Annals of Botany **91**: 807–816, 2003
doi:10.1093/aob/mcg085, available online at www.aob.oupjournals.org

Evidence for Ovarian Self-incompatibility as a Cause of Self-sterility in the Relictual Woody Angiosperm, *Pseudowintera axillaris* (Winteraceae)

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Received: 22 November 2002 Returned for revision: 16 December 2002 Accepted: 6 February 2003 Published electronically: 3 April 2003

Species within the genus *Pseudowintera* exhibit high rates of self-sterility. Self-sterility in the genus has been previously posited—but not confirmed—to be the result of late-acting ovarian self-incompatibility (OSI) functioning within nucellar tissue of the ovule to prevent self pollen tubes from entering the embryo sac. Structural and functional aspects of pollen–carpel interactions and early seed development following cross- and self-pollination were investigated in *P. axillaris* to determine the site, timing and possible mechanisms of self-sterility. No significant differences were observed between pollen tube growth, ovule penetration and double fertilization following cross- and self-pollination. Pollen tubes exhibited phasic growth in an extracellular matrix composed of proteins and carbohydrates, as well as arabinogalactans/arabinogalactan proteins. A uniform failure in embryo sac development prior to division of the zygote was apparent within 15 d following double fertilization by self gametes. Results indicate that SI mechanisms in *P. axillaris* do not prevent double fertilization from occurring. Instead, mechanisms of self-sterility affect post-zygotic development of the embryo sac. Although self-sterility may be attributed to inbreeding depression, given the post-zygotic nature of failure in embryo sac development, the possibility of late-acting OSI is discussed.

Key words: Embryo sac, late-acting self-incompatibility, ovarian self-incompatibility, early-acting inbreeding depression, pollination-regulated development.

INTRODUCTION

Early-acting inbreeding depression and self-incompatibility (SI) are the two main causes of reduced seed set following self-pollination in flowering plants. Early-acting inbreeding acts post-zygotically and results in abortion of progeny homozygous for deleterious recessive alleles (Charlesworth and Charlesworth, 1987; Husband and Schemske, 1996). In comparison, self-incompatibility is a genetically based self-recognition system that reduces the frequency of self seed set through rejection of self pollen. Known SI systems function primarily pre-zygotically with arrest of pollen function at the stigma or style (see Matton *et al*., 1994; Franklin *et al*., 1995; de Nettancourt, 1997), although it has been hypothesized that some species may exhibit post-zygotic SI (see Seavey and Bawa, 1986; Gibbs and Bianchi, 1993, 1999; Sage *et al*., 1994; Gibbs *et al*., 1999).

The vessel-less Winteraceae family of the subclass Magnoliidae has received considerable attention in recent decades as a result of the apparent primitiveness of many of its vegetative and floral features and its long fossil history (Bailey and Swamy, 1951; Sampson, 1980; Cronquist, 1981; Walker *et al*., 1983; Friis and Endress, 1990; Brenner and Bickoff, 1992; Suh *et al*., 1993). One genus within the family, *Pseudowintera*, exhibits high rates of self-sterility (Norton, 1980; Godley and Smith, 1981; Lloyd and Wells, 1992). Qualitative studies on pollen tube growth in *Pseudowintera* have led to the conclusion that self-rejection occurs in the ovary with genetic recognition/rejection of self pollen in ovules at the nucellus prior to embryo sac entry (Norton, 1980; Godley and Smith, 1981; Lloyd and Wells, 1992). Classification of self-sterility as pre-zygotic in ovules, particularly involving the nucellus, of *Pseudowintera* is still tentative. Characterization of self-sterility in *Pseudowintera* requires quantitative analysis of pollen tube growth following cross- and self-pollination and detailed structural studies to assess pollen–carpel interactions and early embryo/seed development. Although previous studies have provided some insights into the pollen tube pathway and pollen–carpel interactions as well as embryo/seed development (Bhandari, 1963; Sampson, 1963; Norton, 1980; Lloyd and Wells, 1992), quantitative comparisons of cross- and self-pollen tube growth, as well as structural information on pollen-carpel interactions and early seed development following cross- and self-pollination are lacking for the genus. The purpose of this study is to provide such information for *P. axillaris*. The primary questions addressed were: (1) do cross and self pollen tubes exhibit differential qualitative and quantitative growth characteristics within the pistil; (2) do self pollen tubes effect double fertilization as successfully as cross pollen tubes; (3) are there differences in ovule/seed development following cross- *vs*. self-pollination; and (4) if there are differences in ovule/seed development, when are they apparent and in what tissues are differences first manifested?

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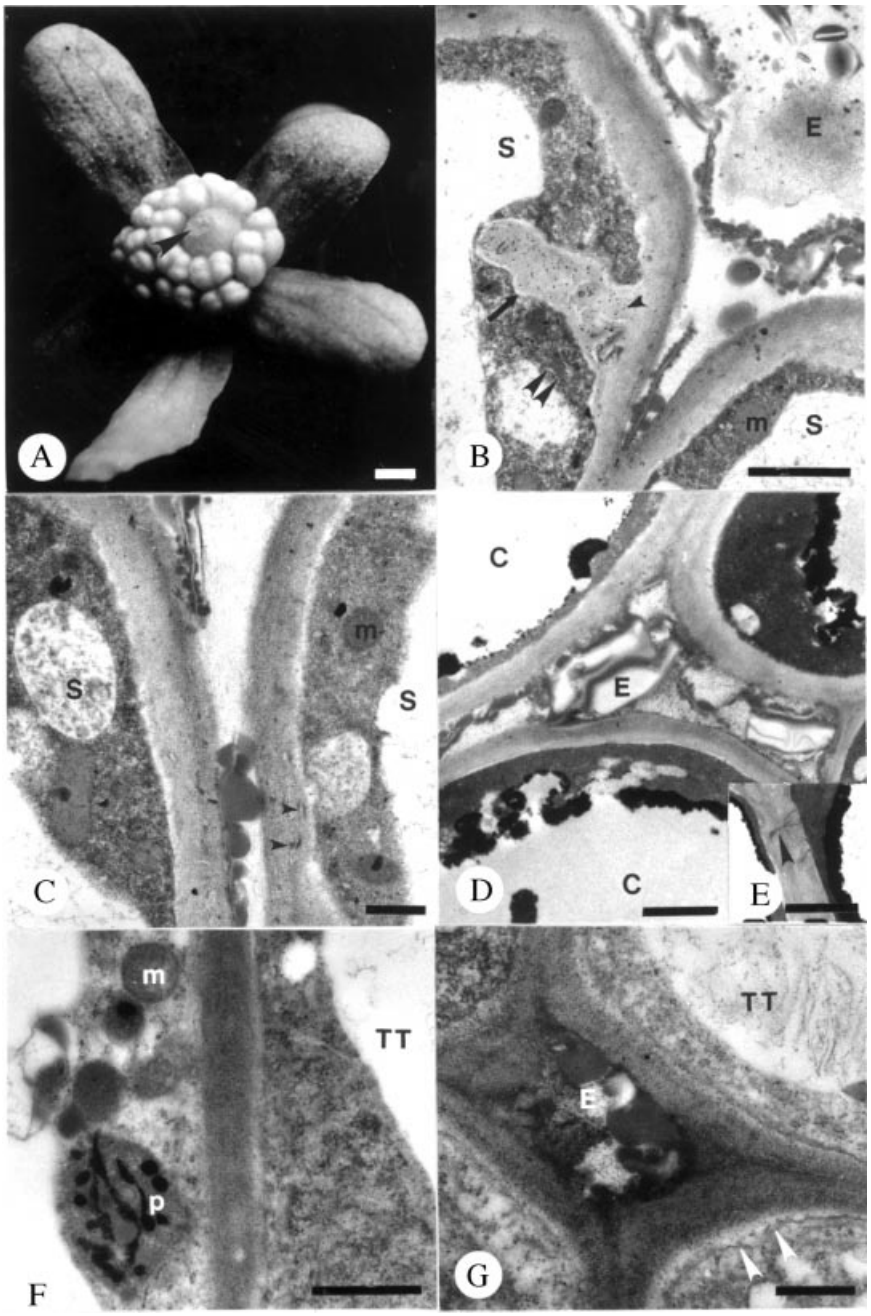

FIG. 1. Unpollinated stigma and sub-stigmatic transmitting tissue of *Pseudowintera axillaris* at anthesis. A, Stigmatic exudate (arrowhead). Bar = 6·67 mm. B, Stigmatic papilla with transfer cell wall (arrow). Note cell wall inclusions (arrowhead) and exudate. Double arrowhead denotes Golgi body. Bar = 2 µm. C, Stigmatic papilla. Note cell wall inclusions (arrowheads). Bar = 0·05 µm. D, Exudate between marginal cleft cells. Bar = 2 µm. E, Secondary plasmodesmata (arrowhead) between marginal cleft cells. Bar = 1·5 µm. F and G, Fine structure of cells in solid core of sub-stigmatic transmitting tissue. F, Bar = 1 µm. G, Note periplasmic exudate adjacent to the invaginated plasmalemma (arrowheads). Bar = 0·05 µm. C, Marginal cleft cell; E, exudate; m, mitochondrion; p, plastid; S, stigmatic papilla; TT, sub-stigmatic transmitting tissue cells.

MATERIALS AND METHODS

Study organism

Experimental material of *P. axillaris* used in this study was located in the Akatarawa region at the southern end of Tararua Forest Park (40°58′S, 175°10′E), North Island, New Zealand. Six flowering trees were selected at random for cross- and self-pollinations. Self-pollinations were made on each of the six flowering trees, and cross-pollinations were conducted on all six flowering trees between individual trees heterozygous at the isozyme locus PGI-2 (determined by W. W. Cole, University of Toronto, Canada). Field and laboratory pollinations were conducted as described by Norton (1980) and Lloyd and Wells (1992). The shoot tip

and leaf apices were removed from branches bearing 15–20 floral buds to allow easy application and removal of nylon mesh pollination bags. Pollinations were made on fully opened flowers with receptive stigmas that had not yet begun to shed pollen. Stigma receptivity was indicated by the presence of stigmatic secretions. Pollinations were performed from 0800 until 1100 h, at which time the secretions dried up. A freshly dehisced anther was removed using fine forceps and brushed on the stigmas of each flower.

Pollen–carpel interactions following cross- and self-pollination

Structural and histochemical features of cross- and self-pollen tube growth and the transmitting tissues encountered by pollen tubes were characterized using light microscopy (LM) and scanning (SEM) and transmission (TEM) electron microscopy as described by Williams *et al*. (1993) and Sage and Williams (1995). SEM observations were made using a Philips 505 SEM at 2–5 kv (La B_6 filament) following gold coating. TEM observations were made using a Philips 201 transmission electron microscope. Unpollinated flowers were sampled at anthesis and 25 h post-anthesis (n = 9 at each time interval). Cross- and self-pollinated flowers were harvested at 5-h intervals for 25 h post-pollination (n = 6–10 at each sample period).

To compare quantitative growth of cross and self pollen tubes, controlled hand-pollinations were conducted in the laboratory as described by Lloyd and Wells (1992). Hand-pollinated flowers were collected at 5-h intervals for 25 h following pollination (n = 9 at each time interval) and prepared for fluorescence microscopy as described by Martin (1959). Parameters measured at each time interval included: (1) the number of germinated pollen grains; (2) the number of pollen tubes at the point of ovary entry; and (3) the number of ovules penetrated by pollen tubes. Mean pollen tube length at each time interval was determined as described by Cruzan (1986). ANOVA was performed to determine whether there were differences over time between (1) mean cross and self pollen tube length; (2) mean cross and self pollen tube density at the point of ovary entry; and (3) percentage ovule penetration by cross and self pollen tubes.

Double fertilization and early embryo/seed development following cross- and self-pollination

Flowers from field pollinated trees were harvested 3 and 15 d after pollination (n = 9–16 for each time interval), fixed in formalin–acetic acid–ethanol (FAA), dehydrated in a tertiary butyl alcohol series, embedded in Paraplast wax, sectioned at 10 μm, and stained in Heidenhain's iron-alum haematoxylin with safranin and fast green as counterstains. Serial sections were scored for double fertilization as described by Sage *et al*. (1998). Double fertilization was indicated by: (1) the absence of unfused sperm nuclei within embryo sacs penetrated by a pollen tube; (2) the presence of a resting zygote; and (3) the presence of one or more endosperm nuclei. Embryo sac development and integument development were quantified using a Zeiss Axiophot microscope equipped with image analysis software (Northern Exposure; Empix Imaging, Ontario, Canada). Mean percentage double fertilization and embryo sac length and width following cross- and self-pollination were contrasted using one-way ANOVA.

RESULTS

Structural features of the pollen tube pathway

An exudate (Fig. 1A–C) that stained positive for protein, lipids and carbohydrates covered papillate stigmatic cells. Stigmatic papillae cells contained prominent vacuoles and dense peripheral cytoplasm with mitochondria and few Golgi (Fig. 1B and C). Endoplasmic reticulum was sparse, as were the basally situated plastids. Stigmatic papillae exhibited cell wall ingrowths (Fig. 1B). Inclusions similar in fine-structure to some components of the extracellular exudate were localized within the cell wall (Fig. 1B and C). Although the winteraceous carpel is style-less, papillate carpellary cleft cells and surrounding ground tissue cells between the stigma and placenta formed a core of cells resembling a solid style, hereafter referred to as sub-stigmatic transmitting tissue. Exudate, similar in histochemistry and fine structure to that of the stigmatic papillate cells, was apparent between appressed papillate adaxial epidermal cells forming the carpel cleft (Fig. 1D). Branched plasmodesmata (secondary plasmodesmata; Lucas *et al*., 1993) were common between cleft cells (Fig. 1E). Cytoplasm of cells comprising sub-stigmatic transmitting tissue contained prominent vacuoles and plastids, ribosomes (Fig. 1F and G) and short profiles of rough endoplasmic reticulum (RER). A granular substance filled the periplasmic space, and fibril strands were located within the vacuoles (Fig. 1F and G). Exudate was apparent in the extracellular matrix (Fig. 1G). This exudate was histochemically positive for proteins, carbohydrates, lipids and arabinogalactans/arabinogalactan proteins (AG/AGPs).

Placental transmitting cells contained protoplasts with prominent nuclei, numerous plastids, mitochondria (Fig. 2A and B) and ribosomes. RER was also present. Vesicles and electron-dense droplets were apparent within the cell wall and periplasmic space adjacent to an invaginate plasmalemma (Fig. 2A and B). A heterogeneous exudate was apparent next to the placental epidermis (Fig. 2A and B) and within the micropyle of the bitegmic, anatropous ovules (Fig. 2C and D). This secretion stained positively for proteins, carbohydrates, lipids and AG/AGPs. Each pollinated ovule of *P. axillaris* contained a seven-celled embryo sac at anthesis. Mean median embryo sac length was 103·19 ± 7·25 μm (n = 8) and mean median embryo sac width was 57·08 ± 6·58 μm (n = 8).

Pollen–carpel interactions following cross- and self-pollinations

No structural differences were observed in pollen tube growth following cross- and self-pollination. The bicellular pollen grain within the coherent tetrad was covered with a

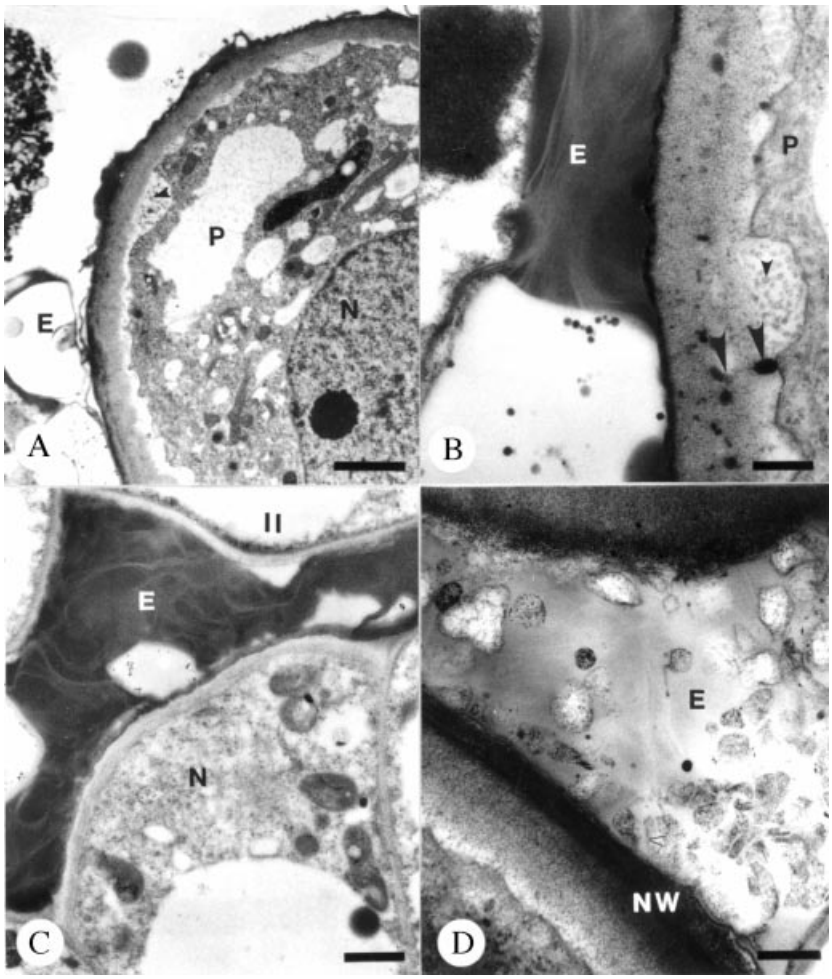

FIG. 2. Unpollinated transmitting tissue and associated exudate of placentae and micropyle of *Pseudowintera axillaris* at anthesis. A and B, Placental epidermal cells. Note vesiculate inclusions in periplasmic space (small arrowheads) and osmiophilic globules grading from periplasmic space into the cell wall (large arrowheads). Bars = 2 μm (A) and 0·05 μm (B). C and D, Micropylar exudate. Bars = 1 μm (C) and 0·03 μm (D). E, Exudate; II, inner integument; N, nucellus; NW, nucellar cell wall; P, placental epidermal cell.

proteinaceous coat (Fig. 3A) that formed an adhesion pad with the stigmatic papillae and intervening exudate (Fig. 3B). Pollen grains of tetrads germinated in the stigmatic crest secretion (Fig. 3C) and pollen tubes subsequently grew intercellularly between stigmatic papillae (Fig. 3D) and between cells of the sub-stigmatic transmitting tissue (Fig. 3E). Upon entry into the ovary locule, tubes tracked along placental epithelial cells (Fig. 4A and B), entered the micropyle (Fig. 4B–E) and subsequently penetrated one synergid of the embryo sac (Fig. 4E). Pollen tubes developed a thin callosic wall during the first 10 h of growth. A ladder-like array of callosic plugs was present by 15 h in sub-stigmatic transmitting tissue. Callose plugs were never observed in tubes in the ovary during the initial 25 h of growth.

No significant differences were observed between cross and self pollen tube length during the 25 h of growth (d.f. = 4,122, $F = 1{\cdot}281$, $P = 0{\cdot}285$). Cross- and self-pollen tubes exhibited biphasic growth (Fig. 5). Furthermore, no significant differences were observed in pollen tube number at the site of ovary entry (Fig. 6A; d.f. = 4,122, $F = 3{\cdot}223$, $P = 0{\cdot}887$) or the percentage of ovule penetration by cross and self pollen tubes during the 25 h of growth (Fig. 6B; d.f. = 4,122, $F = 1{\cdot}126$, $P = 0{\cdot}347$). All ovules appeared receptive at the time of pollen tube entry as indicated by the presence of a seven-celled embryo sac and micropylar secretions.

Double fertilization and early seed development following cross- and self-pollination

There was no significant difference in the frequency at which double fertilization occurred following cross- and self-pollination (cross-pollination, 84·56 ± 3·04 %; self-pollination, 80·00 ± 6·18 %; d.f. = 1,108, $F = 0{\cdot}439$, $P = 0{\cdot}513$). Double fertilization occurred within 3 d following pollination, as indicated by the presence of a zygote (Fig. 7A) and primary endosperm nucleus (Fig. 7B). The first primary endosperm division occurred by 15 d post-pollination (Fig. 7C). Fifteen days after pollination, mean median embryo sac (ES) length was significantly greater following cross-pollination (128·30 ± 3·08 μm) than

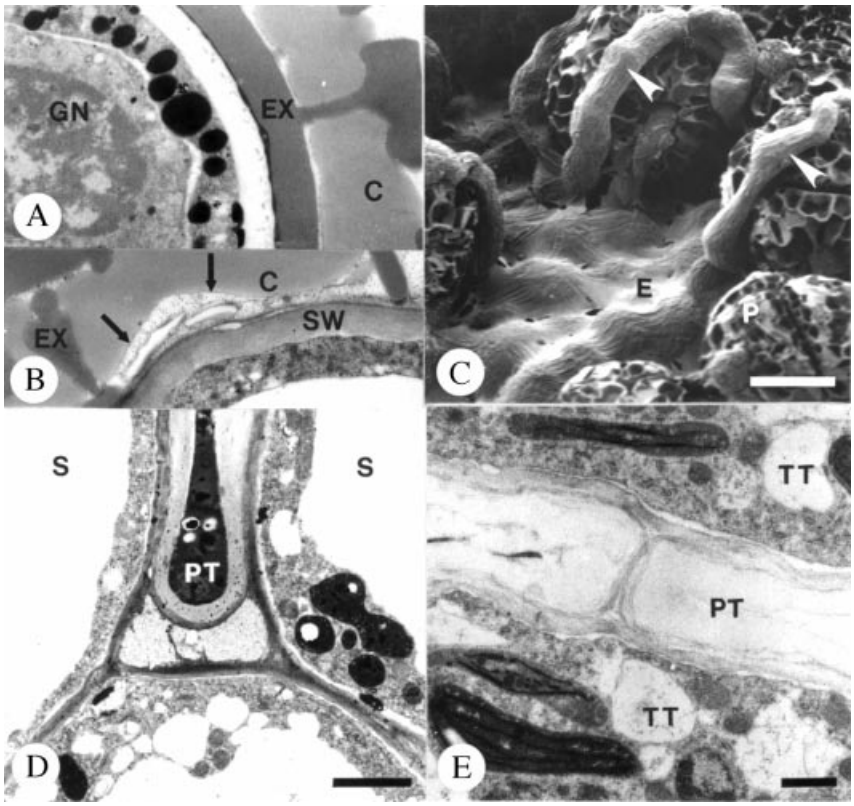

FIG. 3. Pollen tube growth on the stigma and in sub-stigmatic transmitting tissue of *Pseudowintera axillaris*. A, Pollen coat of bicellular pollen grain. Bar = 1 μm. B, Adhesion zone between pollen grain and exudate of stigma papilla. Arrows denote the boundary. Bar = 3 μm. C, Germinating pollen tubes (arrowheads) on stigma. Bar = 10 μm. D, Pollen tube growing between stigmatic papillae towards sub-stigmatic transmitting tissue. Bar = 2 μm. E, Pollen tube in solid sub-stigmatic transmitting tissue. Bar = 1 μm. C, Pollen coat; E, exudate; EX, exine; GN, generative cell nucleus; P, pollen grain; PT, pollen tube; S, stigma papilla cells; SW, stigmatic papilla cell wall; TT, cells of sub-stigmatic transmitting tissue.

following self-pollination (116·54 ± 1·99 μm) (d.f. = 1,108, $F = 11{\cdot}01$, $P < 0{\cdot}001$). Similar observations were made for median embryo sac width (cross-pollination, 71·667 ± 2·07 μm; self-pollinationl, 63·24 ± 1·98 μm; d.f. = 1,108, $F = 8{\cdot}66$, $P = 0{\cdot}004$). No differences were noted in integument structure following either pollination treatment.

DISCUSSION

It is apparent from structural observations of the present study that sterility mechanisms in *P. axillaris* are not acting to reduce self pollen tube growth prior to embryo sac entry at the nucellus, as was hypothesized by earlier workers (Norton, 1980; Lloyd and Wells, 1992). Rather, we note that double fertilization is similar following cross- and self-pollination and there are no differences between cross and self pollen tube growth. Characterization of embryo/seed development indicates that self-sterility in *P. axillaris* is manifested at the microscopic level by a significant uniform suppression in selfed embryo sac enlargement during the zygotic phase of embryogeny. We begin our discussion by placing the results of present study and of other studies on self-sterility in the Winteraceae in the context of criteria proposed to distinguish a potential post-zygotic SI from early-acting inbreeding depression. Since *P. axillaris* fulfils some of the characteristics proposed for the presence of SI, we review mechanisms that may be functioning in self recognition and rejection, focusing on some of our structural–functional observations on the pollen tube pathway in *P. axillaris*. We emphasize that although self rejection results in post-zygotic differences in embryo sac development, self recognition, as well as initial rejection, may be pre-zygotic. We conclude by addressing the possible evolutionary significance of SI in *P. axillaris* and the Winteraceae given the primitive nature of floral features of the taxon and its long fossil history.

Inbreeding depression or SI?

The most widely held views regarding SI and early-acting inbreeding depression contend that SI is a strictly pre-zygotic phenomenon and that post-zygotic self-sterility is due to early-acting inbreeding depression leading to embryonic abortion (Seavey and Bawa, 1986). However, it has been hypothesized that post-zygotic SI may be present in flowering plants, and criteria to test the presence of post-zygotic SI have been set forth (Charlesworth, 1985; Seavey and Bawa, 1986). In summary, these criteria are: (1) the timing of abortion. Uniform failure at a single stage of development may indicate SI mechanisms, whereas a continuum of failure throughout the life cycle of the new

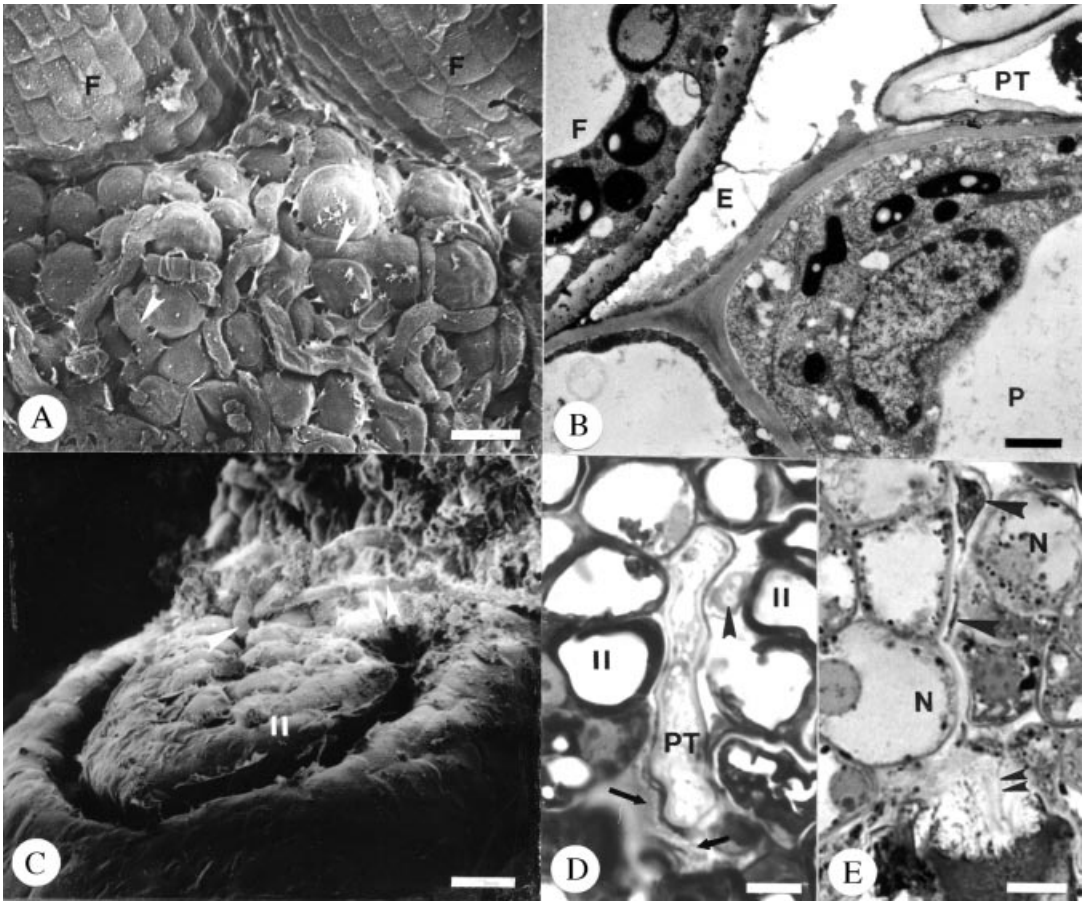

FIG. 4. Pollen tube growth in the ovary of *Pseudowintera axillaris*. A and B, Pollen tubes (arrowheads) growing between papillate epidermal cells of the placenta. Bars = 11 μm (A) and 2 μm (B). C, Arrowheads denote pollen tubes. Bar = 10 μm. D, Micropyle exudate (arrows). Arrowhead marks second pollen tube in micropyle. Bar = 2 μm. E, Intercellular growth of pollen tube in nucellus (large arrowheads) and penetrating synergid (double arrowheads). Bar = 8 μm. E, Exudate; F, funiculus; II, inner integument; N, nucellus; P, placenta; PT, pollen tube.

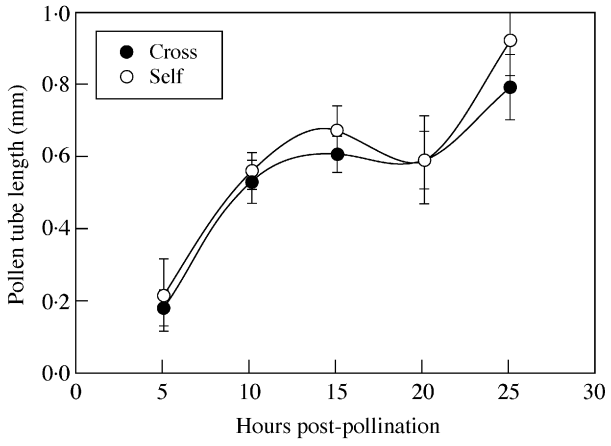

FIG. 5. Changes in cross- and self-pollen tube length over time for *Pseudowitnera axillaris*. Data are mean values ± s.d. See Results for details of the statistics.

generation would be expected if early-acting inbreeding depression was the primary cause of abortion. (2) The amount of variability in self seed set among individuals of a population. Variation in self seed set among individuals would be expected if inbreeding mechanisms are operating, whereas zero or near zero self seed set by most individuals in a genetically variable population suggests that SI is involved. (3) Dependence of abortion on the paternal *vs.* the progeny genotype. Evidence of cross-incompatibility among siblings, or parent and offspring, with a limited number of cross- compatible groups indicative of segregating alleles would be evidence for SI with a genetic basis not dependent upon progeny genotype. (4) Response of embryos to rescue in tissue culture. Embryos carrying homozygous lethal genes would not survive, whereas those aborting as a result of incompatibility might have a chance of survival if the expression of incompatibility did not interfere with their autonomous physiology. The present study and others on self-sterility in the Winteraceae address the first two criteria.

Self-sterility in *P. axillaris* appears to occur uniformly at the zygotic stage of embryogeny in the absence of differential rates of pollen tube growth, ovule penetration and double fertilization, thereby fulfilling the first criterion that differentiates SI from inbreeding depression. Similar observations have been documented for another winteraceous species, *Drimys winteri* (Sage *et al.*, 1998). Failure of development during the zygotic phase of embryogeny in the

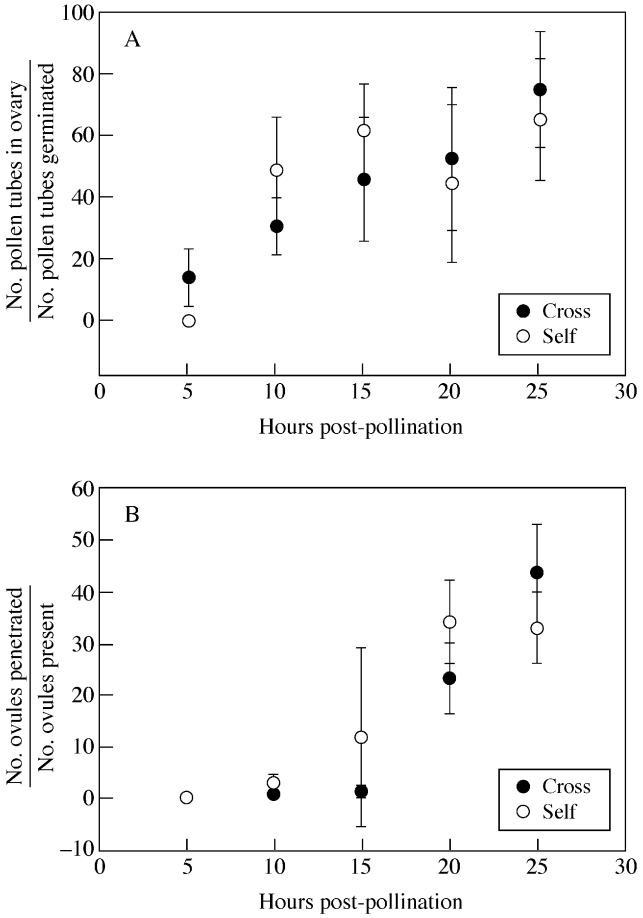

FIG. 6. Graphs illustrating pollen tube density over time at the ovary entrance (A) and ovule penetration over time (B) following cross- and self-pollination for *P. axillaris*. Data are mean values ± s.d. See Results for details of the statistics.

absence of differential cross- and self-pollen tube growth and/or ovule penetration and double fertilization rates has also been reported in other species from diverse groups of derived angiosperm families, including *Gasteria verrucosa* (Liliaceae; Sears, 1937), *Asclepias* spp. (Asclepiadaceae; Sparrow and Pearson, 1948; Sage and Williams, 1991), *Rhododendron* spp. (Ericaceae; Williams *et al*., 1984; Kaul *et al*., 1986), *Chorisia* spp. and *Tabebuia* spp. (Bombacaceae and Bignoniaceae; Gibbs and Bianchi, 1993, 1999), and *Hymenaea stigonocarpa* and *Caesalpinia* spp. (Leguminoseae; Lewis and Gibbs, 1999; Gibbs *et al*., 1999). Failure of development during the zygotic phase of embryogeny following selfing in numerous species provides increasing support for the presence of post-zygotic SI using the first criterion.

Studies examining self seed set within populations of *Pseudowintera* and other genera in the Winteraceae address the second criterion and also provide support for the action of SI. Although limited in sample size, pollination studies on individuals within populations of *P. axillaris*, *P. colorata* and *Drimys* spp. have indicated that self-pollination results in the absence of seed/fruit set [Norton, 1980; Godley and Smith, 1981; Thien, 1982; Lloyd and Wells, 1992; however, see Gottsberger *et al*. (1980) for *D. brasiliensis*]. The absence of seed/fruit production following selfing in populations of some species of the Winteraceae is in contrast to the wide range of seed set between individuals within populations of the self-sterile *Epilobium obcordatum* (Onagraceae), a species once suspected of exhibiting late-acting ovarian SI (OSI) but since demonstrated to experience early-acting inbreeding depression (Seavey and Carter, 1994). And, while the rare palaeoendemic shrub *Dedeckera eurekensis* exhibits uniformly low seed set within populations following selfing, embryonic failure occurs at a variety of developmental stages indicative of genetically mediated embryonic abnormalities (Wiens *et al*., 1989). Furthermore, cross-pollination in *D. eurekensis* does not result in significant increases in seed set over self-pollination, suggesting the presence of high segregational genetic load. In contrast to pollination studies in *D. eurekensis*, cross-pollination in *P. axillaris* and *P. colorata* results in significant increases in seed set over self-pollination (Norton, 1980; Godley and Smith, 1981). Since self-sterility in *P. axillaris* fulfils two of the criteria in support of SI, we cautiously speculate that self-sterility in this species is due to SI rather than early-acting inbreeding depression.

Mechanisms of OSI

A variety of mechanisms have been proposed for species exhibiting late-acting OSI following double fertilization (Seavey and Bawa, 1986; Gibbs and Bianchi, 1993; Sage *et al*., 1994, 2000). Two major points focus on the timing of recognition with respect to rejection, and the sites of recognition and rejection. Although it is possible to posit that both self recognition and rejection function post-zygotically in OSI, an alternative mechanism supports a pre-zygotic recognition of self and is thus consistent with more traditional views on temporal aspects of SI. In contrast to commonly cited SI systems whereby recognition results in failure of pollen tube growth at the stigma or style (de Nettancourt, 1997), rejection reactions in some OSI systems are hypothesized to result in the modification of one or more post-pollination stimulatory functions of pollen tube growth on carpel tissue development. Recognition and rejection may be temporally and spatially separated. The latter concept is supported by studies on *Gasteria verrucosa* (Sears, 1937), *Theobroma cacao* (Cope, 1962) and *Asclepias exaltata* (Sage and Williams, 1991). In all of these species, integument growth fails to proceed normally after self pollen tubes enter ovules. This led Sears (1937) to propose that SI reactions involved pollen tube–integument interactions. He suggested that interaction of compatible pollen tubes with integuments might be important for stimulation of normal seed development. Post-pollination stimulation of carpel tissue development following cross-pollination is well documented in flowering plants (Pimienta and Polito, 1983; Gilissen and Hoekstra, 1984; Singh *et al*., 1992; Sekhar and Heij, 1995; O'Neill, 1997; Pontieri and Sage, 1999). Significantly, with respect to post-pollination signalling following selfing in an OSI species, Sage *et al*. (1999) reported that self-sterility in *Narcissus triandrus* resulted from a pre-zygotic failure in embryo sac

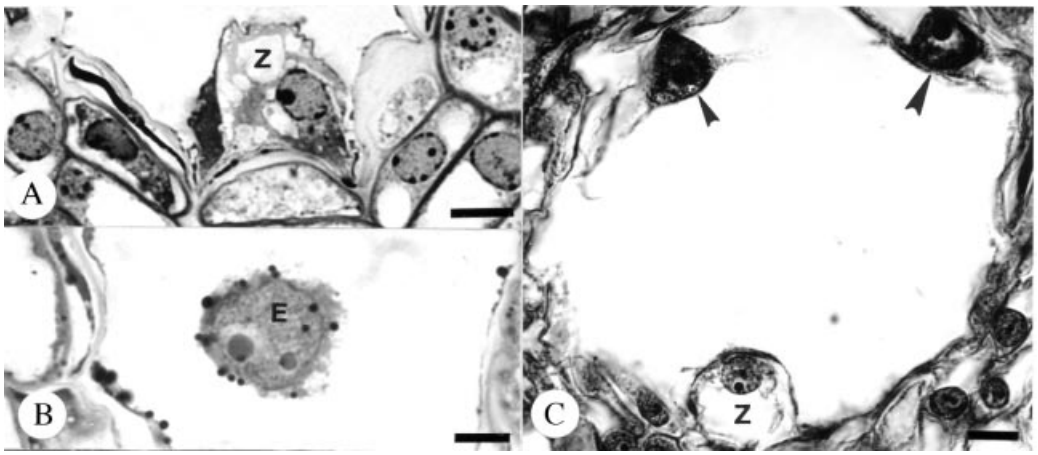

FIG. 7. Embryo sac contents after double fertilization following self-pollination in *Pseudowintera axillaris*. A, Zygote, 3 d post-pollination. Bar = 7·5 μm. B, Primary endosperm nucleus, 3 d post-pollination. Bar = 7·5 μm. C, Zygote and endosperm nuclei (arrowheads), 15 d post-pollination. Bar = 10 μm. E, Primary endosperm nucleus; Z, zygote.

development (Sage *et al*., 1999). Cross-pollination in *N. triandrus* stimulated ovule development, and hence ovule receptivity. A similar induction was not observed following self-pollination, even though self pollen tubes exhibited the same growth characteristics as cross pollen tubes. In *N. triandrus*, recognition and rejection are pre-zygotic and rejection reactions are manifested in the ovary while pollen tubes are still in the style. Similar phenomena may also be functioning in *Ipomopsis aggregata* (Waser and Price, 1991).

Although the present study does not provide data for or against a pre-zygotic self recognition affecting embryo sac development in *P. axillaris*, it does provide researchers with the structural framework for elucidation of the biology of a potential pre-zygotic self recognition and rejection. Notably, we report the presence of extracellular matrix components that may function in recognition of self pollen. We show: (1) the presence of a pollen coat that forms an adhesive zone or foot with stigmatic secretions, and (2) the presence of transmitting tract secretions in the micropyle. Pollen coat in angiosperms contains compounds essential for adhesion, recognition, hydration and germination (Dickinson, 1995; Taylor and Hepler, 1997; Wolters-Arts *et al*., 1998). Although the pollen coat has been demonstrated to contain the S-gene products in *Brassica* (SSI with dry-type stigmas; Schopfer *et al*., 1999), Willemse (1996) speculates that in the OSI species *Gasteria verrucosa* it may influence ovule receptivity, and hence play a role in self recognition. Micropylar secretions have been implicated in pollen tube guidance phenomena in flowering plants (Knox, 1984; Sanders and Lord, 1992; Hülskamp *et al*., 1995; Cheung, 1997; Lennon *et al*., 1998) and have been documented to occur in a number of phylogenetically older and younger taxa (for a review, see Pontieri and Sage, 1999). Significantly, with respect to OSI species, it has been suggested that micropylar secretions may contain S-gene products (Sage *et al*., 1994). Confirmation of the role of pollen coat and micropylar secretions in temporal and spatial aspects of recognition and rejection of self in *P. axillaris* awaits further study.

In addition to the structural observations on components of the extracellular matrix of pollen grains and micropyle, we also note that pollen tubes of *P. axillaris* penetrate and grow in a solid transmitting tract en route to the ovary after stigmatic germination. The solid core of transmitting tissue encompasses ground tissue as well as marginal cleft cells that are post-genitally fused, as indicated by the presence of branched ('secondary'; Lucas *et al*., 1993) plasmodesmata. These results are significant since they suggest additional sites for pollen–carpel interactions that may be important in self *vs*. cross recognition. Similar growth patterns of pollen tubes in a solid transmitting tract en route to the ovary have been reported for another genus within the Winteraceae, *Drimys winteri* (Sage *et al*., 1998), the primitive angiosperm *Saururus cernuus* (Pontieri and Sage, 1997, 1999) and *Nymphaea capensis* (Orban and Bouharmount, 1995) of the Nymphaeaceae, a member of the basal ANITA clade (Qiu *et al*., 1999; Graham and Olmstead, 2000). Growth of pollen tubes following stigmatic germination within a solid transmitting tract in basal angiosperms may be important in the evolution of SI (see below) and other pollen–carpel signalling phenomena, and may have important implications for the reconstruction of the early evolution of the pollen tube pathway, a topic of long-standing interest (Bailey and Swamy, 1951; Lloyd and Wells, 1992; Bell, 1995; Endress and Igersheim, 2000).

Evolutionary relationships of OSI and other forms of SI

Recent molecular data support the hypothesis that SI has evolved on multiple occasions throughout the history of flowering plant evolution (Matton *et al*., 1994; Franklin *et al*., 1995; de Nettancourt, 1997). However, the timing of evolution of SI with respect to angiosperm origins remains an unanswered question, as does the nature of primitive SI mechanisms and site-specific origins of SI (Whitehouse, 1950; Bernhardt and Thien, 1987; Olmstead, 1989; Sage *et al*., 1994, 2000; Read *et al*., 1995; Weller *et al*., 1995; Weller and Sakai, 1999). Both stylar gametophytic SI (GSI) and stigmatic sporophytic SI (SSI) have been implicated as

the first SI mechanisms to evolve (Zavada, 1984, 1990; Gibbs, 1986, 1991; Bernhardt and Thien, 1987). An intriguing hypothesis is that primitive SI mechanisms may have been some form of OSI (Kenrick *et al*., 1986; Barrett, 1988; Lloyd and Wells, 1992). Kenrick *et al*. (1986) hypothesized that SI mechanisms may have evolved and been operative on the adaxial surface of an open carpel in association with any structure which at the time was functionally important in pollen germination and transmission of the male gametophyte to the female gametophyte. Bell (1995) noted that SI reactions evolved in association with penetration of pollen tubes into ground tissue as well as with closure of the carpel.

The present study on *P. axillaris* and a recent study on self-sterility in another winteraceous species, *Drimys winteri* (Sage *et al*., 1998), suggest the possible presence of OSI in a relictual flowering plant family. Given the primitive standing of Winteraceae, we support the ideas of Kenrick *et al*. (1986) and Barrett (1988) that some form of OSI may have been functional during the early stages of angiosperm evolution. However, we do not conclude that OSI was the only self recognition and rejection mechanism operating during the early evolution of angiosperms. Reports of a potential stigmatic SI in *Austrobaileya scandens* (Prakash and Alexander, 1984) and *Trimenia moorei* (T. L. Sage, pers. comm.), both woody members of the basal ANITA grade (Qiu *et al*., 1999; Graham and Olmstead, 2000), and stigmatic SI in the Saururaceae (Pontieri and Sage, 1997, 1999) indicate that angiosperms may have rapidly co-opted many forms of SI. This scenario is consistent with multiple origins of SI and maintains the importance of SI as a player in the early success of angiosperms.

ACKNOWLEDGEMENTS

This research was funded by a grant from the Natural Sciences and Engineering Research Council of Canada to T.L.S. and a grant awarded to F.B.S. from Victoria University of Wellington. The authors thank Len Thien and Shirley Tucker for helpful comments on the manuscript, and Bill Cole for technical assistance. This manuscript is dedicated to the memory of Professor Hugh D. Gordon, Head of Botany 1947–1977, Victoria University of Wellington, New Zealand.

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