Pollen–pistil interactions and self-incompatibility in the Asteraceae: new insights from studies of *Senecio squalidus* (Oxford ragwort)

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• *Background* Pollen–pistil interactions are an essential prelude to fertilization in angiosperms and determine compatibility/incompatibility. Pollen–pistil interactions have been studied at a molecular and cellular level in relatively few families. Self-incompatibility (SI) is the best understood pollen–pistil interaction at a molecular level where three different molecular mechanisms have been identified in just five families. Here we review studies of pollen–pistil interactions and SI in the Asteraceae, an important family that has been relatively understudied in these areas of reproductive biology.

• *Scope* We begin by describing the historical literature which first identified sporophytic SI (SSI) in species of Asteraceae, the SI system later identified and characterized at a molecular level in the Brassicaceae. Early structural and cytological studies in these two families suggested that pollen–pistil interactions and SSI were similar, if not the same. Recent cellular and molecular studies in *Senecio squalidus* (Oxford ragwort) have challenged this belief by revealing that despite sharing the same genetic system of SSI, the Brassicaceae and Asteraceae molecular mechanisms are different. Key cellular differences have also been highlighted in pollen–stigma interactions, which may arise as a consequence of the Asteraceae possessing a 'semi-dry' stigma, rather than the 'dry' stigma typical of the Brassicaceae. The review concludes with a summary of recent transcriptomic analyses aimed at identifying proteins regulating pollen–pistil interactions and SI in *S. squalidus*, and by implication the Asteraceae. The *Senecio* pistil transcriptome contains many novel pistil-specific genes, but also pistil-specific genes previously shown to play a role in pollen–pistil interactions in other species.

• *Conclusions* Studies in *S. squalidus* have shown that stigma structure and the molecular mechanism of SSI in the Asteraceae and Brassicaceae are different. The availability of a pool of pistil-specific genes for *S. squalidus* offers an opportunity to elucidate the molecular mechanisms of pollen–pistil interactions and SI in the Asteraceae.

Key words: Asteraceae, *Senecio*, pistil, stigma, pollen, pollen–pistil interactions, self-incompatibility, transcriptome.

INTRODUCTION

The evolution of the carpel had profound consequences for the reproductive biology of angiosperms by encasing the ovule and its egg-containing female gametophyte (embryo sac) within a mass of maternal sporophytic tissue, thereby denying the sperm-dispensing male gametophyte (pollen) easy access to its mate. This physical separation of female and male gametophytes by the carpel (= pistil) gave greater powers of maternal mate discrimination to the flower and resulted in the evolution of the pollen–pistil interaction (Heslop-Harrison, 1975), a complex series of cellular and molecular interactions that effectively constitute a form of 'courtship' between the haploid pollen and the diploid pistil. During this molecular courtship process various recognition processes take place, often associated with active processes of discrimination and rejection of 'incompatible' pollen at interspecific and intraspecific levels (Hiscock and Allen, 2008). Additionally, with ovules often in limited supply, 'compatible' pollen tubes have to compete for ovules leading to an additional level of selection, on the male gametophyte, a consequence of the carpel that is thought to have been a major factor in the evolutionary success of angiosperms (Mulcahy, 1979; Hormazo and Herrero, 1992).

The pollen–pistil interaction is thus a fundamental process in the reproductive biology of flowering plants and has been the subject of intense research for many decades (for recent review see Hiscock and Allen, 2008). In recent years there has been much progress in identifying molecules that mediate specific events during the pollen–pistil interaction, such as pollen adhesion and hydration, pollen tube growth and navigation through the pistil, and self-incompatibility (SI) in particular (Edlund *et al.*, 2004; Swanson *et al.*, 2004; Takayama and Isogai, 2005; Hiscock and Allen, 2008). Nevertheless most studies have been confined to a few model species, and even among these few well-studied

species there does not appear to be any general consensus among the types of molecules regulating a common programme of cellular pollen–pistil interactions necessary for compatibility (Lord, 2003; Hiscock and Allen, 2008). Such a lack of consensus is not surprising because proteins that regulate sexual reproductive processes evolve more rapidly than proteins that regulate other cellular processes (Swanson and Vacquier, 2002). This is endorsed by molecular studies of SI, the best understood pollen–pistil interaction, in which the pistil recognizes and actively rejects self-pollen or pollen from genetically closely related individuals. Here, despite identical genetic determination, different molecules have been identified regulating gametophytic SI (GSI) in the Solanaceae and Papaveraceae (see Meng *et al.*, 2011; McClure *et al.*, Franklin-Tong *et al.*, 2011), whilst molecules regulating sporophytic SI (SSI) in the Brassicaceae (Takayama and Isogai, 2005) are not encoded at the S (self-incompatibility) locus of *Ipomoea trifida* (Convolvulaceae) (Rahmann *et al.*, 2007).

Pollen–pistil interactions and SI have been most widely studied in species from the Brassicaceae [SSI, and self-compatible (SC)], Solanaceae (GSI and SC), Rosaceae (GSI and SC), Plantaginaceae (GSI and SC), Papaveraceae (GSI and SC), and the Liliaceae (SI and SC), where SI has been more intensively studied at a molecular level than 'compatibility' (self- and cross-). In the Brassicaceae SSI is regulated by a pistil-expressed S-receptor kinase (SRK), which interacts with its cognate pollen ligand SCR (S-cysteine-rich protein) to initiate recognition and subsequent rejection of self-pollen (Takayama and Isogai, 2005). In the Solanaceae, Rosaceae and Plantaginaceae, GSI is triggered by an interaction between a pistil-expressed S-RNase and a pollen-expressed F-box protein (SLF) although the details of this molecular interaction and how it triggers pollen rejection remain to be determined (Meng *et al.*, 2011; McClure *et al.*). Interestingly, despite sharing identical genetic determination, GSI in poppy (Papaveraceae) is mediated by a calcium-based signalling pathway probably triggered by interaction between a pollen-expressed Ca^{2+}-channel receptor and its cognate ligand, a stigma-expressed S-glycoprotein (Franklin-Tong *et al.*, 2011). Compatibility has been studied most extensively in species from the Solanaceae, where in *Nicotiana* and *Petunia* it has been demonstrated that stigmatic lipids are essential for pollen development and pollen tube guidance on the stigma and once growing within the pistil other molecules such as receptor kinases and their ligands, lipid-transfer proteins (LTPs), and arabinogalactan glycoproteins variously impact on pollen tube growth and guidance. In the Brassicaceae studies of compatibility in SC *Arabidopsis thaliana* have identified an oleosin and LTPs as important for the initial stages of the pollen–pistil interaction (Mayfield *et al.*, 2001), while GABA and cysteine-rich proteins have been shown to function in pollen tube guidance and attraction, respectively (reviewed in Hiscock and Allen, 2008). Interestingly, two small proteins, SCA and chemocyanin (a planticyanin), first identified in *Lilium* (Liliaceae; Park and Lord, 2003; Kim *et al.*, 2003) and shown to play a role in pollen tube growth in the pistil, have now been identified in *Arabidopsis*, where they also appear to function in pollen–pistil interactions (reviewed in Hiscock and Allen, 2008). SCA and planticyanins thus represent the first consensus molecules potentially involved in a common compatibility pathway across diverse plant families spanning the monocot–eudicot divide.

To extend our understanding of the molecular regulation of pollen–pistil interactions involved in compatibility and SI it is important to extend our studies of these key reproductive processes to species in other families from the monocots and eudicots. With this in mind a study of pollen–pistil interactions and SI was initiated in *Senecio squalidus* as a model species in the Asteraceae, a family that had hitherto received relatively limited study in these areas of reproductive biology. Here we review our current understanding of these processes in the Asteraceae generally, and *S. squalidus* specifically.

POLLEN–PISTIL INTERACTIONS AND SI IN THE ASTERACEAE

The Asteraceae is the second largest family of flowering plants, containing approximately 1620 genera and 22 750 species (APG III, 2003). The family includes many important crop plants (e.g. sunflower, lettuce and chicory), ornamental plants (e.g. 'daisies', gerberas and chrysanthemums), as well as some invasive weedy species (e.g. centaurea and star thistle). Early studies of pollen–pistil interactions in the Asteraceae were directed exclusively at SI species. Indeed, the first genetic accounts of SSI were made from studies in *Crepis foetida* (Hughes and Babcock, 1950) and *Parthenium argentium* (Gerstel, 1950) and then extended through a subsequent study of *Cosmos bipinnatus* (Crowe, 1954). These pioneering studies of SSI showed the system to be controlled by a single *S* locus with multiple *S* alleles that could display dominance-recessive relationships in both pollen and pistil – the latter property being one of the confounding factors in the 'delayed' discovery of this system relative to the simpler GSI system, first described by East and Mangelsdorf (1925). Extensive confirmation of SSI in the Brassicaceae then followed with the classic studies of *Brassica* by Bateman (1952). From this point on genetic and, later, molecular genetic studies of SSI have tended to focus on species in the Brassicaceae whilst similar studies in the Asteraceae did not occur until fairly recently (Hiscock, 2000*b*; Allen *et al.*, 2010*b*). A recent review of the phylogenetic distribution of SSI within the Asteraceae (Ferrer and Good-Avila, 2007) estimated 63 % of species to be SI (presumably all SSI), with the remaining species a mixture of pseudo-self-incompatibility (PSI, 10 %) and SC (27 %). This high percentage of SI species in the Asteraceae suggests that SI is the ancestral breeding system within the family although the phylogenetic support for this assumption is inconclusive (Ferrer and Good-Avila, 2007).

Structural and cytological studies of compatible and incompatible pollen–pistil interactions in species from the Asteraceae (*Cosmos*, *Ambrosia* and *Helianthus*) and the Brassicaceae (*Brassica* and *Raphanus*) identified similarities between their shared SSI systems at a cellular level (Knox, 1973; Howlett *et al.*, 1975; Dickinson and Lewis, 1975; Vithanage and Knox, 1977). These similarities included: the release of exine-held pollen coat soon after contact was made between pollen and stigma – this being a consequence of both compatible and incompatible pollinations; the arrest

of incompatible pollen at the stigma surface soon after germination; and the deposition of callose in incompatible pollen tubes and in stigma cells adjacent to the arrested pollen tubes.

Electron microscopy studies of the stigma surface of *Helianthus* (Vithanage and Knox, 1977) and various other Asteraceae species (Heslop-Harrison and Shivanna, 1977) reported it to be of the 'dry' type – another commonality with the Brassicaceae (Heslop-Harrison and Shivanna, 1977). Angiosperm stigmas have been classified into two broad categories, 'wet' and 'dry', depending on whether or not they possess a surface secretion (Heslop-Harrison and Shivanna, 1977; Heslop-Harrison, 1981). Wet stigma species include members of the Solanaceae, Rosaceae and Liliaceae, while dry stigmas, as well as being typical of the Brassicaceae, are also found in the grasses (Poaceae) and Papaveraceae. This fundamental difference in stigma type has been found to correlate with broad differences between the pollen–pistil interaction in species with wet vs. dry stigmas (Heslop-Harrison, 2000; Johnson and Preuss, 2003; Lord, 2003; Edlund *et al.*, 2004). For instance, in species with wet stigmas pollen capture is non-specific and pollen hydration within the secretion is passive and unregulated, whereas in species with dry stigmas (e.g. *Arabidopsis*) pollen capture and adhesion show a degree of species specificity (Zinkl *et al.*, 1999; Zinkl and Preuss, 2000) and pollen hydration on the stigma is a highly regulated process (Dickinson, 1995). Epidermal cells of wet stigmas tend to lack a continuous cuticle, so penetration of the stigma by the pollen tube is fairly easy, whereas species with dry stigmas usually possess a continuous cuticle which presents a major barrier to pollen tube penetration that must be overcome by pollen secreting hydrolytic enzymes, such as cutinase (Hiscock *et al.*, 1994, 2002*a*).

STUDIES OF REPRODUCTION, POLLEN–PISTIL INTERACTIONS AND SI IN *S. SQUALIDUS*

Senecio squalidus has an intriguing evolutionary history and population biology that make it a unique 'model' offering unconventional opportunities for studies of plant reproductive biology, particularly SI (see Hiscock, 2000*a*; Hiscock *et al.*, 2003). *S. squalidus* is a recent diploid hybrid species derived from a cross between *S. aethnensis* and *S. chrysanthemifolius* that occur on Mt Etna, Sicily, where hybrids still flourish today (James and Abbott, 2005; Abbott *et al.*, 2009). Material from this hybrid zone was introduced to Britain around 1690, where it was cultivated in the Oxford Botanic Gardens (Harris, 2002). UK *S. squalidus* evolved from this founder population after its escape and subsequent spread from Oxford during the late eighteenth century. Genetic studies confirmed that, like other Asteraceae species, SI in *S. squalidus* is controlled sporophytically by a single *S* locus (Hiscock *et al.*, 2000*b*), and population genetic studies predict that between seven and eleven *S*-alleles are present in UK populations, with two to six per local population (Brennan *et al.*, 2006). One aspect of studying SI in *S. squalidus* is to discern how the species has been able to maintain a strong system of SI and yet colonize the UK so rapidly with such a small reserve of *S*-alleles (Brennan *et al.*, 2005). Unusually high levels of dominance interactions between *S*-alleles and pseudo-self-compatibility are together predicted to facilitate maintenance of SI whilst allowing effective mating and seed production for colonization (Brennan *et al.*, 2005, 2006, 2010). In addition to its intriguing population biology, which offers unique opportunities for studies of the population genetics and evolution of SSI, *S. squalidus* has many other attributes that make it a good model for studies of pollen–pistil interactions and plant reproductive biology more generally: (1) it is easily grown in a glasshouse, taking approximately 6 months to reach flowering from seed; (2) under glasshouse conditions it flowers continuously producing large numbers of flower heads (capitula); and (3) it can be propagated clonally by cuttings, facilitating maintenance of defined *S* genotypes.

Reproductive development in S. squalidus

Senecio squalidus possesses a capitulum-type inflorescence, typical of the Asteraceae. The inflorescence consists of an outer whorl of carpellate ray florets and inner whorls of cosexual disc florets (Fig. 1). The individual disc florets develop sequentially, with florets from the outer whorls maturing before those in the centre of the inflorescence (Fig. 1). The pistil possesses a bi-lobed semi-dry stigma, a style and a single ovary. The receptive surface of the stigma, the papillae cells, is protected in immature pistils where the two stigmatic lobes are pressed tightly together (Fig. 2A). As the pistil matures and grows past the anthers, sterile pseudo-papillae at the ends of the stigmatic lobes collect pollen from the anthers and present this to pollinators (Figs 2B and 3). At maturity the two lobes of the stigma come apart to reveal the receptive papillae cells (Fig. 3).

S. squalidus *possesses a semi-dry stigma*

Because early studies of stigma surfaces in the Asteraceae reported the dry type it was always assumed that this was the case and it was not until Elleman *et al.* (1992) carried out a comparative study of pollen–stigma interactions in *Brassica*, poppy, *Cosmos* and *Helianthus*, using novel anhydrous fixation conditions, from which it emerged that stigmas of Asteraceae species were not entirely dry, but rather appeared to produce a small amount of surface secretion. This finding was subsequently confirmed by Hiscock *et al.* (2002*b*) in an extensive structural and cytological study of the pollen–stigma interaction in *S. squalidus*, and other Asteraceae, which led to a reclassification of the Asteraceae stigma as 'semi-dry', reflecting the fact that small amounts of a lipid-rich secretion were always present in the basal regions of stigmatic papillae where the cuticle was absent (Hiscock *et al.*, 2002*b*; Fig. 4). This study revealed that the stigma of *S. squalidus* shows characteristics of both dry- and wet-stigma surfaces. The stigmatic papillae possess a cuticle on their surface, but unlike in dry stigma species (e.g *Brassica* sp.) this is not continuous, and does not extend to the base of the cells (Hiscock *et al.*, 2002*b*). The mature semi-dry surface of the *S. squalidus* stigma produces small amounts of an extracellular secretion, on the surface of the papillae cells and secretion of this lipid-rich material is enhanced when pollen makes contact with the stigma, irrespective of whether the pollen is compatible or incompatible (Hiscock *et al.*, 2002*b*).

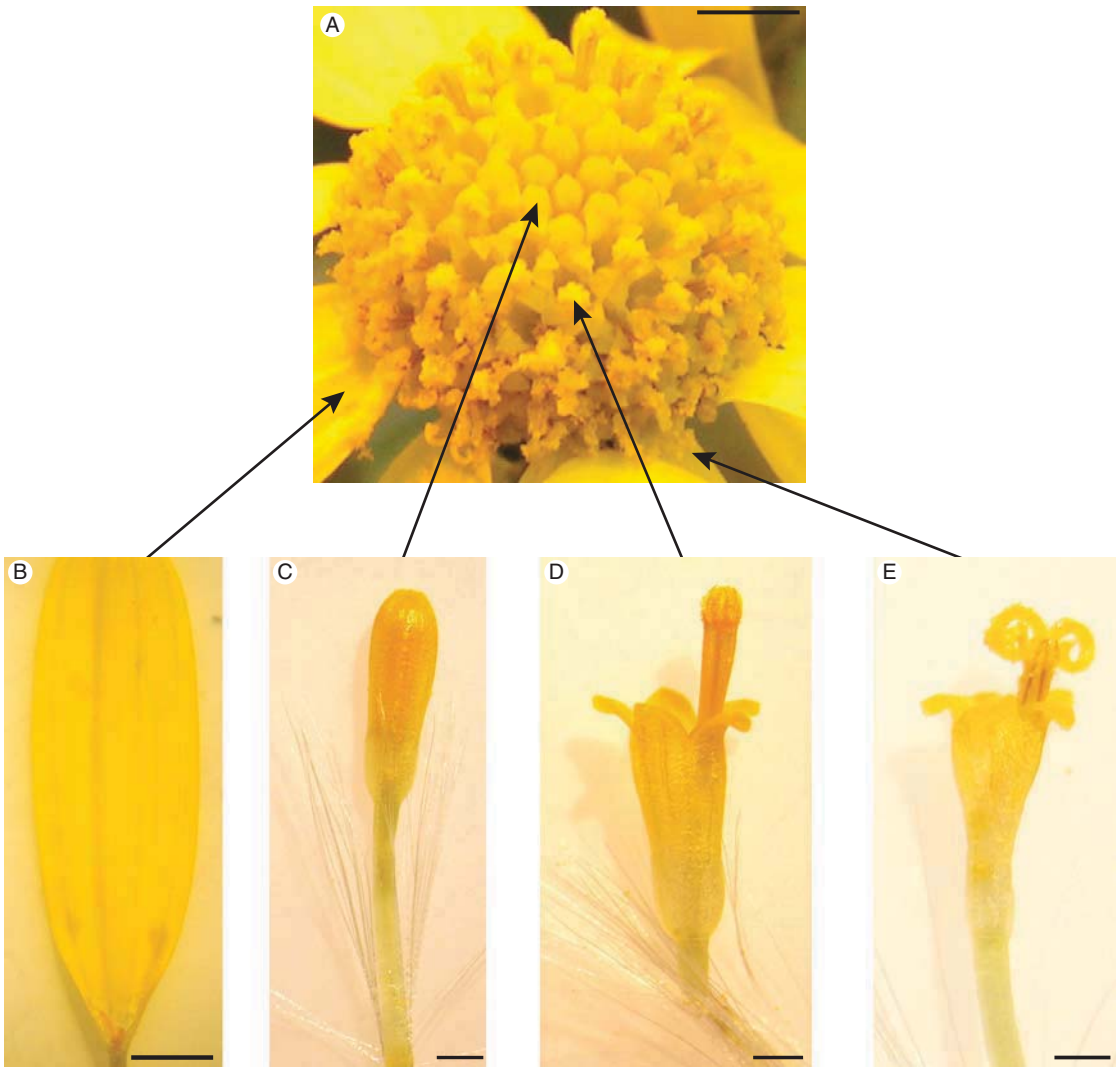

FIG. 1. Stages of floret development in *S. squalidus*. (A) Developing captiulum; arrows indicate florets of different developmental stages; scale bar = 2 mm. (B) Ray floret; scale bar = 1 mm. (C) Immature disc floret. (D) Disc floret stage 1 (anther mature). (E) Disc floret stage 2 (pistil mature). Scale bars in (C–E) = 0·5 mm.

The stigma surface stains positive for the presence of lipids, carbohydrate, protein, reactive oxygen species (ROS) and peroxidases, components that have been identified as important for stigma function (McInnis *et al.*, 2006; Fig. 4). Since the discovery of high levels of ROS, mainly in the form of hydrogen peroxide, in *Senecio* stigmas, it has been shown that accumulation of high levels of ROS is a general feature of angiosperm stigmas when they reach maturity and are optimally receptive to compatible pollen (McInnis *et al.*, 2006). Nevertheless, the biological function of these ROS remains a mystery. Two possible functions of stigmatic ROS might be: (1) protecting stigmas from pathogen attack in a similar way to that proposed for ROS in protecting nectar from infection by bacteria and fungi (Carter and Thornburg, 2004), and (2) as a signal to germinating pollen (McInnis *et al.*, 2006).

Pollen–pistil interactions in S. squalidus

Upon landing on the stigma, the pollen grain rapidly releases pollenkitt (pollen coat) from the exine onto the stigma surface, leading to the formation of an 'attachment foot' beneath the pollen grain (Hiscock, 2000*a*; Hiscock *et al.*, 2002*b*). The attachment foot is structurally complex, and is formed of a mixture of pollen-wall material and stigmatic extracellular secretion. The cytoplasm of the papillae cells directly below pollen grains has been observed to contain large numbers of vesicles, suggesting that these cells are actively producing secretion (Hiscock, 2000*a*; Hiscock *et al.*, 2002*b*). During a compatible pollination, after the pollen grain hydrates (within 15–30 min of landing on the stigma surface), a pollen tube emerges and grows between the papillae cells (Fig. 5). The pollen tube penetrates the stigma and grows intercellularly through the cells of the stigmatic cortex, before turning 90 ° and growing parallel with the transmitting cells of the stigmatic lobe and style, towards the ovary (Hiscock *et al.*, 2002*b*).

The SI response in S. squalidus

The site of the SI response in *S. squalidus* is variable, but typically occurs at the stigma surface, where incompatible

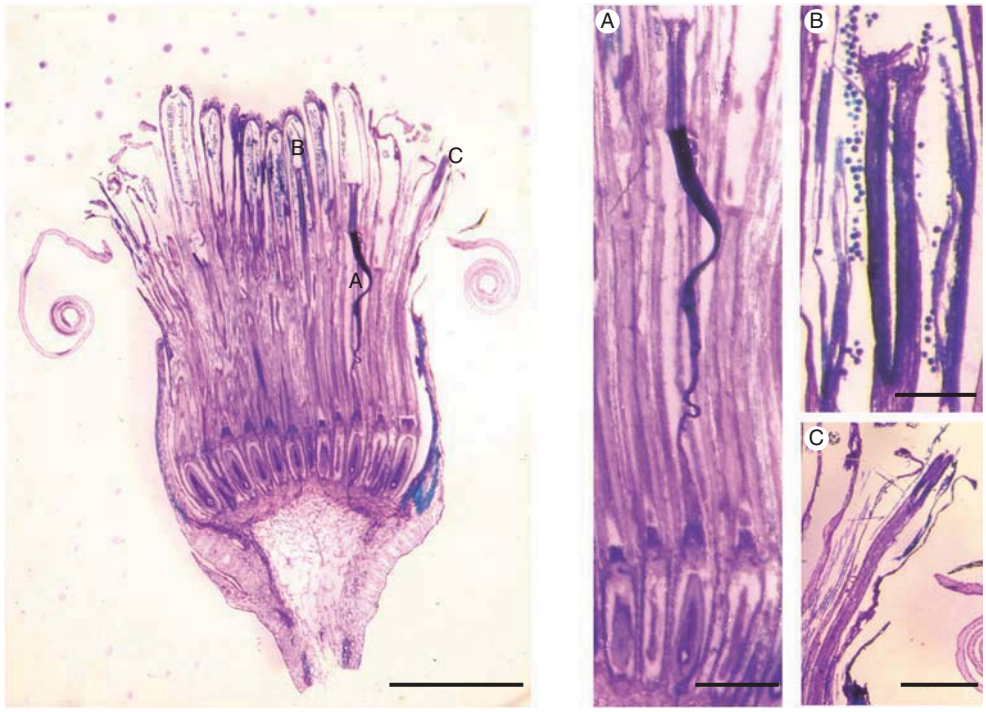

FIG. 2. Section through a *Senecio squalidus* capitulum, showing individual disc florets in different stages of development. Inset pictures show detail of pistils: (A) entire pistil; (B) developing pistil; (C) emerging pistil. Section stained with Toluidine blue. Scale bars = 5 mm (main image), and 1 mm (insets).

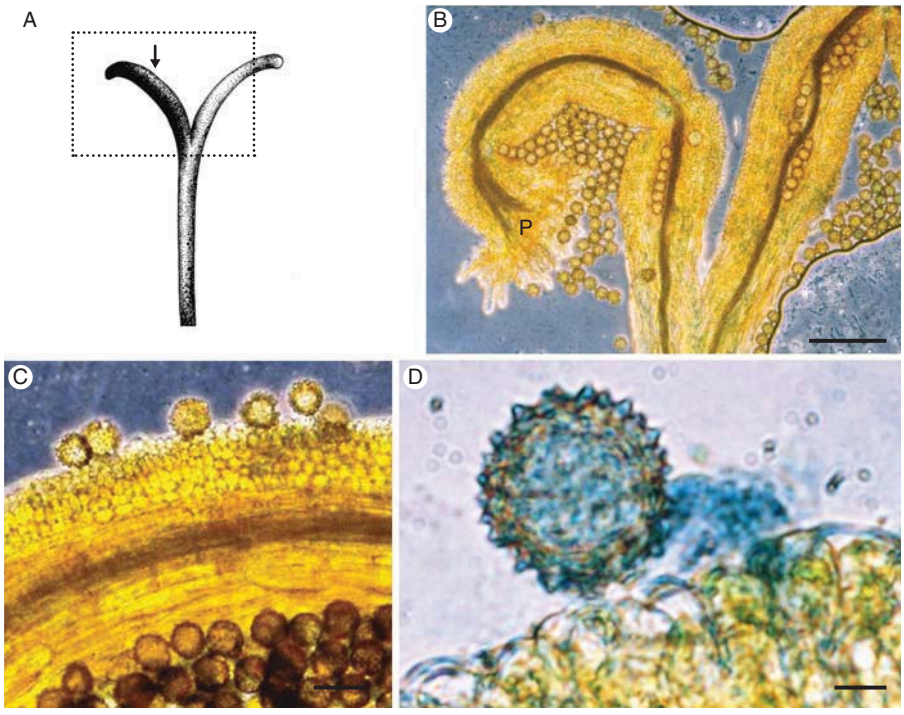

FIG. 3. (A) Illustration of *Senecio squalidus* pistil. (B) Squash preparation of *S. squalidus* stigma, stained with aniline blue (section of pistil indicated by hatched line in A). P, pseudo-papillae cells; scale bar = 0·1 mm. (C) Detail of stigma papillae cells with pollen grains attached (indicated by black arrow in A); scale bar = 25 μm. (D) Detail of pollen tube penetrating papillae cells; scale bar = 5 μm.

pollen tubes are often arrested prior to germination. However, some incompatible pollen grains do produce pollen tubes; these are arrested on the stigma surface or, on rare occasions, after penetration of the stigma (Hiscock *et al.*, 2002*b*). Pollen tube arrest is accompanied by the accumulation of vesicles, and deposition of callose plugs in papillae cells directly

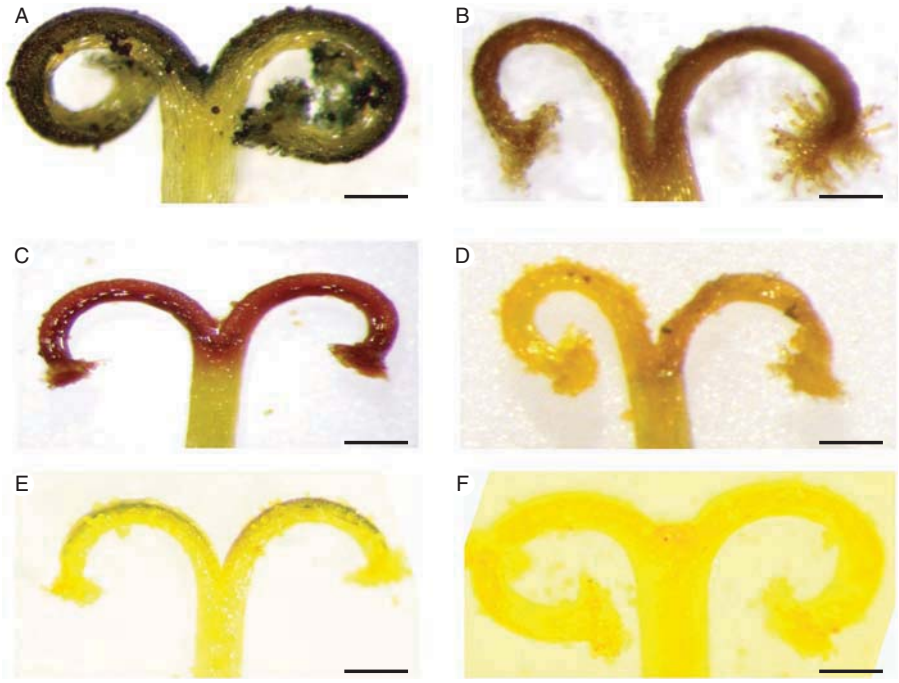

FIG. 4. The semi-dry stigma of *Senecio squalidus* stained for the presence of lipids, peroxidase and reactive oxygen species (ROS). (A) Stigma stained with Sudan black b to visualize the presence of lipids, indicated by black staining. (B) Control stigma stripped of lipids by placing in 10 % SDS prior to staining. (C) Stigma stained with 0·1 M guaiacol, 0·1 M H_2O_2, in 20 mM phosphate buffer, pH 4·5, to visualize peroxidase activity. (D) Control of C. (E) Stigma stained with TMB-HCl (3,39,5,59-tetramethylbenzidine-HCl, 0·1 mg ml^{-1} in TRIS acetate, pH 5·0) showing the localized activity of ROS in papillae cells, indicated by blue staining. (F) Control of (E). Scale bars = 50 μm.

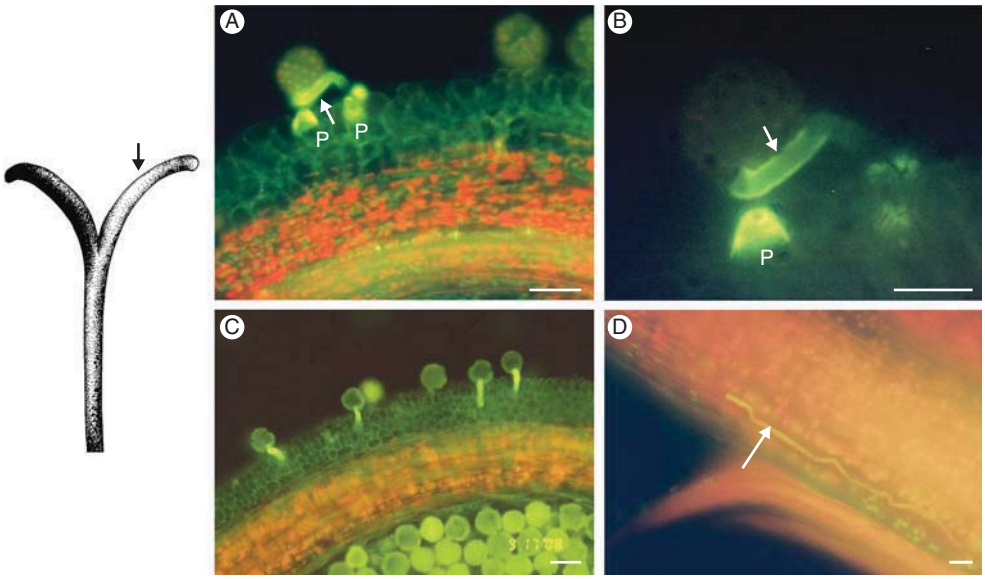

FIG. 5. Incompatible and compatible pollinations in *Senecio squalidus*. Squash preparations of stigmas stained with aniline blue and viewed under UV light. (A,B) Incompatible pollination; pollen tube (arrow) blocked from entering papillae (P). (C) Compatible pollination; pollen tubes penetrating stigma tissue. (D) Compatible pollen tube growing through transmitting tissue (arrow). Scale bars = 0·25 μm.

below incompatible pollen tubes (Hiscock, 2000*a*; Hiscock *et al.*, 2002*b*; Fig. 5).

Despite the extensive research of SSI in population genetic studies of *S. squalidus*, the genetic *S*-locus controlling SSI remains unidentified. Previous studies of *S. squalidus* have shown that orthologues of the *Brassica S*-gene, SRK, are not expressed exclusively in the stigma, or linked to the *S*-locus (Hiscock *et al.*, 2003; Tabah *et al.*, 2004). From this evidence it has been concluded that SSI in *Senecio* species operates through a different molecular mechanism from that found in *Brassica* (Hiscock *et al.*, 2003). Subsequent studies using isoelectric focusing identified stigma-specific peroxidases (SSPs),

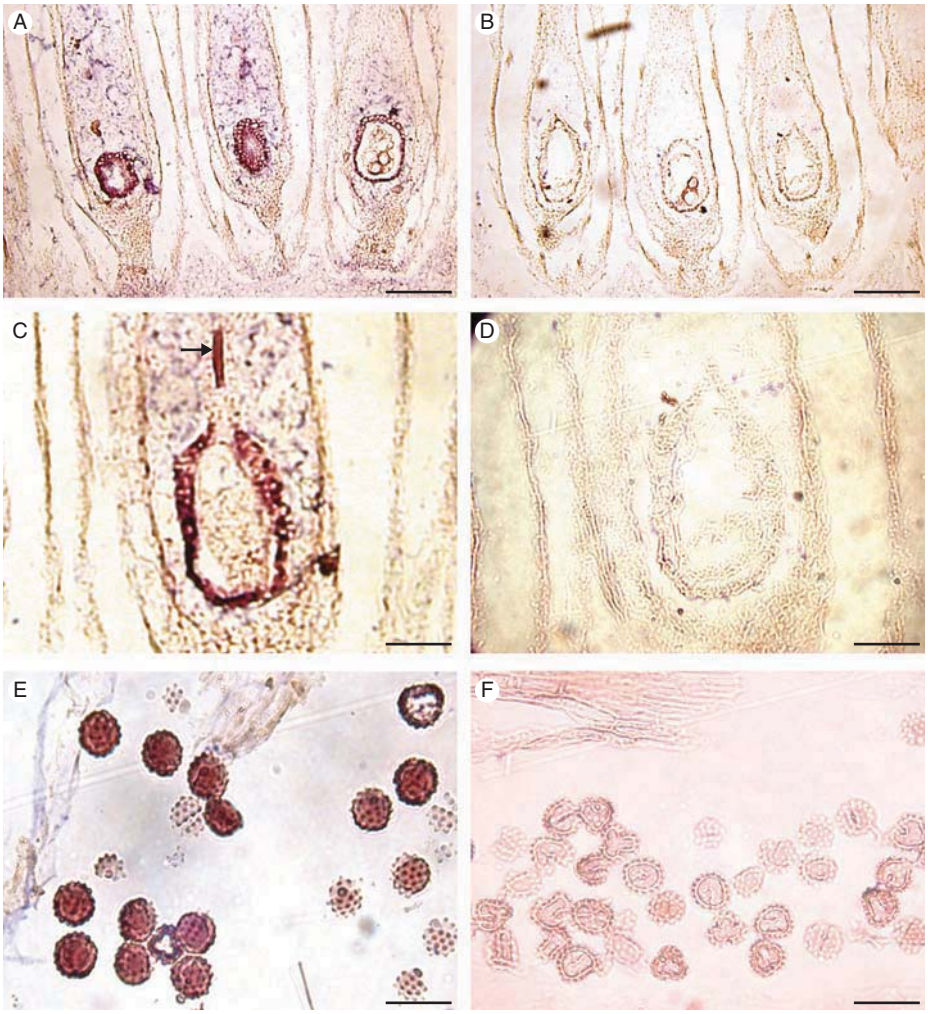

FIG. 6. *In situ* hybridizations on longitudinal sections of *Senecio squalidus* pistils. Pistils at ovule developmental stages 2 and 3 hybridized with SF21 antisense probe (A,C,E) and hybridized with sense probe (B,D,F). (A) Base of pistil with staining in ovules. (C) Expression is localized to the integument cells surrounding the embryo sac and transmitting tissue immediately above ovule (black arrow). (E) Expression was also detected in mature pollen grains. Scale bars: (A,B) = 50 μm; (C–F) = 25 μm.

which were associated with defined *S*-alleles and expressed exclusively in the pistil (Hiscock *et al.*, 2003). Expression of these was developmentally regulated with maximal expression occurring at anthesis when SI is functioning. *In situ* hybridizations revealed that expression of SSP was specific to the stigmatic papillae cells. However, the SSP gene did not segregate with defined *S*-alleles and so tight linkage cannot be established between the SSP genes and the *S*-locus (McInnis *et al.*, 2005).

TRANSCRIPTOMIC ANALYSIS OF THE *S. SQUALIDUS* PISTIL

During its colonization of the UK, *S. squalidus* hybridized with native *S. vulgaris* (groundsel) to form the allohexaploid hybrid species *S. cambrensis* (Welsh ragwort) within the last 60 years (Abbott and Lowe, 2004). Despite their close evolutionary relationships these three *Senecio* species are highly divergent in flower head morphology and mating system. *Senecio squalidus* has large outer ray flowers in its capitulum and is SI while *S. vulgaris* has no ray flowers and is SC, and *S. cambrensis* has short or variable ray flowers and is usually SC, although functional SI has been found in synthetic hybrids (Brennan *et al.*, 2010). These three *Senecio* taxa therefore offer unique opportunities to study the regulation of ray flower development and SI/SC. To further study these developmental processes anonymous cDNA microarrays were created using floral tissue from the three taxa and the primary triploid hybrid of *S. vulgaris* × *S. squalidus*, *S.* × *baxteri* (Hegarty *et al.*, 2005).

Great variation in gene expression was detected between the three floral transcriptomes (Hegarty *et al.*, 2005, 2006) and sequencing identified potential candidate genes for pollen–pistil interactions and SI (Allen *et al.*, 2010*a*). 'Virtual subtraction' analyses identified genes up-regulated in SI *S. squalidus* compared with SC *S. vulgaris* and *S. cambrensis*. Northern blot analysis confirmed tissue-specific expression in pollen or pistil (or both) and directed identified genes of interest for

more detailed study. One such gene showed a particularly interesting expression profile, being developmentally regulated with transcripts present in both pollen and pistil (Allen *et al.*, 2010*a*). This gene showed significant orthology to *SF21*, a gene of unknown function originally identified in *Helianthus annuus* (sunflower) (Kräuter-Canham *et al.*, 1997). The *SF21* gene family is widespread in plants (Okuda and Kondoh, 1999), and there is evidence of specialization of *SF21* genes to function in reproductive processes, particularly in the Asteraceae, where *Helianthus* and *Senecio SF21* genes show pistil- and pollen-specific expression. *S. squalidus SF21* has multiple copies with one gene copy expressed in pollen, and the other in pistil tissues (Fig. 6; Allen *et al.*, 2010*a*). In *Senecio SF21* has been proposed to act in compatible pollen tube guidance from the stigma to the ovary (Allen *et al.*, 2010*a*). If *SF21* does function in pollen tube guidance, this important role may explain the high degree of conservation of gene sequence across the angiosperms. Certainly, the *SF21* gene family represents a rare example of a family of highly conserved pistil- and pollen-expressed genes.

To identify additional genes potentially involved in pollen–pistil interactions and SI in *S. squalidus* we recently created a pistil-enriched cDNA library using suppression subtractive hybridization (SSH) (Allen *et al.*, 2010*b*). The resulting data set contained both novel genes to *S. squalidus* and genes previously identified in similar studies of other unrelated species from the Brassicaceae, Poaceae, Solanaceae and Liliaceae. The *Senecio* pistil data set was expected to contain genes potentially involved in mediating the female side of SI, including primary *S*-recognition genes. Thus, any novel pistil genes identified could be considered as potential candidate SI genes. Novel genes identified in this study included a WNK (with no K/lysine) kinase with a putative calcium-binding domain, a kinase-interacting protein, a membrane-associated protein, a nematode resistance protein and several hypothetical proteins of unknown function (Allen *et al.*, 2010*b*; Table 1). These novel pistil-specific genes may be candidate SI genes as the pistil data set is expected to contain genes potentially involved in mediating the female side of SI, including primary *S*-recognition genes.

Two pistil-expressed genes were of particular interest as candidates for pollen–pistil interactions and SI. First, the *Senecio* pistil-specific *membrane associated protein* (*MAP*) was found to be expressed in the papillar cells and transmitting tissue of the stigma (Fig. 7). The MAP protein is predicted to have three transmembrane helices, with most of the protein lying extracellularly. In *S. squalidus* the nucleotide sequence of *MAP* exhibits relatively high *S*-genotypic polymorphism, which is elevated in the extracellular region (Allen *et al.*, 2010*b*). The closest homologue in *Arabidopsis*, AtMAMI (*Arabidopsis thaliana* membrane-associated mannitol induced) is related to vesicle-associated membrane proteins (VAMPs), which were also returned on a BLAST-X search using *MAP* (Galaud *et al.*, 1997). VAMPs are predicted to function in membrane trafficking of molecules from vesicles to the cell membrane. Mannitol is a potent ROS quencher and a chemical commonly produced by fungi to suppress ROS-mediated plant defence systems (Jennings *et al.*, 1998). ROS are present at high levels in the receptive cells of the stigma, offering a potential obstacle to pollen tube development; it is possible that the production of mannitol suppresses ROS, to allow pollen tube penetration and growth. If the *Senecio* MAP was also induced by mannitol, the presence of this chemical would potentially signal the presence of a pollen grain and stimulate membrane trafficking in the papillar cells of the stigma during the pollen–stigma interaction. The cytoplasm of *S. squalidus* papillae directly below pollen grains has been observed to contain large numbers of vesicles, suggesting that these cells are actively producing secretion (Hiscock *et al.*, 2002*b*), and pollen tube arrest during the SI response is characterized by pronounced 'swellings' in cell walls of papillae cells in direct contact with incompatible pollen tubes (Hiscock, 2000*a*; Hiscock *et al.*, 2002*b*). The *Senecio* AtMAMI might well be involved in these secretion activities in papillae.

The second candidate gene, a pistil-specific nodulin/mtn3 gene (*Nod*), is expressed exclusively in the papillar cells of the *S. squalidus* stigma, where it appears to be developmentally regulated, reaching maximal expression as the stigmatic lobes reflex to expose the papillar cells (Fig. 8). Originally identified in *Medicago trunculata* root nodule tissue, mtn3 is predicted to be involved in a hypersensitive defence response (Gamas *et al.*, 1996). The nodulin/mtn3 gene family includes a number of genes that play specialized roles in reproductive tissues (Yang *et al.*, 2006; Guan *et al.*, 2008). The role of pollen-expressed members of this gene family has been investigated in *Arabidopsis* and rice. In rice, *OS8NG* (a gene expressed primarily in pollen but also in leaves) appears to have a dual function; it has been implicated in blight resistance in leaves and also functions in pollen development (Yang *et al.*, 2006). In *Arabidopsis*, RPG1 has been shown to be essential for pollen wall development (Guan *et al.*, 2008). Despite the large number of pistil-specific nodulin/mtn3 genes in *Arabidopsis* and other plant species, the role of these genes in pistils has not been studied. Nod possesses seven predicted transmembrane regions, with one variable and two highly conserved intracellular domains. The conserved domains are essential for function in RPG1, which is predicted to act by regulating membrane traffic in the pollen tapetum (Guan *et al.*, 2008). A similar role for *Nod* in *S. squalidus* is proposed; as putative regulators of membrane traffic in papillae cells these proteins could be important mediators of pollen–stigma interactions. The presence of pollen- and pistil-specific nodulin/mtn3 genes in the pollen tapetum and stigmatic papillae cells has intriguing implications, raising the possibility that these proteins could play similar or complementary roles in the primary receptive tissue of pollen grains and stigmas.

Comparative analysis of the *Senecio* pistil transcriptome with the four other available pistil transcriptomes – *Oryza sativa* (rice), *Crocus sativus* (crocus), *Arabidopsis thaliana* and *Nicotiana tabacum* (tobacco) – allowed us to identify key similarities and differences between these diverse species which represent the three major clades of the angiosperms (Asterids, Rosids and Monocots) and three stigma states (wet, dry and semi-dry). Our data showed that the pistil transcriptome of *S. squalidus* is generally more similar to the dry stigma transcriptome of *Arabidopsis* but also contains genes expressed in wet stigmas (Allen *et al.*, 2010*b*). Additionally, our analysis indicated that certain classes of

TABLE 1. *Putative function and expression characteristics of candidate SI/pollen–pistil genes in* Senecio squalidus

Accession number	Gene description	Gene annotation	Putative function	Site of expression
GO255172	Fbox	*Fbox*	Protein fate	Pollen
GO255101	ABC transporter	*ABC*	Transport	Pistil
GO255139	NECIV	*NECIV*	Defence	Pistil
GO255232	Integral membrane protein	*IMP*	Cell wall	Pistil
GO255201	CBS-domain	*CBS*	Unknown	Pistil
GO255154	Calcium-kinase	*CaKin*	Signalling	Pollen
GO255182	Nodulin/mtn3	*Nod*	Signalling/transport	Pistil
GO255107	Membrane-associated protein	*MAP*	Signalling/transport	Pistil
GO255153	Kinase interacting protein	*KIP*	Signalling	Pollen
GQ227732	Sunflower 21a	*SF21a*	Signalling	Pollen/pistil
GQ227733	Sunflower 21b	*SF21b*	Signalling	Pollen
GO255242	Sunflower 3	*SF3*	Transcription	Pollen
GO255239	Sunflower 16	*SF16*	Transcription	Pollen
GO255140	Stigma specific peroxidase	*SSP*	Defence	Pistil
GO255135	Putative binding protein	*Bind*	Transport	Pistil
GO255123	*myo*-Inositol oxygenase	*Myo*	Metabolism	Pistil
GO255100	UDP glycosyltransferase	*UDP*	Defence	Pistil
GO255085	Nematode resistance protein	*NR*	Defence	Pistil
GO255235	Unknown protein	*Unk*	Unknown	Pistil
GO255177	Dehydration sensitive	*Dehyd*	Stress	Pistil

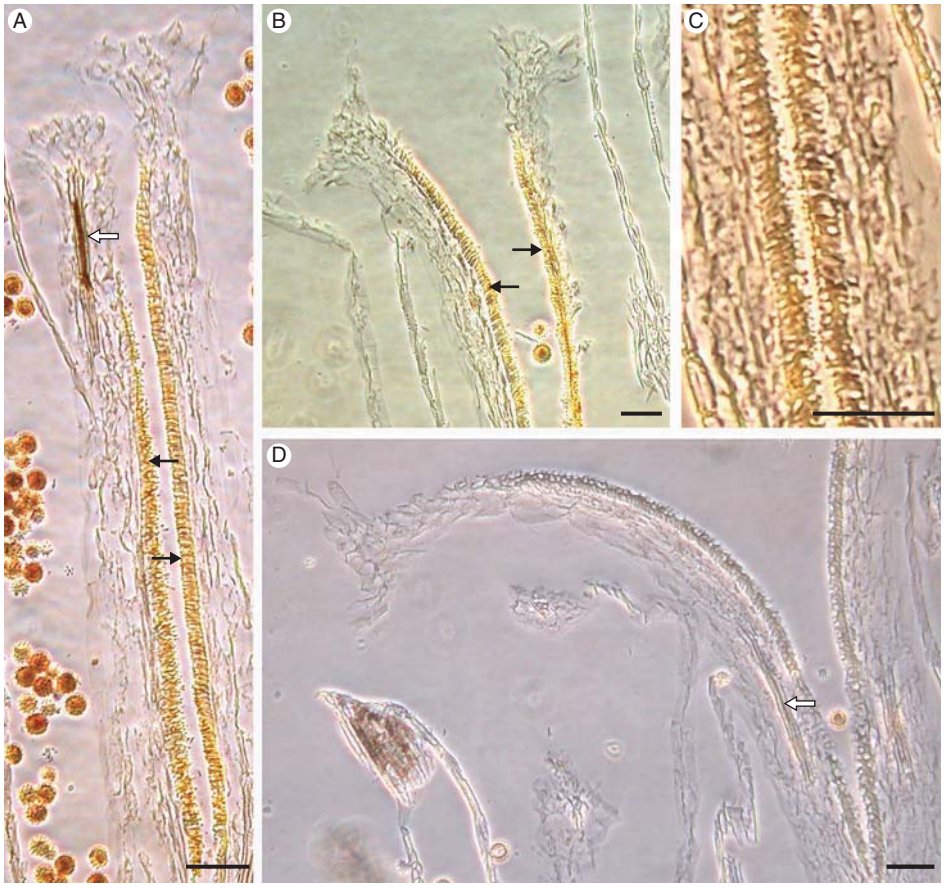

FIG. 7. *In situ* hybridizations on longitudinal sections of mature *Senecio squalidus* pistils hybridized with *MAP* antisense probe (A–C) and hybridized with sense probe (D). (A) Upper section of emerging pistil showing expression in papillar cells of stigma (black arrows) and transmitting tissue (white arrow). (B) Fully mature and reflexed pistil exhibiting expression in papillar cells (black arrows). No expression was detected in pseudopapillae cells at the tips of the stigma. (C) Close-up of papillar cells. (D) Corresponding sense control showing no expression in papillar cells (black arrow) or transmitting tissue (white arrow). Scale bars = 50 μm.

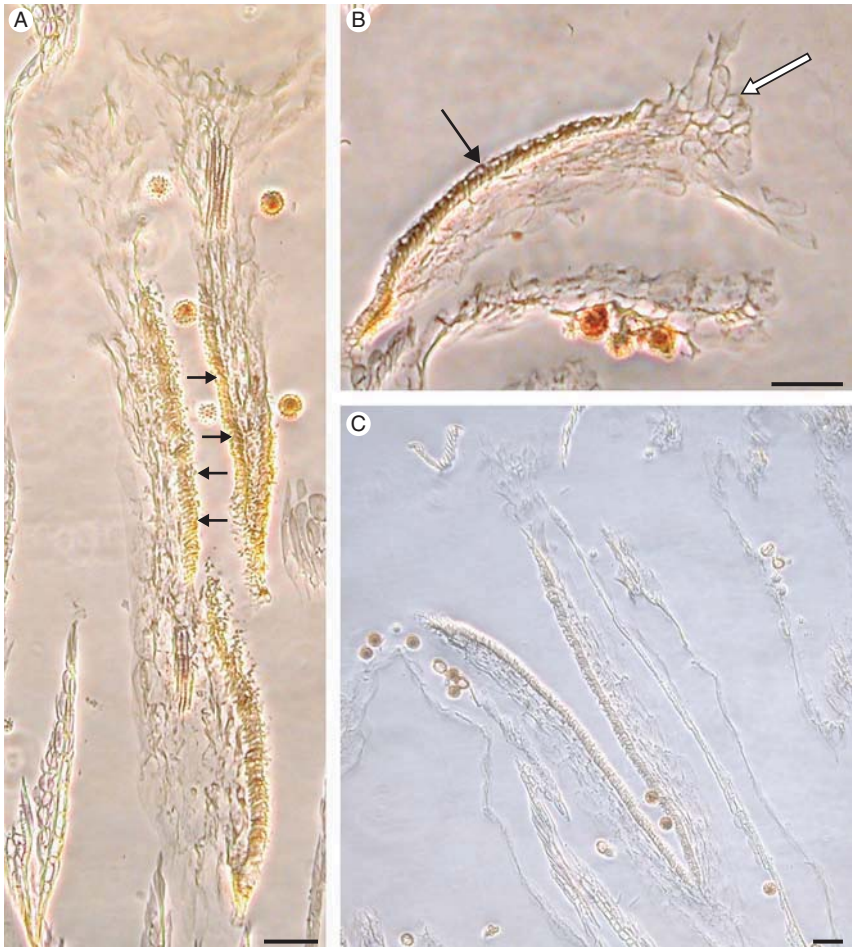

FIG. 8. *In situ* hybridizations on longitudinal sections of mature *Senecio squalidus* pistils hybridized with Nodulin antisense probe (A,B) and hybridized with sense probe (C). (A) Upper section of emerging pistil showing expression in papillar cells of stigma (black arrows). (B) Fully mature and reflexed pistil exhibiting increased expression in papillar cells (black arrow). No expression was detected in pseudopapillae cells (unfilled arrow). Staining of pollen grains occurred due to high levels of intrinsic pollen cytosolic alkaline phosphatase activity (Knox and Heslop-Harrison, 1969); no expression of *Nod* was detected in pollen via northern blot analysis (Fig. 7B). (C) Corresponding sense control showing no expression in papillar cells. Scale bars = 50 μm.

genes were common to the pistil transcriptomes of the five angiosperm species sampled to date, suggesting they are important for pistil function and hence are conserved between diverse angiosperm groups (Allen *et al.*, 2010*b*). This suggests that some of the complex interactions underlying pistil function in diverse species with wet, dry and semi-dry stigmas are shared and have been inherited from the common ancestor of monocots and eudicots (Hiscock and Allen, 2008).

CONCLUSIONS AND PROSPECTS

Despite previously perceived similarities in stigma structure and SI between the Brassicaceae and Asteraceae, recent studies in *Senecio* have shown that both are fundamentally different: *Senecio*, and other Asteraceae species sampled, possess a semi-dry stigma (Hiscock *et al.*, 2002*b*) rather than a completely dry (*Brassica*-type) stigma, and the sporophytic SI system of *Senecio* (and by implication other Asteraceae) does not operate through the *Brassica*-type SRK/SCR(SP11) molecular machinery (Hiscock *et al.*, 2003). To understand evolutionary homologies between Asteraceae pollen–pistil interactions and SSI and those of other families it is therefore important to identify and characterize molecules regulating these critical reproductive processes in this large and important family.

Our recent transcriptomic studies in *Senecio* have identified numerous genes potentially involved in pollen–pistil interactions and SI in the Asteraceae. Some pistil-specific and pollen-specific genes are novel to *Senecio*, whereas others have been identified previously in pollen or pistils of unrelated species (Allen *et al.*, 2010*a*, *b*). Novel *Senecio* pollen and pistil genes are particularly interesting given that the *Senecio* SSI system has yet to be characterized at a molecular level. Indeed, research on another elusive SSI system in *Ipomoea* (Convolvulaceae) has recently identified putative *S*-genes, which have no homologous matches in the databases (Rahmann *et al.*, 2007). Similarly the recently identified *Papaver* pollen determinant is also encoded by a novel gene (Franklin-Tong, 2008). Of the pistil-specific candidates in *S. squalidus*, two genes, *MAP* and *Nod*, expressed in stigmatic papillae may play important roles in the recognition of pollen

grains, so are the focus of ongoing studies. Both *MAP* and *Nod* are localized to the cell membrane, where they could potentially regulate membrane traffic during pollen hydration and germination. Comparative transcriptome analysis has also highlighted potentially important conserved pistil genes, such as *SF21*, and phylogenetic analysis of this gene family indicates these genes are ancient and show a high degree of sequence conservation across different angiosperm taxa (Allen *et al.*, 2010*a*); this suggests a fundamental role for *SF21* in reproduction. Determining the precise function of *SF21* and other candidate genes will be a vital step in assessing their importance in controlling pollination in *Senecio* and other angiosperm taxa.

Despite many years of research, relatively little is known about general mechanisms regulating pollen–pistil interactions. Current research indicates a high diversity of molecules recruited for the same processes in different species, but there are fewer examples of conserved pollen–pistil genes (Hiscock and Allen, 2008). More comparative studies of pollen and pistil transcriptomes are therefore needed, especially in basal angiosperms, to identify further conserved and unique genes potentially involved in angiosperm reproductive processes.

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