance to the material contained within wall bodies. Wall swellings were observed in material fixed anhydrously using osmium tetroxide vapor and in material fixed conventionally using modified Karnovsky's fixative. Interestingly, in Karnovsky-fixed material, wall swellings often appeared to be partly electron translucent and partly electron opaque, as though some of the material within the wall swelling had been washed away during fixation and subsequent processing (fig. 6*E*). An unexpected finding was that in some incompatible pollinations, a number of pollen tubes were seen penetrating the stigma surface (fig. 6*F*). These tubes clearly passed into the stigmatic cortex, having grown through the basal region of the papillae. Further development of the pollen tube was then arrested, with a characteristic swelling of the pollen tube tip and deposition of callose within the tip and within the stigmatic cells around it (fig. 6*F*).

Discussion

This study has shown that the stigma surface of *Senecio squalidus* has characteristics associated with both the dry and the wet type of stigma (*sensu* Heslop-Harrison et al. 1975). This finding therefore confirms earlier suggestions that species from the Asteraceae possess a stigma type that is somewhat intermediate between the extremes of dry (as typified by *Brassica*) and wet (as typified by the Solanaceae) (Elleman et al. 1992). In common with species possessing dry stigmas, a surface cuticle overlaid by a proteinaceous pellicle covers the stigmatic papillae of *S. squalidus*. As in previous studies (reviewed in Heslop-Harrison et al. 1975), the presence of a surface pellicle on the stigma of *Senecio* was inferred by the strong nonspecific esterase activity at the stigma surface. The esterase reaction layer detected in assays (fig. 1*F*, 1*G*) characteristically did not appear following treatment of stigmas with pronase, indicating that the esterase activity is associated directly with

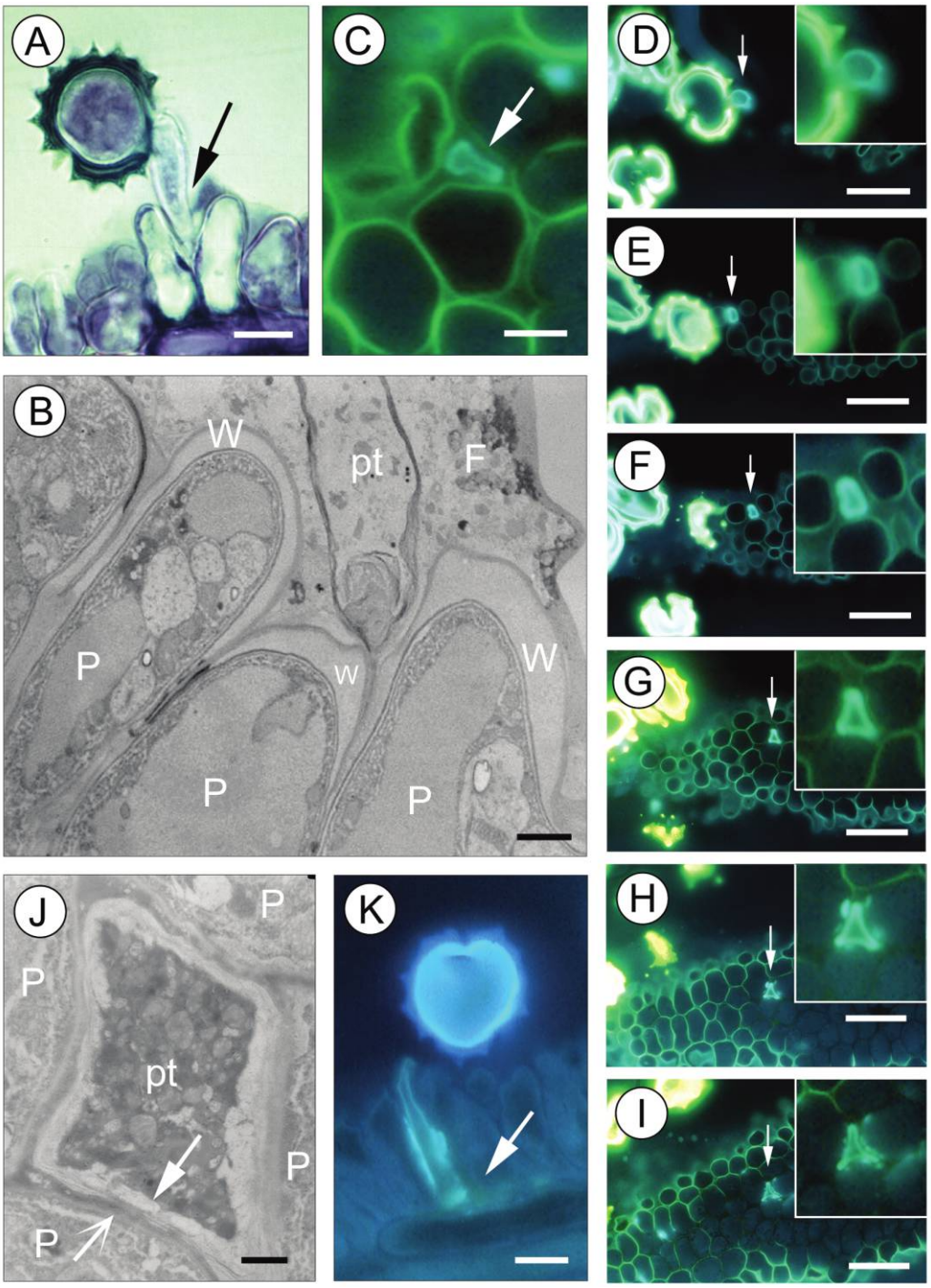

Fig. 5 Pollen-stigma interactions in *Senecio squalidus* following compatible pollination. *A*, Section through pollen grain with pollen tube penetrating the stigma between two papilla cells (arrow), stained with toluidine blue; bar = 10 μm. *B*, TEM of oblique section through pollen tube (*pt*) entering the stigma between two papilla (*P*) following growth through the attachment foot (*F*), 2 h after pollination; fixed in modified Karnovsky's buffer. Note the pronounced expansion of the papilla cell walls (*W*); bar = 1 μm. *C*, Transverse section through pollen tube (arrow) growing between stigmatic papillae, double-stained with aniline blue to visualize the pollen tube wall (blue) and auramine O to visualize the cuticle of the papillae (green); bar = 5 μm. *D–I*, Serial transverse sections through stigma to follow the path taken by a growing pollen tube (arrow). Sections double-stained as in *C*. Right-hand boxes show detail of pollen tube (original × 5). At no point is there direct penetration of a papilla cell or penetration of its wall by the pollen tube. In *E–G*, the cuticle of papillae between which the pollen tube is growing can be seen fluorescing green, whereas in *H* and *I*, farther into the stigma, the cuticle of the papillae is no longer visible around the tube. Interestingly, in *H* and *I*, small deposits of callose (fluorescing blue) can be seen within papillae appressed to the pollen tube; bar = 30 μm. *J*, TEM of transverse section through pollen tube (*pt*) growing between stigmatic papillae (*P*). Note that the pollen tube wall (filled arrow) and the walls of the papillae (arrow) are distinct, indicating that there has been no direct penetration of the papilla cell wall by the pollen tube; bar = 0.5 μm. *K*, Oblique section through pollen grain with pollen tube growing downward into the stigma between papillae and then turning through 90° at the base of the papillae (arrow) before growing farther through the stigma toward the style; stained with aniline blue; bar = 10 μm.

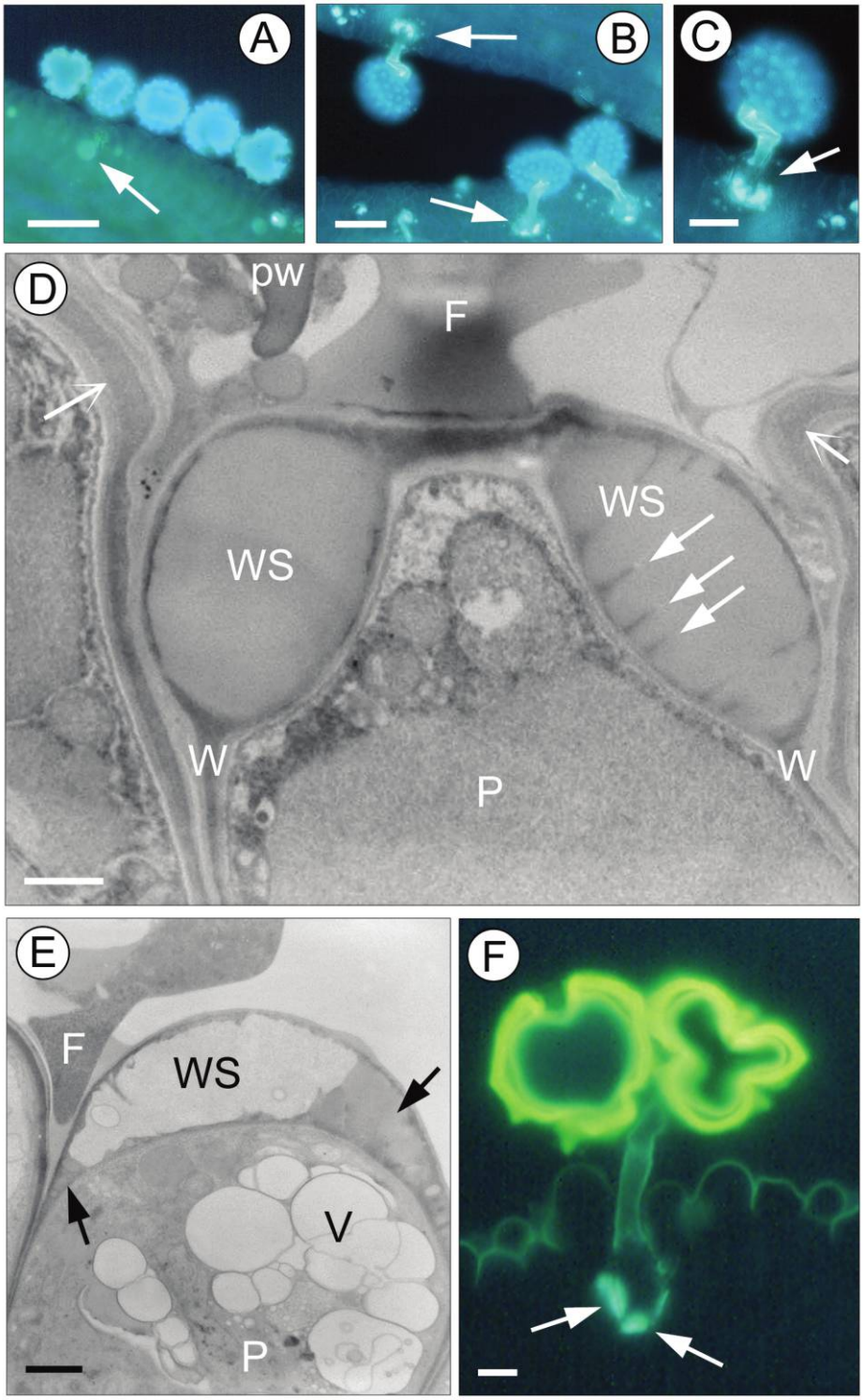

Fig. 6 Pollen-stigma interactions in *Senecio squalidus* following incompatible pollination. *A*–*C*, Squash preparations of incompatible pollinated stigmas stained with aniline blue. *A*, Self pollination of *S*2*S*2 individual. In this "strong" incompatibility reaction, pollen grains have failed to germinate, and callose can be seen in stigmatic papillae (arrow); bar = 25 μm. *B*, Self pollination of *S*1*S*1 individual. Pollen grains have germinated and produced tubes that are inhibited as they grow between stigmatic papillae. Callose deposits can be seen around the sites of pollen tube inhibition (arrows); bar = 25 μm. *C*, Inverted detail of left grain from *B* showing ring of callose around the inhibited pollen tube. *D*, TEM of oblique section through a stigmatic papilla (*P*) directly beneath an incompatible pollen grain, 1 h after self pollination of an *S*4*S*4 individual. The cell wall (*W*) of the papilla is greatly expanded by the presence of two large wall swellings (*WS*). Elongations of electron-opaque material within the right wall swelling (filled arrows) indicate that these swellings may result from coalescence of wall bodies because of the similarity in electron density of material contained within wall swellings and some wall bodies (fig. 4). The cell walls of adjacent papillae have expanded

the proteinaceous pellicle (Mattson et al. 1974; Heslop-Harrison and Heslop-Harrison 1975; Knox et al. 1976; Heslop-Harrison and Shivanna 1977). Despite these features of the dry stigma, TEM observations revealed that *Senecio* stigmas also bear a small quantity of secreted material at the base of their papillae. Comparable TEM observations of stigmas from typical dry stigma species *Brassica*, *Raphanus*, and *Arabidopsis* show no such surface secretions, and the cuticle can be clearly seen extending to the most basal regions of the papillae (Dickinson and Lewis 1973; Elleman et al. 1988, 1992). Standard cytochemical techniques were used to show the presence of lipid, carbohydrate, and protein in the stigmatic secretion of *Senecio*, all of which are components (in very variable proportions) of the secretions of wet stigmas (Konar and Linskens 1966; Dumas et al. 1988; Wolters-Arts et al. 1998). For comparison with similar cytochemical observations of wet stigma species, see Konar and Linskens (1966), Dumas (1974), Dickinson and Lawson (1975), Kristen et al. (1979), Sedgley and Scholefield (1980), Sedgley (1981, 1982, 1983), Sedgley and Blesing (1982), Schou (1984), Heslop-Harrison and Heslop-Harrison (1980, 1985), and Heslop-Harrison (1990), and for comparison with dry stigmas, see Dickinson and Lewis (1973), Pettitt (1980), Heslop-Harrison and Heslop-Harrison (1980, 1981), Schou (1984), and Heslop-Harrison (1990). Even though the secretion of the *Senecio* stigma is small compared with the copious secretions of wet stigmas (Dickinson and Lawson 1975; Heslop-Harrison 1979; Sedgley and Scholefield 1980; Kenrick and Knox 1981; Sedgley and Blesing 1982; Heslop-Harrison and Heslop-Harrison 1985), the *Senecio* stigma cannot be described as dry in the same sense as the *Brassica* stigma, which bears no surface secretion, bar the pellicle (Elleman et al. 1988, 1992). We therefore suggest that semidry best describes the stigma surface of *S. squalidus*. Further observations of stigmas from other species in the Asteraceae—*Senecio vulgaris*, *Senecio laxifolius*, *Cosmos bipinnatus*, *Hieracium aurantiacum*, and *Agyranthemum* sp. (S. Hiscock and K. Hoedemaekers, unpublished observations)—indicate that the semidry stigma is likely to be a general feature of the Asteraceae.

The secretory nature of the *Senecio* stigma was further highlighted by events at the stigma surface following pollination. Within 30 min of a compatible or incompatible pollination, a prominent attachment foot became established at the point of contact between the pollen grain and stigmatic papillae. The attachment foot was well preserved in material fixed with osmium vapor or Karnovsky's fixative but appeared to be dissolved away during ethanol- or methanol-based fixation techniques (fig. 3*D*, 3*E*,) indicating that lipids form a significant component of the attachment foot—a conclusion confirmed by cytochemical analysis. Although the attachment foot appeared to be composed predominantly of pollenkitt derived from the pollen wall, material of identical appearance to the stigmatic secretion was also a distinct component of the heterogeneous matrix constituting the attachment foot. There was no indication that the attachment foot of *Senecio* underwent any structural changes akin to the "coat conversion" phenomenon associated with formation of the attachment foot in *Brassica* (Elleman and Dickinson 1986, 1990). During the period of attachment foot formation in *Senecio*, vesicle-like inclusions—wall bodies—were prominent features of the papilla cell walls through which they appeared to be moving and then extruding their contents onto the stigma surface. These wall bodies had a similar appearance to structures observed in the papilla walls of *Brassica* ("wall vesicles") after application of purified pollen coatings to stigmas and following some incompatible pollinations (Elleman and Dickinson 1990, 1994). No structures comparable to wall bodies or wall vesicles have been recorded previously in the literature, so it is likely that these structures are part of a very specific cellular response to pollination in plants with dry (*Brassica*) or semidry (*Senecio*) stigmas. Even though intact vesicles have been observed discharging their contents into cell walls (Dickinson and Bell 1970), it is far from clear how vesicles or vesicle-like structures could move through a cell wall. Thus, the secretory nature of these structures remains obscure. Significantly, wall bodies were never observed in unpollinated stigmas or in the walls of papillae away from the zone of direct contact with a pollen grain.

In *Senecio*, the appearance of wall bodies was frequently accompanied by an expansion, or loosening, of the papilla cell wall. Wall expansion is also a feature of the pollination response in *Brassica*, in which it appears to be an essential prerequisite for penetration by the pollen tube (Elleman and Dickinson 1994). In *Senecio*, as in *Brassica*, wall expansion was confined to papillae in direct contact with the pollen grain. Despite the apparent similarities between stigmatic responses to pollination in *Senecio* and *Brassica*, the *Senecio* response was consistently more pronounced and more predictable than that found in *Brassica* (Elleman and Dickinson 1994), and unlike the *Brassica* case, the *Senecio* response was accompanied by enhanced secretory activity of the stigma. Indeed, the *Senecio* response bore many similarities to certain pollination responses described in species with wet stigmas (Kenrick and Knox 1981; Sedgley and Blesing 1982; Sedgley 1983).

The function of the wall expansion in *Senecio* stigmatic papillae appears somewhat obscure, given that pollen tubes were never seen penetrating the wall or growing within it. In *Bras-*

(arrows) but contain no wall swellings. Material osmium vapor fixed. *pw* = pollen wall; *F* = attachment foot; bar = 0.5 μm. *E*, As in *D*, but material fixed in modified Karnovsky's buffer. The wall swelling (*WS*) contains only a small amount of material of similar electron density as that found within wall swellings of *D*, with most of the wall swelling being more or less electron translucent, indicating that the more electron-opaque material may have been washed away during fixation. The more heterogeneous nature of the electron-dense material within the wall swelling indicates its probable origin from coalesced wall bodies (arrows). *V* = vesicle-like bodies in the papilla cytosol; bar = 1 μm. *F*, Self pollination of *S1S1* individual. Section through two incompatible pollen grains double-stained with aniline blue and auramine O. One grain has failed to germinate, whereas the other has produced a pollen tube that has penetrated the stigma; inhibition of the incompatible tube is accompanied by swelling of the tube tip and deposition of callose within the region of contact between the swollen tube tip and the stigma cells around it; bar = 5 μm.

sica, expansion of the papilla cell wall is assumed to facilitate entry of the pollen tube into the middle lamella region, where it grows following direct penetration through the cuticle (Elleman et al. 1988). In *Senecio*, however, pollen tubes always grew between the closely packed stigmatic papillae and penetrated the stigma through the basal region of the papilla epidermis, where the cuticle was no longer a barrier, indicating that stigmatic penetration by the pollen tube is physical and does not involve enzymic degradation of the cuticle by a pollen-held cutinase (Hiscock et al. 1994). Such a conclusion does not rule out a role for other hydrolytic enzymes (such as pectinases) in the *Senecio* wall expansion response. Interestingly, in *Senecio*, as in *Brassica* and *Arabidopsis* (Elleman et al. 1992), pollen tubes appeared always to grow through the matrix of the attachment foot and to enter the stigma directly beneath it. In *Brassica* and *Arabidopsis*, it has been proposed that lipids within the attachment foot (derived from the pollen coating) provide a gradient of increasing water concentration that is believed to direct the growing pollen tube toward the stigma (Wolters-Arts et al. 1998; Lush et al. 2000). In *Senecio*, the attachment foot presumably fulfills a similar function, but the lipidic components are provided by a combination of the stigmatic secretion and the exine-derived pollenkitt, extra stigmatic secretion presumably being added through the activity of wall bodies.

Although it is clear that the constitutive stigmatic secretion and the attachment foot both contain lipids, the chemical composition of the material within the wall bodies is not clear. The osmiophillic nature of some highly electron-opaque wall bodies indicates that they may contain lipid; however, the resolution of the light microscopy employed for cytochemical staining was not sufficient to detect wall bodies. Nevertheless, in sections stained with lipophillic dyes, strong staining was frequently observed within the cytosol of papillae directly beneath pollen grains (fig. 4*E*, 4*F*), indicating that lipids are abundant in papillae after pollination, at a time when vesicles and vesicle-like structures are also very prominent in papillae in contact with pollen grains. These observations may reflect the mobilization of lipids within papillae for secretion onto the stigma surface. The fact that wall bodies were also observed during incompatible pollen-stigma interactions in *Senecio* indicates that they are part of a general stigmatic response to pollination that may be important for recognition events that establish compatibility or incompatibility between pollen and stigma.

It was evident from observations of incompatible pollinations that the site of the incompatibility response in *Senecio* is the stigma surface. This would be expected for a species with sporophytic SI and was previously reported for the Asteraceae in *Cosmos* and *Helianthus* (Knox 1973; Vithanage and Knox 1977). However, the stage of pollen development at which inhibition took place in *Senecio* was very variable, ranging from inhibition prior to germination to inhibition after penetration of the stigma by the pollen tube. Preliminary observations indicate that variability in the *Senecio* SI response may be correlated with the presence of specific *S* (self-incompatibility) alleles and also with genetic background. For instance, a higher proportion of incompatible pollen tubes were observed penetrating stigmas after self pollination in plants homozygous for the *S*1 allele than in plants homozygous or heterozygous for the *S*2, *S*3, and *S*4 alleles (S. Hiscock, unpublished observation; but see fig. 6). The *S*1 allele is recessive to the three other *S* alleles in the stigma and shows variable codominance and recessiveness in pollen (Hiscock 2000*a*). In addition, incompatible pollen tubes that penetrate the stigma were observed most frequently in anomalous semicompatible pollinations that could not be explained by a sporophytic model for SI and were attributed to other genetic factors, such as modifier loci that influence the penetrance of *S* alleles (Hiscock 2000*a*, 2000*b*).

The strength of the incompatibility response in *Brassica* is also somewhat variable, but not to the same extent as is observed in *Senecio*. In *Brassica*, so-called strong *S* alleles (usually dominant *S* alleles) are able to inhibit pollen before it has hydrated properly, whereas other weak *S* alleles permit more extensive pollen development, usually as far as germination and production of a short pollen tube (Elleman et al. 1988). Even so, incompatible pollen tubes of *Brassica* have only very rarely been observed penetrating the stigma, and inhibition is most typically at the hydration stage (Dickinson 1995). This highlights yet another subtle, but important, difference between the pollen-stigma interaction in *Senecio* and *Brassica*, indicating that there may be mechanistic differences in the way sporophytic SI operates in the Asteraceae and Brassicaceae. Such a hypothesis is supported by the curious finding in *Senecio* of large swellings in the cell walls of stigmatic papillae after incompatible pollination. These wall swellings were only present in papillae beneath incompatible pollen grains that failed to germinate or that produced only short pollen tubes. Similar structures have never been observed in *Brassica* or, to our knowledge, in any other species and bear no resemblance to callose deposits (Elleman and Dickinson 1986). Material contained in wall swellings appeared very similar to the material contained in the much smaller wall bodies, which was presumed to be lipidic. Indeed, the detailed structure of certain wall swellings gave the distinct impression that they were the result of coalescence of wall bodies, because the partial remains of extensions of the cell wall gave the appearance of once having formed distinct boundaries between regions of the swelling. We considered the possibility that the wall swellings might be artifacts arising from the anhydrous fixation technique, but the fact that they were only seen after incompatible pollinations indicated that this was unlikely, a conclusion subsequently confirmed when material fixed conventionally was observed. It still remains to be determined whether wall swellings are a cause or a consequence of pollen inhibition in *Senecio*.

In the past, correlations have been made between stigma type, pollen type, and the type of self-incompatibility system present (Heslop-Harrison et al. 1975; Heslop-Harrison and Shivanna 1977; Zavada 1984; Dickinson 1994, 1995; Wolters-Arts et al. 1998), and speculation has been made as to which combination of the three reflects the more evolutionary "advanced" state. Even though there have been no comprehensive phylogenetic studies of these characters (but Heslop-Harrison 1981), the *Brassica* combination of dry stigma, complex pollen coating, and sporophytic SI has generally been regarded as most likely representing an advanced angiosperm condition (Dickinson 1994, 1995). What, then, is the evolutionary significance of the semidry stigma of *Senecio*, and does it represent a transition of stigma type from wet to dry or a transition

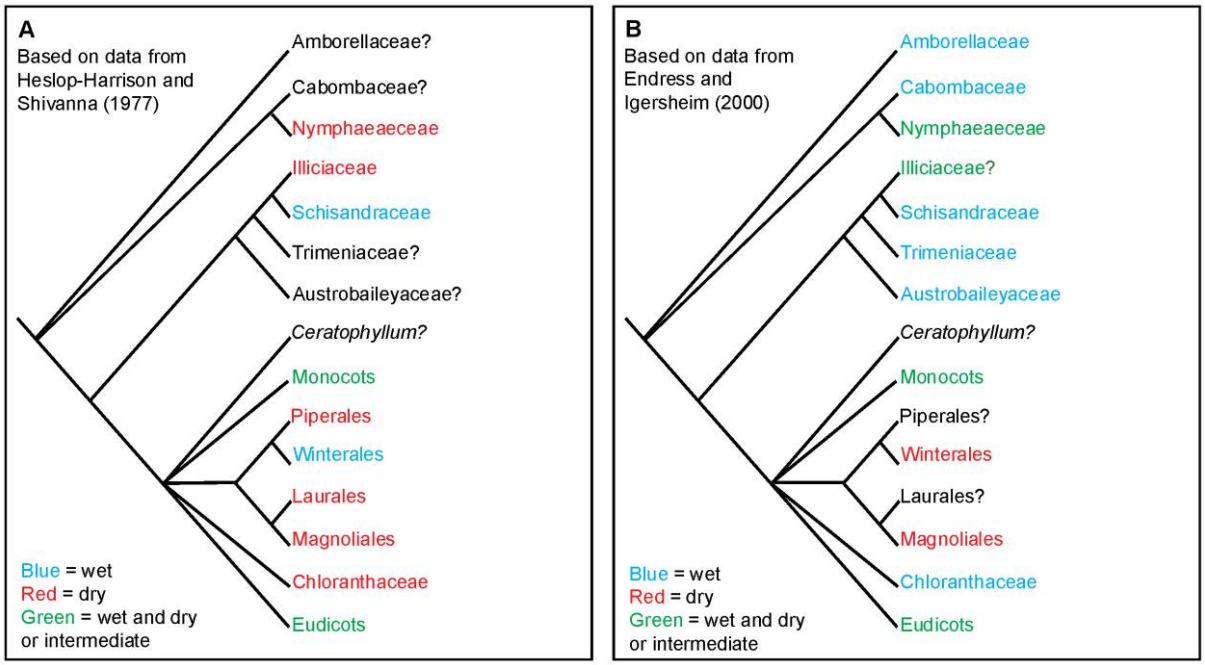

Fig. 7 The distribution of wet and dry stigmas among basal angiosperms. *A*, Stigma types designated by Heslop-Harrison and Shivanna (1977) mapped onto the most recent phylogeny of basal angiosperms adapted from Qiu et al. (1999). *B*, As in *A*, but with stigma types designated by Endress and Igersheim (2000).

from dry to wet? The Asteraceae can be considered phylogenetically advanced, and members of the family possess a complex pollenkitt (equivalent to pollen coat) and sporophytic SI, so perhaps one would suppose that the transition, if transition is what is reflected in the semidry stigma, is from wet to the supposedly more advanced dry type. However, if the phylogenetic distribution of stigma type is analyzed by mapping wet and dry stigma types (according to the methods of Heslop-Harrison and Shivanna 1977) onto current angiosperm phylogenies (Qiu et al. 1999; Soltis et al. 1999), there is no apparent correlation between stigma type and phylogenetic position (fig. 7*A*). Indeed, according to the Heslop-Harrison and Shivanna (1977) classification, dry stigmas appear equally abundantly in lineages considered to be basal (e.g., Nymphaeaceae, Illiciaceae, Chloranthaceae, and Magnoliaceae), as do wet stigmas (e.g., Schisandraceae and Winteraceae), and some lineages have representatives with dry and wet stigmas (e.g., Ranunculaceae). Given that the carpel is believed to have evolved by enclosure of ovules by a leaflike structure (Corner 1964; Takhtajan 1991), one might speculate that the ancestral stigmatic surface was probably dry and possibly cuticularized and that wet stigmas represent a derived condition. However, in extant angiosperms, the process of carpel closure is hypothesized to have been the result of either postgenital fusion or secretion or a combination of the two, and, in those lineages considered most basal (including *Amborella*, Cabombaceae, and Austrobaileyaceae), secretion appears to have been the most likely route to carpel closure (Endress and Igersheim 2000). Thus, Endress and Igersheim (2000, p. S218) conclude that stigmas of basal angiosperms are "more or less secretory" (fig. 7*B*), a conclusion that is slightly at odds with the survey of Heslop-Harrison and Shivanna (1977), which points to a more scattered distribution of wet and dry stigmas among these basal groups (fig. 7*A*). It is possible that these inconsistencies in stigma classification could be a consequence of differences in the methods used to fix and prepare material. Nevertheless, it is interesting to note that according to Heslop-Harrison and Shivanna (1977), the stigmas of Nymphaeaeceae and Illiciaceae are dry, whereas Endress and Igersheim (2000) report them to be intermediate between wet and dry; Chloranthaceae, on the other hand, are classified as dry by Heslop-Harrison and Shivanna (1977) but as wet by Endress and Igersheim (2000). Clearly, a thorough systematic reappraisal of stigma type in basal angiosperms is needed to address this fundamental area of the evolution of angiospermy. Among higher monocots and Eudicots, the distribution of wet and dry stigma types is extremely scattered and unpredictable, and some families even have representatives with wet and dry stigmas (Heslop-Harrison and Shivanna 1977; Heslop-Harrison 1990), indicating that stigma type may be in a state of flux, with wet, dry, and semidry stigmas evolving through a continuous process of gain and loss during angiosperm diversification.

Whatever the evolutionary history of dry and wet stigmas, it is clear that there is a strong correlation between possession of dry stigmas and the presence of complex pollen coatings

on pollen grains (Dickinson 1994, 1995; Dickinson et al. 2000). Pollen coatings have thus been hypothesized to perform a role analogous to that of stigmatic exudates (Dickinson 1994; Wolters-Arts et al. 1998). Support for this hypothesis is strong, because lipids present in stigmatic exudates can restore the ability of coatless pollen to penetrate stigmas and also the ability of secretionless stigmas to support normal pollen development (Wolters-Arts et al. 1998). However, we do not know whether stigmatic secretions were present before the evolution of complex pollen coats or whether the reverse is the case, so the evolutionary relationship between dry stigmas and complex pollen coatings remains obscure. Clearly more work is needed on the phylogenetic distribution of (*a*) stigma types and (*b*) complex pollen coatings before we can speculate objectively on the evolutionary origins of dry and wet stigmas and their association with particular pollen types.

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