ANNALS OF
BOTANY
Founded 1887

Arabinogalactan-protein secretion is associated with the acquisition of stigmatic receptivity in the apple flower

Juan M. Losada* and María Herrero

Pomology Department, Aula Dei Experimental Station – CSIC, Zaragoza 50080, Spain
* For correspondence: E-mail juanlosada@eead.csic.es

• *Background and Aims* Stigmatic receptivity plays a clear role in pollination dynamics; however, little is known about the factors that confer to a stigma the competence to be receptive for the germination of pollen grains. In this work, a developmental approach is used to evaluate the acquisition of stigmatic receptivity and its relationship with a possible change in arabinogalactan-proteins (AGPs).
• *Methods* Flowers of the domestic apple, *Malus × domestica*, were assessed for their capacity to support pollen germination at different developmental stages. Stigmas from these same stages were characterized morphologically and different AGP epitopes detected by immunocytochemistry.
• *Key Results* Acquisition of stigmatic receptivity and the secretion of classical AGPs from stigmatic cells occurred concurrently and following the same spatial distribution. While in unpollinated stigmas AGPs appeared unaltered, in cross-pollinated stigmas AGPs epitopes vanished as pollen tubes passed by.
• *Conclusions* The concurrent secretion of AGPs with the acquisition of stigmatic receptivity, together with the differential response in unpollinated and cross-pollinated pistils point out a role of AGPs in supporting pollen tube germination and strongly suggest that secretion of AGPs is associated with the acquisition of stigma receptivity.

Key words: AGPs, arabinogalactan proteins, apple, *Malus × domestica*, pollen, pollen tube, stigma, stigmatic receptivity, flower receptivity.

INTRODUCTION

Flower receptivity plays a crucial part in pollination dynamics, reproductive success and plant productivity. The stigma is the first surface of the pistil to make contact with the pollen grains, and pollen grain adhesion on the stigma is followed by pollen hydration and germination (Lord and Russell, 2002; Hiscock and Allen, 2008). Stigmatic receptivity is the temporal interval in which the stigma provides an adequate environment for pollen germination (González *et al.* 1995a; Sanzol *et al.*, 2003), and this parameter has significant biological implications. In dichogamous species it has a clear role separating the female and male phase and encouraging out-breeding (Lora *et al.*, 2011). Also, in hermaphrodite flowers, a late acquisition of stigmatic receptivity favours pollen gathering in the stigma prior to pollen germination, and encourages pollen competition (Hormaza and Herrero, 1992). Finally, stigmatic receptivity is susceptible to temperature fluctuations (Hedhly *et al.*, 2003, 2005; Lora *et al.*, 2011), and is a clear candidate vulnerable to climate change (Hedhly *et al.*, 2009; Hedhly, 2011), since a short period of stigmatic receptivity may hinder crop production (Sanzol *et al.*, 2003). But in spite of the widespread biological implications of stigmatic receptivity, very little is known about what confers on a stigma the capability to be receptive for pollen grain germination.

Stigmatic receptivity is evaluated in an indirect way by pollen-germination ability, the capacity to promote fertilization, or some enzymatic activities (Edlund *et al.*, 2004). Attention has been focused on the nature of stigmatic secretions and their genetic control (Verhoeven *et al.*, 2005), and an interaction between stigmatic secretions and pollen tubes has been shown (Wolters-Arts *et al.*, 1998; Inamura *et al.*, 2007), opening a search for the components of the stigmatic secretion that may play a role in stigmatic receptivity. At maturity stigmas have high levels of esterase (Hiscock *et al.*, 1994, 2002) and also peroxidase activity (Dupuis and Dumas, 1990; McInnis *et al.*, 2006b).

The association of peroxidase activity with stigmatic receptivity prompted an investigation of the activity of reactive oxygen species and reactive nitrogen species in stigmas and pollen, which revealed that receptive stigmas accumulate high levels of reactive oxygen species, particularly hydrogen peroxide, suggesting a possible role in stigma receptivity (McInnis *et al.*, 2006 *a*, *b*; Hiscock and Allen, 2008). Some glycoproteins such as arabinogalactan-proteins (AGPs) (Quiapim *et al.*, 2009) are also potential candidates for markers of stigma receptivity, pointing to a co-ordinated cell response on the stigma; however, evidence of their biological roles remains elusive.

AGPs are a superfamily of highly glycosylated hydroxyproline-rich glycoproteins which are constituents of cell walls of many flowering plants in different tissues of every taxonomic group studied (Clarke *et al.*, 1979; Seifert and Roberts, 2007), from some early angiosperms (Prychid

et al., 2011) to eudicots (Vaughn *et al.*, 2007). They are classified into two main groups (classical or non-classical AGPs), depending on the presence or absence of a hydrophobic glycosylphosphatidilinositol-lipid membrane anchor at the C terminus. This glycosylphosphatidilinositol anchor makes AGPs capable of being secretory molecules involved in signalling (Ellis *et al.*, 2010). While the Hyp-rich protein backbone is fairly well conserved, the complex carbohydrate moiety that occupies 90 % of the molecule varies between AGPs types and determines their biological function (Ellis *et al.*, 2010). AGPs participate in several physiological processes in plant growth and development of both monocots (Ma and Zhao, 2010) and dicots (Majewska-Sawka and Nothnagel, 2000; Showalter, 2001; Seifert and Roberts, 2007), as cell expansion and proliferation (Yang *et al.*, 2007), programmed cell death and xylem differentiation (Zhang *et al.*, 2003; Motose *et al.*, 2004), or somatic embryogenesis (van Hengel *et al.*, 2002).

In reproductive tissues, the expression of AGPs is associated with the sporophyte–gametophyte transition (Acosta-García and Vielle-Calzada, 2004), or components of pollen-tube wall elongation (Wu *et al.*, 2001; Pereira *et al.*, 2006; Coimbra *et al.*, 2010). Several AGP-like molecules have also been reported along different pistil structures of lily (Kim *et al.*, 2003), tobacco (Wu *et al.*, 2000; Hancock *et al.*, 2005), and arabidopsis (Lee *et al.*, 2009; Coimbra *et al.*, 2010). Their abundance and generalized presence along the pistil makes AGPs clear candidates for pollen–pistil interactions, but their possible function remains to be deciphered. The difficulty in unveiling the role of AGPs is partly due to the fact that it is difficult to find a system where an on/off function can be found. In this sense, the stigma that becomes receptive at a particular stage of development offers the opportunity to look for the possible implications of AGPs on pollen germination.

This work investigates the possible role of AGPs on the acquisition of stigmatic receptivity in the flowers of apple (*Malus × domestica*). There has been extensive research on agronomic properties of apple, but there is relatively little information on the reproductive process. The anatomy of the pistil (Cresti *et al.*, 1980) and gynoecial structure (Sheffield *et al.*, 2005) have been examined. Some aspects referring to flower receptivity and fruit set have further been considered (Williams *et al.*, 1984; Buszard and Schwabe, 1995; Sheffield *et al.*, 2005). Apple flowers have a wet stigma (Heslop-Harrison and Shivanna, 1977), but the nature of secretions contributing to the acquisition of stigmatic receptivity is lacking. To gain insight into the key steps of stigmatic receptivity competency, in this work we combined a developmental approach to evaluate the functional capacity of the stigmas, with detailed anatomical observations and the cytoimmunodetection of AGP epitopes.

MATERIALS AND METHODS

Plant materials

Flowers from apple trees *Malus × domestica,* Borkh 'Golden Delicious Spur' grown in the province of Huesca (Spain), 461 m a.s.l. were used in this study. To evaluate at which developmental stage the stigma acquires the competence to promote pollen grain germination, flowers from different phenological stages were emasculated and placed in damp florist foam for hand pollinations. Five phenological stages were chosen (Fig. 1A).

Since apples are self-incompatible, anthers from the compatible 'Royal Gala' were collected from flowers at an advanced balloon stage and left to dry on a paper at room temperature around 20 °C for 24–48 h until dehiscence. Pollen was sieved with a mesh with a diameter pore of 0·26 mm and then stored at −20 °C until required.

Pollination experiments

Stigmatic receptivity was evaluated through the capacity of stigmas to offer support for pollen germination and pollen tube growth (González *et al.*, 1995b). Following hand pollinations with a paintbrush, six flowers – 30 gynoecia – from each phenological stage were sampled 1 d after pollination and fixed in FAA (formalin : acetic acid : 70 % ethanol) (1 : 1 : 18) (Johansen, 1940). Once fixed, the pistils were washed three times in distilled water for 1 h each, and left in 5 % sodium sulfite for 24 h. Samples were autoclaved for 10 min at 1 kg cm^{-2} in 5 % sodium sulfite (Jefferies and Belcher, 1974), and then the individual styles were squashed onto glass slides with 0·1 % aniline blue in 0·1 N K_3PO_4 to visualize callose (Currier, 1957) and pollen tubes (Linskens and Esser, 1957) with a LEICA DM2500 fluorescence microscope with 340/400-nm filter. The percentage of stigmas bearing either adhered or germinated pollen grains was subjected to a chi-square homogeneity test at $P \leq 0.05$. Then the mean number of adhered and germinated pollen grain/tubes on the stigma was compared by one-way ANOVA, and groups separated by the Duncan multiple-range test at $P \leq 0.05$. Statistical analysis was performed with the SPSS software (SPSS Inc., Chicago, IL, USA).

Histochemical preparations

Flowers for histochemical examination were selected according to the stigmatic receptivity results. Pistils from three phenological stages (3, 4 and 5) were fixed in 2·5 % glutaraldehyde in 0·03 M saline phosphate buffer pH 7·3 for 4 h (Sabatini *et al.*, 1963). Then the gynoecia were washed in 0·03 M saline phosphate buffer and sequentially dehydrated in an ethanol series (30 %, 50 %, 70 %, and 96 %) leaving them for 1 h in each ethanol concentration. The pistils were left for 5 d in the embedding solution at 4 °C, and then embedded in JB4 plastic resin (0226A; Polysciences Inc.). Sections, 2 μm thick, were cut on a LEICA EM UC6 ultramicrotome with a glass knife and then placed onto distilled water on a glass slide previously coated with 1 % gelatine.

For general structure and presence of polyphenols, 0·02 % toluidine blue in water was used (Feder and O'Brien, 1968). Insoluble polysaccharides were stained with periodic acid Shiff reagent (Feder and O'Brien, 1968). Cellulose was stained with 0·07 % calcofluor white (Hughes and McCully, 1975) and cutin and lipids with 0·01 % auramine in 0·05 M phosphate buffer, pH 7·8 (Heslop-Harrison, 1977). General structure and secretions were observed following staining with 0·01 % acridine orange in 0·03 % phosphate buffer, pH
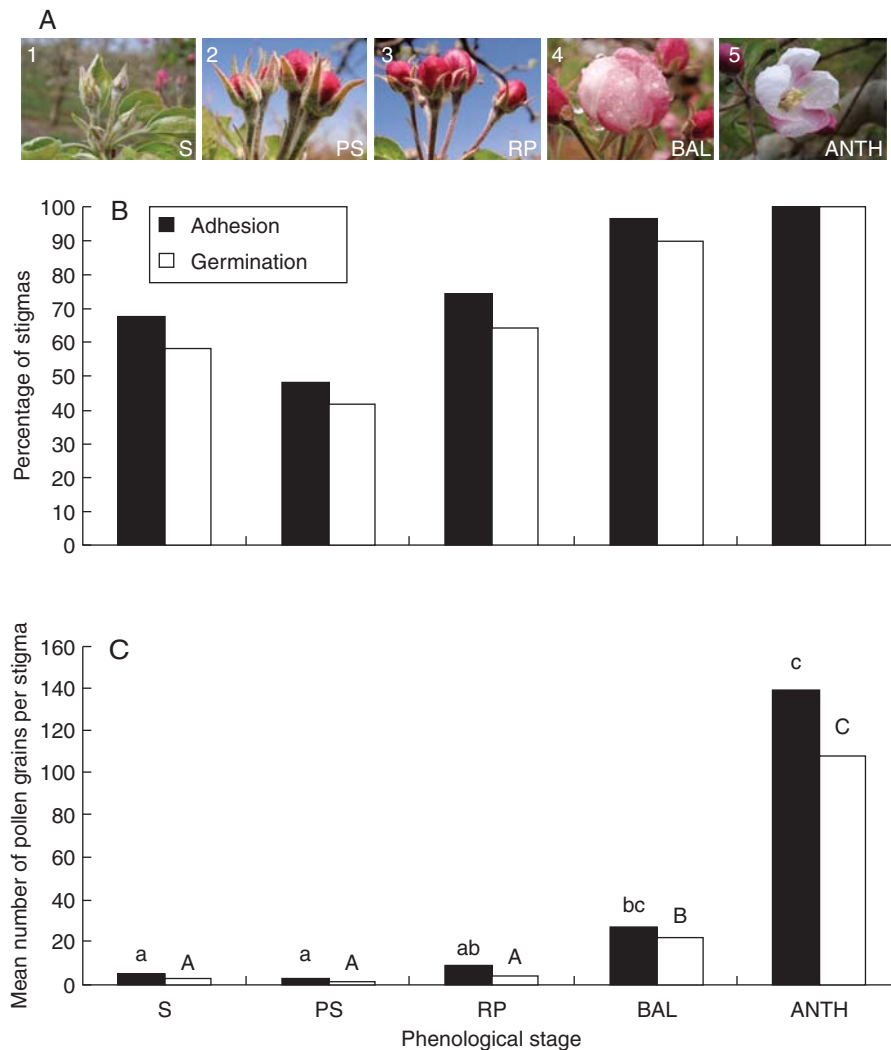
FIG. 1. Evaluation of stigmatic receptivity in different apple-flower phenological stages. (A) Flower phenology: (1) petals enclosed by sepals (S); (2) petals show between sepals (PS); (3) petals protruding and showing red colour – red-petal stage (RP); (4) balloon stage (BAL) where petals are pink; and (5) anthesis (ANTH). (B) Percentage of stigmas supporting at least one pollen grain adhered and germinated (as indicated) in each phenological stage. (C) Mean number of adhered and germinated pollen grains per stigma on each phenological stage, showing that only stages close to flower opening reached a high number of adhered and germinated pollen grains. Letters over the columns show differences at a $P \leq 0.05$ on either adhesion (lower-case letters) or germination (upper-case letters).

7·4 (Nicholas *et al.*, 1987). Slides were observed under bright field LEICA DM2500 microscope carrying a 100-W light source, and photographs were obtained with a Leica DFC320 camera linked to the software Leica Application Suite. Fluorescence microscopy was developed with the same device provided with an epifluorescence source and connected to a CANON Power Shot S50 camera linked to the CANON Remote Capture software. Filters used were 355/455 nm for calcofluor white, and 450/510 nm for auramine- or acridine orange-stained sections.

In situ *detection of AGPs*

To detect the presence of AGPs in fresh tissue, Yariv reagents were used, both β-D-glucosyl Yariv reagent (β-GlcYR), which specifically binds to and precipitates

AGPs, giving a red to brown colour, and α-D-galactosyl Yariv reagent (α-GalYR) as a negative control (Yariv *et al.*, 1967; Anderson *et al.*, 1977; Capataz-Tafur *et al.*, 2010). Following the manufacturer's instructions, 2 mg of Yariv powder reactive (Biosupplies, Australia) were dissolved in 1 mL of 0·15 M NaCl. After that, aliquots of 20 μL of the stock solution were dissolved in 500 μL of distilled water. While this reaction has been previously used in a biochemical context, here the presence of AGPs on the stigmatic surface was evaluated in fresh tissue. For this purpose, batches of five flowers – 25 stigmas – per phenological stage were emasculated, and the stigmas were placed in contact with the solutions for 5 h at room temperature. The presence of AGPs was detected through the presence of a red to brown colouration on the stigma, which was observed under a binocular Leica MZ16 with a light source Leica

KL1500LCD. Photographs were taken with the bright-field system mentioned above. Reaction for both stains was quantified, scoring the percentage of stigmas showing colouration on the stigma surface.

Immunolocalization of AGPs

Two monoclonal antibodies (mAbs), Jim13 (Knox *et al.*, 1991) and Jim8 (Pennell *et al.*, 1991), against AGPs glycosyl moeties were obtained from Carbosource Services (University of Georgia, USA). As a secondary antibody, Alexa 488 fluorescein isothiocyanate (FITC)-conjugated anti-rat IgG was used (F-1763; Sigma).

Gynoecia from flowers of developmental stages balloon (stage 4), anthesis (stage 5), 1 d after anthesis (1DAA), 1 d after pollination (1DAP) and 3 d after pollination (3DAP) were fixed in 4 % paraformaldehyde in phosphate-buffered saline (PBS), pH 7·3, and left overnight at 4 °C. Samples were dehydrated in an acetone series (30 %, 50 %, 70 %, 90 %, 100 %) and embedded in Technovit 8100 (Kulzerand Co., Germany) for 2 d. The resin was polymerized at 4 °C, and slices sectioned at 4 μm width. Sections were placed in a drop of water on a slide covered with 2 % (3-aminopropyl) triethoxysilane (Sigma-Aldrich) and dried at room temperature.

Sectioned samples were soaked in PBS, pH 7·3, for 5 min followed by 5 % bovine serum albumin in PBS for 5 min. Then, they were incubated at room temperature for 1 h with both primary mAbs, Jim8 and Jim13, followed by three washes in PBS. After that, samples were incubated with a 1/25 dilution of the secondary anti-rat antibody conjugated with FITC in 1 % bovine serum albumin in PBS for 45 min in the dark, followed by three washes in PBS (Solís *et al.*, 2008). Controls were performed for each sample, by incubating with the blocking solution instead of the primary antibody. For better localization of the epitopes, some sections were counterstained with calcofluor white for cellulose (Coimbra *et al.*, 2007), mounted in PBS or Mowiol and examined under a LEICA DM2500 epifluorescence microscope connected to a LEICA DFC320 camera. Filters were 355/455 nm for calcofluor white and 470/525 nm for the Alexa488 fluorescein label of the antibodies. Overlapping photographs were obtained with the Leica Application Suite software.

RESULTS

Acquisition of stigmatic receptivity

Five phenological stages where chosen from the sepals stage through to anthesis (Fig. 1A): (1) petals enclosed by sepals (S); (2) petals show between sepals (PS); (3) petals protruding and showing red colour – red-petal stage (RP); (4) balloon stage (BAL) where petals are pink; and (5) anthesis (ANTH). The style was fully formed at stage 1; however, the stylar length varied from 6 mm at this stage to 11 mm at stage 5. The different phenological stages were hand pollinated and evaluated for their ability to adhere and germinate pollen grains. Whereas in early stages (1 and 2) a proportion of the stigmas were able to support adhesion and germination of at least one pollen grain (Fig. 1B), no significant differences were recorded between them, and only advanced stages (4 and 5) showed significant differences with earlier stages 1 and 2 ($P \leq 0.05$). But the number of pollen grains adhering and germinating gave a more precise picture and a better estimate of stigmatic receptivity. The number of pollen grains adhering and germinating per stigma increased at later developmental stages (Fig. 1C). Few pollen grains (under ten) were able to germinate in the early developmental stages (1–3) compared with 40 pollen grains per stigma at stage 4, and 100 at stage 5. Significant differences were recorded in the number of adhered and germinated pollen grains between early (1–3) and later (4 and 5) stages ($P \leq 0.05$). At stage 4, germinating pollen grains were localized mainly to the outermost edges, marking the first receptive area in the stigma. By stage 5, they included the whole stigma. Therefore, the acquisition of stigmatic receptivity evaluated by pollen behaviour began at balloon stage (4), but a spatial distribution was observed and the stigmas started to be receptive at the stigmatic edges, progressing then centripetally to the inner stigma.

Developmental changes in the stigma

To evaluate the changes in the stigma associated with the acquisition of stigmatic receptivity, stigma development was characterized. A thick cuticle covered the whole stigmatic area and underlined the pistil suture line in young stigmas (Fig. 2A); below the cuticle, an incipient vacuolation was initiated at the papillae cells (Fig. 2B). With development, at stage 3, the papillae increased in size, and a large central vacuole developed, as the cuticular layer appeared thinner (Fig. 2C). At this stage, no secretion was observed in whole mounts stained with acridine orange (Fig. 2D), and papillae had a turgid appearance (Fig. 2E). Close to anthesis, at stage 4, a lipoid secretion was secreted from the stigmatoid cells located below the papillae (Fig. 2F). This secretion was clearly observed on the surface in fresh whole mounts of stigmas (Fig. 2G). Secretion release coincided with papillae loosing turgidity (Fig. 2H).

Not only papillae developed, but also the stigmatoid tissue below papillae further vacuolated at stage 3 (Fig. 3A), and the vacuolar content reacted for polyphenolic compounds showing green to blue colour when stained with toluidine blue. At stage 5, secretion increased in the intercellular spaces (Fig. 3B). Interestingly, not all cells of the stigma reacted in a similar way, and before flower opening, at stage 3, some cells located in the inter-papillae valleys did not vacuolate and kept a dense cytoplasm (Fig. 3C). These cells became autolytic with stigma maturation at stage 4 and released their contents in the stigmatic secretion (Fig. 3D). Neighbouring cells in the stigmatoid tissue further accumulated starch, which soon vanished at stage 5, as a secretion reacting for insoluble polysaccharides was apparent in the intercellular spaces (Fig. 3E). As a consequence, the apple stigma was covered at anthesis (stage 5) with a lipoid secretion on the surface and a carbohydrate-rich secretion filled the intercellular spaces of the stigmatoid tissue.
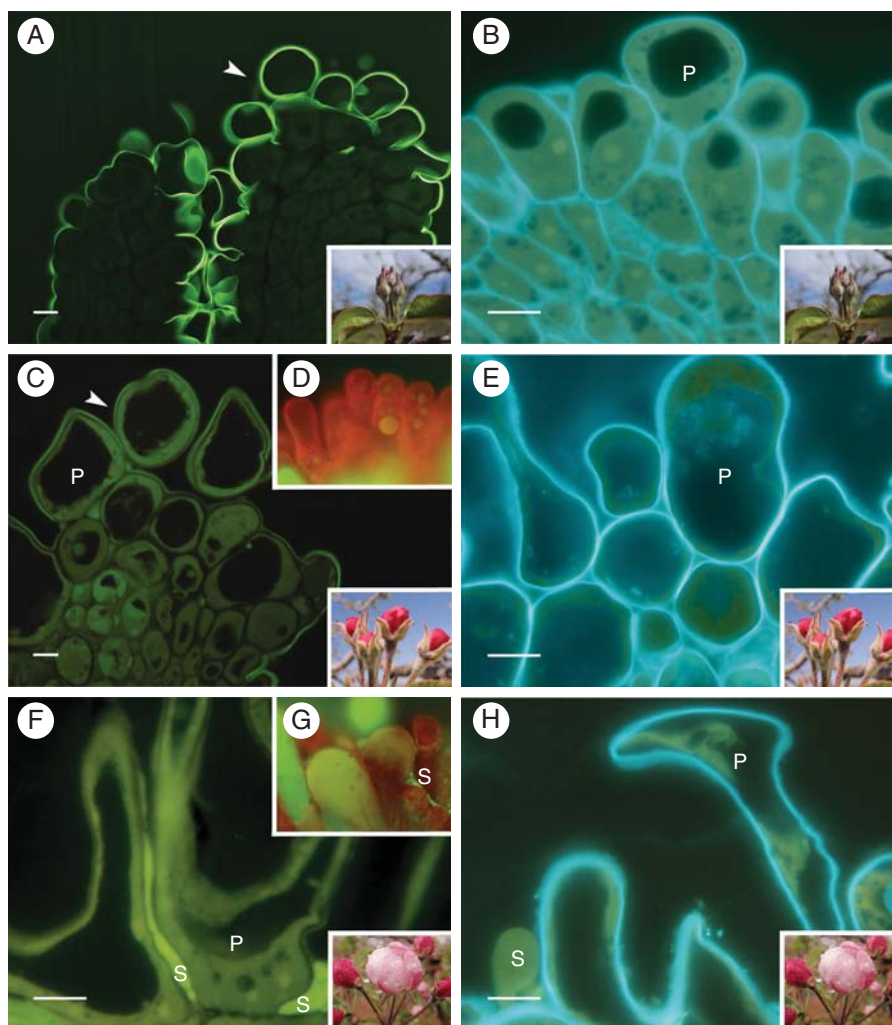
FIG. 2. Papillae development in the apple flower stigma: (A) thick cuticle layer (arrowhead) covering the stigma and suture line along the short style at stage 1; (B) undifferentiated stigma at this stage showing papillae with small vacuoles; (C) stigma with a continuous cuticle layer covering papillae at stage 3; and (D) whole mounts without secretion; (E) turgid papillae after the vacuoles enlarged at stage 3; (F) lipidic secretions from stigmatoid tissue cells merged between papillae at stage 4; (G) stigma whole mount with secretions at this stage; (H) papillae loose turgidity at stage 4. (A, C, F) Auramine O staining. (B, E, H) calco-fluor white for cellulose; 2-μm sections embedded in JB4 resin; (D, G) acridine orange of squashed preparations. Abbreviations: p, papilla; s, secretions. Scale bars = 10 μm.

AGPs in the stigma

AGPs were monitored in fresh tissues by staining with the specific β-GlcYR (Fig. 4), that shows the presence of AGPs with an intense brown colour. As suggested by the manufacturer, α-GalYR, which stains for polysaccharides other than AGPs, was used as a negative control. Albeit the presence of polysaccharides other than AGPs could be observed in a small proportion of stigmas, a sequential reaction was conspicuous for AGPs with development. While no colour reaction was observed at early stage 1 (Fig. 4A), AGPs were first detected at the stigmatic edges at stage 3 (Fig. 4B). As the stigma matures, the reaction to colour of the AGPs increases in intensity and is widespread in the area covered (Fig. 4C). At anthesis, AGPs were localized across the entire stigma (Fig. 4D). Within a particular developmental stage, variability was observed between stigmas in their reaction, and the percentage of reactive stigmas was quantified. Although few reactive stigmas could be seen at the early phenological stage (Fig. 4E), about 50 % of the stigmas were positive for AGPs at stages 3 and 4. At stage 5, close to 100 % of the stigmas were positive for AGPs. Additionally, at this stage, when the stigmas were removed from the solution, a precipitate could be seen, which could result from leaking of soluble AGPs into the solution.

Since the staining with the β-GlcYR reacts with all AGPs, two mAbs, Jim8 that localizes an unknown epitope in the glycan side and Jim13 against the epitope βGlcA1 → 3αGalA1 → 2Rha (Knox *et al.*, 1991), were used to specifically detect them. Both antibodies have been used previously in different tissues of other species without distinction. While in negative controls no labelling was detected, both antibodies were constantly labelling cell walls of the stylar cortical tissue in all phenological stages studied (Fig. 5). In contrast,
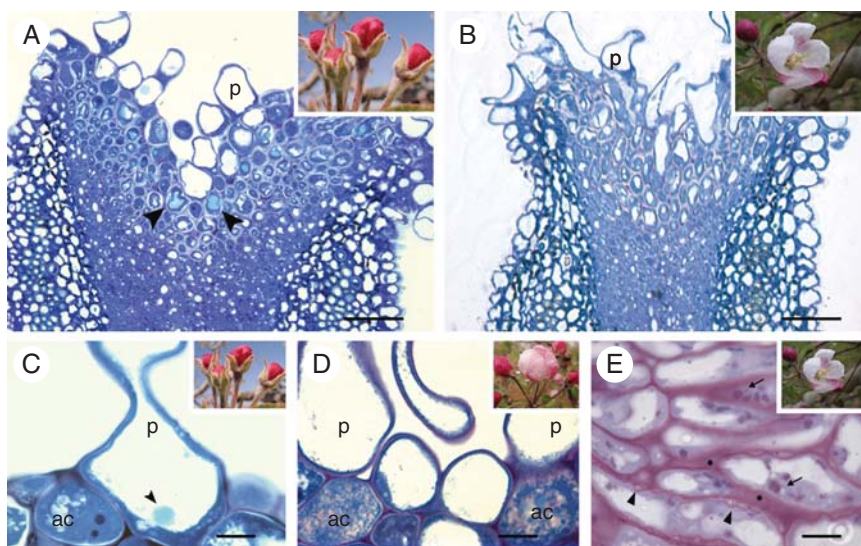
Fɪɢ. 3. Changes in the stigmatoid tissue with development in the apple flower stigma: (A) general view of the stigma at stage 3 showing a vacuolar reaction for polyphenolic compounds (arrowheads) in the cells underneath papillae; (B) at stage 5, stigmatoid cells without phenolic compounds, as the intercellular substance appeared in the stigmatoid tissue; (C) phenolic bodies were conspicuous within papillar vacuoles (arrowhead) at stage 3 (autolytic cell at the interpapillar valley with a dense cytoplasm); (D) autolytic cell degeneration at stage 4; (E) at stage 5, stigmatoid cells contained starch (arrows) and wall ingrowths (arrowheads), as the intercellular substance (*) stained for carbohydrates. (A–D) Toluidine blue staining for general structure showing metachromasia. (E) periodic acid Shiff reagent staining for insoluble carbohydrates. 2-μm sections embedded in JB4 resin. Abbreviations: ac, autolythic cells; p, papilla. Scale bars: (A, B) = 50 μm; (C–E) = 10 μm.

the stigmatic cells showed different staining with development, and at early phenological stages there was no epitope detection in the stigma of either antibody (Fig. 5A, B). The signal for Jim8 epitopes appeared at stage 4, but just at the papillae base (Fig. 5C). But epitopes recognized by Jim13 mAb were located more conspicuously in the stigmatoid tissue (Fig. 5D) and, although mainly inside the cells, they started to appear in the intercellular spaces of the stigmatoid tissue at the stigmatic edges, which is the area where the stigmas start to be receptive to pollen. At stage 5, while Jim8 epitopes remained inside the papillae (Fig. 5E), Jim13 epitopes continued to be secreted and conspicuously reacted in the intercellular spaces of the stigmatoid tissue at the receptive stigmatic edges (Fig. 5F). They further flooded in the stigmatic surface covering some papillae at this mature stage. Therefore localization of AGPs labelled by Jim13 was temporarily and spatially regulated along stigma development. Moreover, these Jim13-labelled AGPs were secreted at the same time and followed the same spatial distribution as the acquisition of stigmatic receptivity to pollen-grain germination.

Changes in AGPs with pollination

To seek for possible AGPs involvement on pollen tube germination and growth, unpollinated and pollinated stigmas were compared (Fig. 6). For unpollinated stigmas, 1 d after anthesis (1DAA), Jim8 epitope detection was very similar to anthesis and was confined within papillae as well as in some cell membranes (Fig. 6A, B). In contrast, Jim13 labelling increased in the whole stigmatoid tissue (Fig. 6C), with higher secretion accumulation outside the cell walls, both in the stigmatoid tissue and on the papillae (Fig. 6D).

Conspicuous changes occurred 1 d following pollination (1DAP). The epitope detected with Jim8 changed and appeared now outside of the papillae (Fig. 6E). In contrast, the epitope detected with Jim13 at this stage (Fig. 6F) showed a weaker reaction at the intercellular spaces and labelling decreased compared with that observed in unpollinated stigmas at the same stage (Fig. 6C, D).

After the passage of the pollen tube, when examining stigmas 3 d after pollination (3DAP), a reaction could be seen on the pollen grain wall and cytoplasm for the Jim8 mAb. But on the stigma, the staining just remained on cell surfaces contacting pollen grains and tubes (Fig. 6G). At this stage the Jim13 label was also much weaker in the intercellular substance of the stigmatoid tissue (Fig. 6H), and appeared to be absent close to the passage of the pollen tube.

DISCUSSION

The results described show that the acquisition of stigmatic receptivity in apple flowers was concomitant with the secretion of AGPs, which further vanished with pollination as pollen tubes grew through the stigmatoid tissue.

The onset of stigma receptivity: cell differentiation and secretions

Apple stigmas are of the wet type (Heslop-Harrison and Shivanna, 1977), and acquired the capability to support pollen germination close to flower opening, being fully receptive at anthesis. Stigmatic receptivity was acquired in a developmentally controlled way, maturing in a basipetal fashion as in other species (Herrero and Arbeloa, 1989), but our results also show a centripetal developmental sequence from the stigmatic edges to the groove of the suture line. Previous reports in
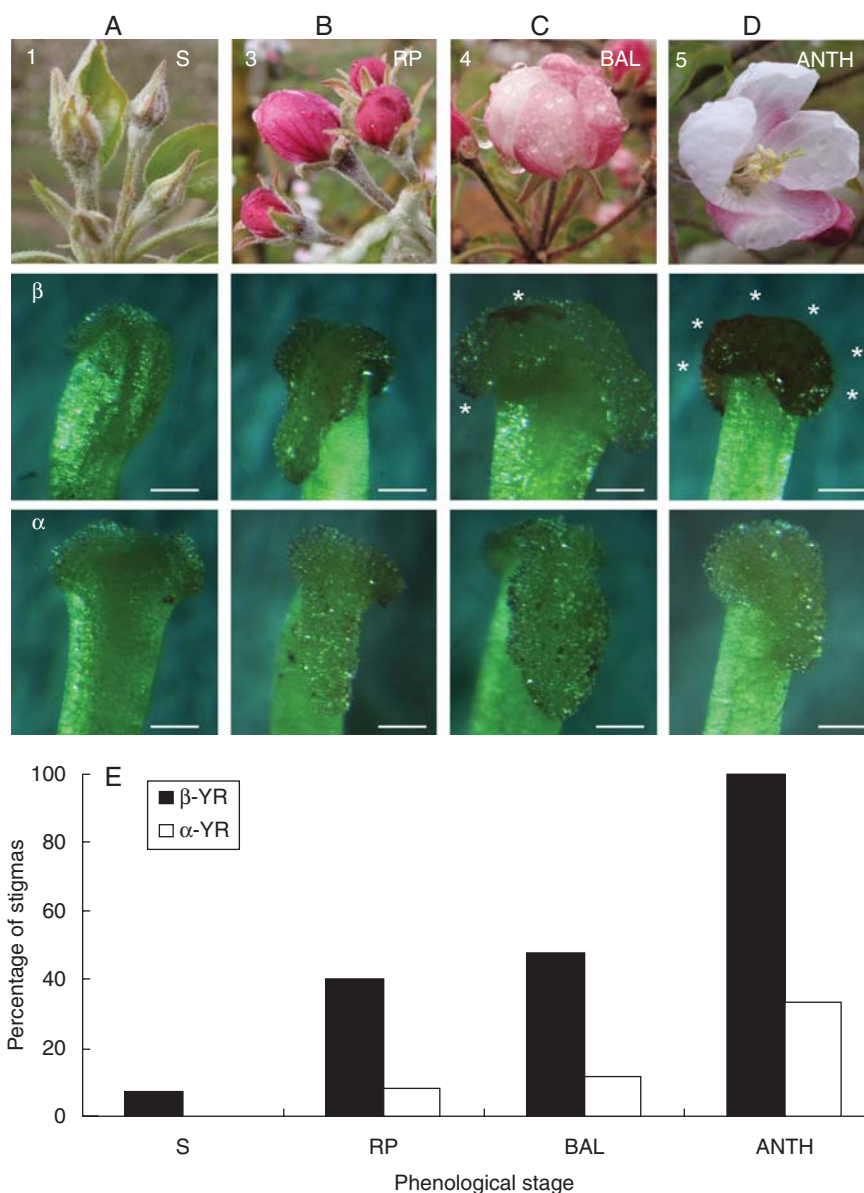
FIG. 4. *In situ* detection of AGPs on whole mounts of the apple flower stigma. Each column corresponds to a flower phenological stage. The first row shows the phenological stages, the second row shows reactions to positive β-glucosyl-Yariv reagent (β-GlcYR) indicating the presence of AGPs, and the third row shows the negative control using α-D-galactosyl-Yariv reagent (α-GalYR). While no reaction appeared on the stigmatic surfaces at the early stage 1 (A), some scattered points stained at stage 3 (B). The colour spreads first to the stigmatic edges at stage 4 (C), but a maximum intensity was clear all over the stigma at stage 5 (D), asterisks). (E) Percentage of stigmas with reaction to β-GlcYR, with almost 100 % of the mature stigmas reacting at anthesis, and the negative control α-GalYR. Scale bars = 0·25 mm.

species with wet stigmas have shown a fairly well-conserved structure at anthesis with papillae covered with secretion and a stigmatoid tissue beneath (Konar and Linskens, 1966; Ciampolini *et al.*, 1990; Heslop-Harrison, 2000). This work shows that three types of cells developed and matured in an orchestrated manner: the stigmatic papillae, cells of the stigmatoid tissue, and some autolythic cells, not previously reported, located at the stigmatoid tissue underneath the interpapillar valleys. These three cell types all contributed to the production of stigmatic secretion. At maturity, as papillae loose turgidity, autolysis of interpapillar stigmatoid cells and secretion by stigmatoid cells resulted in a copious secretion

located both on the stigma surface and also in the stigmatoid intercellular spaces. The contribution of different types of cells to a common stigmatic secretion may be in line with the different functions of a stigma, from pollen recognition (Dickinson, 2000) and support for pollen germination (Herrero and Dickinson, 1979), to xenobiotic resistance (Heslop-Harrison, 2000; McInnis *et al.*, 2006a).

Also in time with these concurrent functions that occur in the small stigma is the varied and rich composition of the stigmatic secretion. Thus stigmatic secretion was rich in polysaccharides and lipids, similar to other wet stigmas (Cresti *et al.*, 1980, 1986). In other species, lipids appear to play a role in
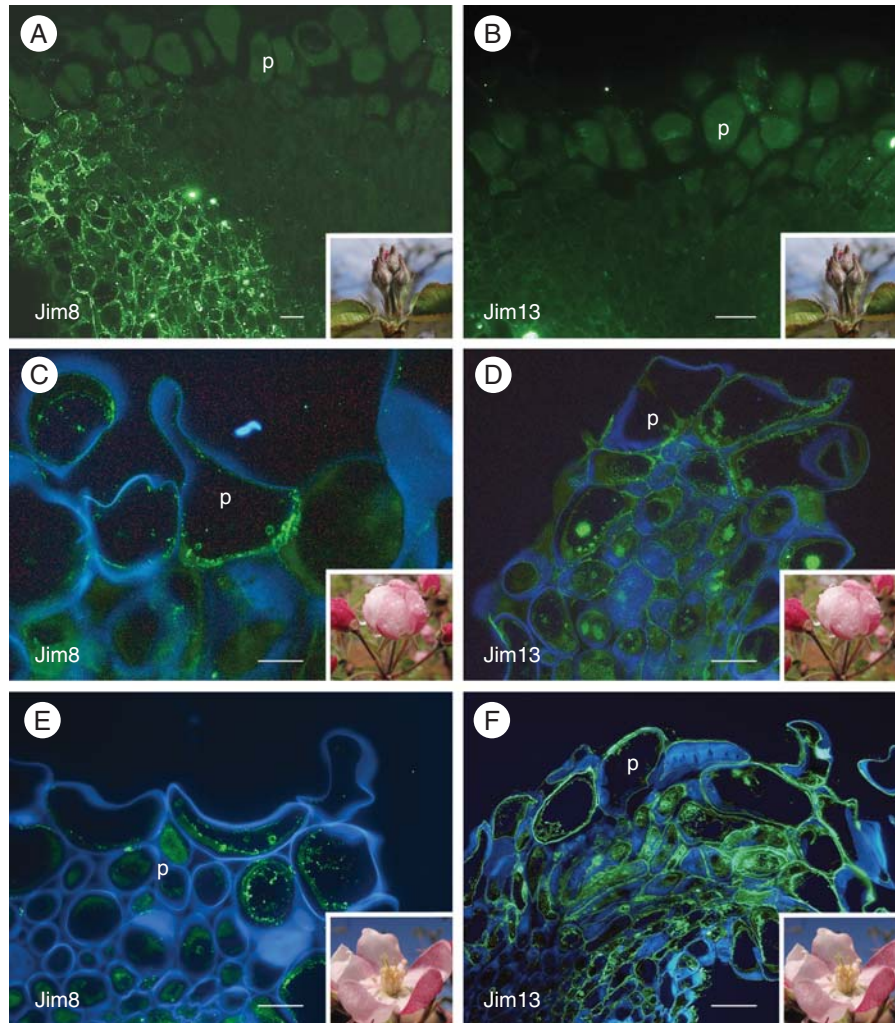
FIG. 5. Changes in AGPs on the apple flower stigma in different phenological stages: stage 1 (A, B), stage 4 (C, D) and stage 5 (E, F). The left column of photographs shows epitopes labelled with Jim8 mAb FITC-conjugated mAb signal in fluorescent green. The right column shows the same staining but labelled with Jim13 mAb. At stage 1, neither Jim8 (A) nor Jim13 mAbs (B) showed labelling on the papillae, only in the stylar cortical tissue. (C) At stage 4, Jim8 epitopes appeared in the papillae cytoplasm. (D) In contrast, Jim13 labelled the stigmatic edges in the stigmatoid tissue, and the intercellular substance. (E) At stage 5, Jim8 epitopes still labelled inside the papillae. (F) Conspicuous signal of Jim13 epitopes in the intercellular substance mainly at the stigmatic edges. (A, B) FITC signal; (C–F) FITC signal counterstained with calcofluor white for cellulose (blue); 4-μm sections embedded in Technovit8100. Abbreviation: p, papilla. Scale bars: (A, B) = 10 μm; (C–F) = 50 μm.

pollen-grain hydration and pollen-tube orientation on the stigma (Wolters-Arts *et al.*, 1998). Sugars have been reported to provide an adequate pollen germination medium (Herrero and Dickinson, 1979). Apple stigmatoid tissue cells also secreted phenolic compounds into the intercellular space, and lipids onto the surface. In some species, secretions of phenolics have been considered as peroxidase substrates (Šukalović *et al.*, 2010), which may act as indicators of stigma receptivity (Shivanna and Sastri, 1981; McInnis *et al.*, 2006 *a*, *b*; Hiscock and Allen, 2008). Phenolic accumulation has also been associated with senescence (Beckam, 2000), and also reported during maturation of floral organs (Wu and Cheung, 2000; Rogers, 2006). In the apple stigma, lipophenolic cell release occurred at a time close to the end of the secretory process, suggesting that they could also be defensive molecules (González *et al.*, 1995*a*), just as the presence of reactive oxygen species and reactive nitrogen species

(McInnis *et al.*, 2006*a*, Hiscock and Allen, 2008), which have been shown to clearly contribute to stigmatic receptivity, could be involved in a prophylactic role (Heslop-Harrison, 2000). Clearly, the rich display of different activities that occur in a receptive stigma support the fact that stigmatic receptivity depends on multiple factors. However, from an interactive perspective, the specific and tuned secretion of AGPs point to them as good candidates playing also a part in the acquisition of stigmatic receptivity.

AGPs were secreted concurrently with the acquisition of stigmatic receptivity

Stigmatic receptivity was acquired concomitantly with the secretion of AGPs. In addition to displaying the same developmental timing, AGP secretion followed the same centripetal and basipetal spatial development pattern. While the use of
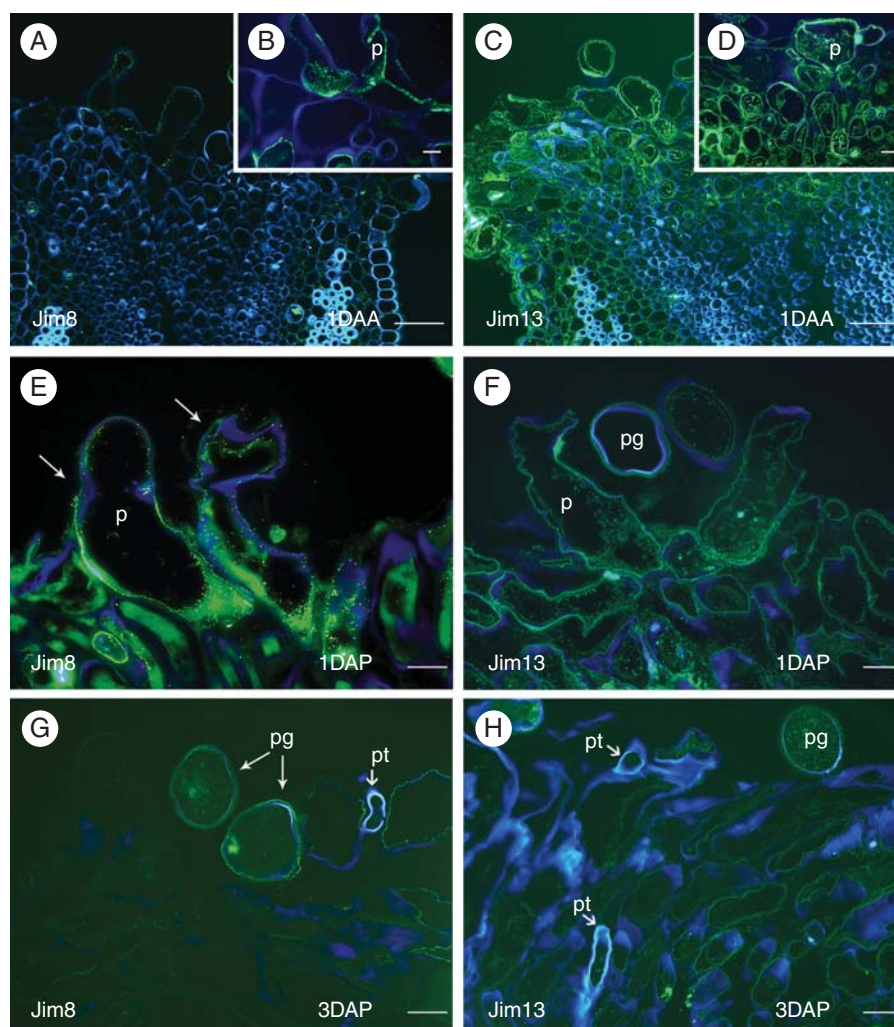
FIG. 6. Changes in apple flower stigmatic AGPs with pollination. Unpollinated stigmas (A–D) 1 d after anthesis (1DAA), and pollinated stigmas (E, F) 1 d after pollination (1DAP) and (G, H) 3 d after pollination (3DAP). (A) Unpollinated stigmas 1DAA showing Jim8 epitopes in the stigmatic papillae. (B) Detail of epitopes inside of a papilla. (C) Jim13 epitopes secreted to the external cell walls in the stigmatoid tissue of unpollinated stigmas, and also (D) on the papillae. (E) Jim8 epitopes 1DAP showed now a reaction outside the papillae cell walls (arrows). (F) Jim13 labelled epitopes decreased 1DAP. (G) Jim8 epitopes recognized only in pollen grains and papillae walls contacting them 3 DAP. (H) Jim13 labelling decreased 3DAP, especially at places of pollen tube passage. FITC fluorescent green counterstained with calcofluor white – in blue; 4-μm sections embedded in Technovit 8100. Abbreviations: p, papilla; pg, pollen grain; pt, pollen tube. Scale bars: A, C = 50 μm; B, D–H = 10 μm.

Yariv reagent to precipitate and quantify AGPs in other systems has been put forward (Baron-Maurer *et al.*, 2010), results in this work show the appearance of AGPs on the stigmatic surface in fresh tissues by a colour reaction. These results are consistent with a previous biochemical approach using Yariv reagent in *Nicotiana* stigmatic extracts (Gell *et al.*, 1986). Our results in apple suggest that this simple procedure could be used to evaluate the acquisition of stigmatic receptivity in additional species, but it requires further evaluation.

The immunocytochemical approach with Jim8 and Jim13 mAbs was consistent with the Yariv biochemical reaction, and confirmed the presence of AGPs on the apple stigma. AGPs have been previously reported in reproductive tissues (Juah and Lord, 1995; Coimbra *et al.*, 2007), but they are also ubiquitous in plant tissues derived from the epidermis

(Vaughn *et al.*, 2007; Qin and Zhao, 2007). While the possible function of AGPs has not been previously considered in a developmental context, their immunolocalization followed the same developmental pattern as the maturing stigma. Furthermore, secretion of AGPs was co-ordinated with the initiation of stigmatic receptivity. A differential expression pattern of proteins recognized by Jim8 and Jim13 mAbs was observed during stigma development. While AGPs recognized by Jim8 appeared to be constitutive of the papillae cell wall at vacuolation developmental stages, those recognized by Jim13 were secreted at the stigmatoid tissue concomitantly with tissue maturation, suggesting an additive role on the acquisition of stigmatic receptivity. The epitopes recognized by both mAbs were located in the glycan side of the glycoproteins, and they represent post-translational modifications of the glycoproteins within the cells (Estévez *et al.*, 2006). This

work shows, for the first time, a different location of developmental tissue for both mAbs that recognize different AGPs. The ubiquitous nature of AGPs combined with their glycan heterogeneity questions their rapid functional adaptability in plant tissues and/or cell types (Ellis *et al.*, 2010), but current results suggest that small changes may have been recruited to fulfil different functions leading to the functional plasticity of these glycoproteins.

Changes in AGPs following pollination

The different nature and function of the different epitopes recognized by Jim8 and Jim13 mAbs was further apparent following pollination. Pollination elicited the secretion of Jim8 epitopes to the papillar cell-wall surface, and their presence was stronger at contact points with pollen tubes, suggesting a possible adhesion cue. The involvement of proteins influencing adhesion in wet stigmas has been previously documented in lily, with a hollow style (Park *et al.*, 2000), as well as in the arabidopsis dry stigma (Dong *et al.*, 2005); of special interest are small cystein-rich proteins that influence pollen-tube behaviour (Kim *et al.*, 2003). Our observations in this work showed an induced expression of Jim8 epitopes by pollen arrival on the papillae, suggesting a potential involvement in an early male–female physical contact on the papillar surface.

A different pattern was followed by Jim13-recognized epitopes, which were abundant in the intercellular substance of unpollinated stigmas, and disappeared as pollen tubes passed by, suggesting a pollen tube–AGP interaction within the stigmatoid tissue in apple. The possible relationship between small, secreted proteins and pollen-tube performance in the pistil has been suggested (Lee *et al.*, 2009; Chae *et al.*, 2010; Higashiyama, 2010; Chae and Lord, 2011). AGPs in the stylar transmitting tissue follow a deglycosylation gradient in the style (de Graaf *et al.*, 2003; Hancock *et al.*, 2005; Kim *et al.*, 2006). Since AGPs exert a crucial control in rapidly expanding tissues such as the stigma, the intrinsic developmental regulation of Jim13 AGP expression and their vanishing after pollen-tube passage further supports a possible contribution to pollen-tube wall-building and orientation towards the style.

This work shows that the developmental regulation of the secretion of AGPs is associated with the onset of stigmatic receptivity in the apple flower. Changes in their labelling following pollination suggest a role in pollen–stigma interaction. The presence of AGPs in the pistil has been shown in divergent species, from monocots (Ma and Zhao, 2010) and eudicots (Coimbra *et al.*, 2007) to early divergent angiosperms (Prychid *et al.*, 2011). This together with their functional plasticity opens a way for an understanding of glycoprotein evolution modulating pollen–pistil interactions in angiosperms.

LITERATURE CITED

Acosta-García G, Vielle-Calzada JP. 2004. A classical arabinogalactan protein is essential for the initiation of female gametogenesis in *Arabidopsis. The Plant Cell* **16**: 2614–2628.

Anderson RL, Clarke AE, Jermyn MA, Knox RB, Stone BA. 1977. A carbohydrate-binding arabinogalactan protein from liquid suspension cultures of endosperm from *Lolium multiflorum. Australian Journal of of Plant Physiology* **4**: 143–158.

Baron-Maurer JB, Pereira-Netto AB, Pettolino FA, Gaspar YM, Bacic A. 2010. Effects of Yariv dyes, arabinogalactan-protein binding reagents, on the growth and viability of Brazilian pine suspension culture cells. *Trees* **24**: 391–398.

Beckam CH. 2000. Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants? *Physiological and Molecular Plant Pathology* **57**: 101–110.

Buszard D, Schwabe WW. 1995. Effect of previous crop load on stigmatic morphology of apple flowers. *Journal of the American Society of Horticultural Science* **120**: 566–570.

Capataz-Tafur J, Trejo-Tapia G, Rodríguez-Monroy M, Sepúlveda-Jiménez G. 2010. Arabinogalactan proteins are involved in cell aggregation of cell suspension cultures of *Beta vulgaris* L. *Plant Cell, Tissue and Organ Culture* **106**: 169–177.

Chae K, Lord EM. 2011. Pollen tube growth and guidance: roles of small, secreted proteins. *Annals of Botany* **108**: 627–636.

Chae K, Gonong BJ, Kim SC, et al. 2010. A multifaced study of stigma/style cysteine-rich adhesin (SCA)-like *Arabidopsis* lipid transfer proteins (LTPs) suggests diversified roles for these LTPs in plant growth and reproduction. *Journal of Experimental Botany* **61**: 4277–4290.

Ciampolini F, Shivanna KR, Cresti M. 1990. The structure and cytochemistry of the pistil of *Sternbergia lutea* (Amaryllidaceae). *Annals of Botany* **66**: 703–712.

Clarke AE, Anderson RL, Stone BA. 1979. Form and function of arabinogalactans and arabinogalactan-proteins. *Phytochemistry* **18**: 521–540.

Coimbra S, Almeida J, Junqueira V, Costa ML, Pereira LG. 2007. Arabinogalactan proteins as molecular markers in *Arabidopsis thaliana* sexual reproduction. *Journal of Experimental Botany* **58**: 4027–4035.

Coimbra S, Costa M, Mendes MA, Pereira AM, Pinto J, Pereira LG. 2010. Early germination of *Arabidopsis* pollen in a doble null mutant for the arabinogalactan protein genes *AGP6* and *AGP11. Sexual Plant Reproduction* **23**: 199–205.

Cresti M, Ciampolini F, Sansavini S. 1980. Ultrastructure and histochemical features of pistil of *Malus communis*: the stylar transmitting tissue. *Scientia Horticulturae* **12**: 327–337.

Cresti M, Keijzer CJ, Tiezzi A, Ciampolini F, Focardi S. 1986. The stigma of *Nicotiana:* ultrastructural and biochemical studies. *American Journal of Botany* **73**: 1713–1722.

Currier HB. 1957. Callose substance in plant cells. *American Journal of Botany* **44**: 478–488.

Dickinson HG. 2000. Pollen stigma interactions: so near yet so far. *Trends in Genetics* **16**: 373–374.

Dong J, Kim ST, Lord EM. 2005. Plantacyanin plays a role in reproduction in *Arabidopsis. Plant Physiology* **138**: 778–789.

Dupuis I, Dumas C. 1990. Biochemical markers of female receptivity in maize (*Zea mays* L.) assessed using *in vitro* fertilization. *Plant Science* **70**: 11–20.

Edlund AF, Swanson R, Preuss D. 2004. Pollen and stigma structure and function: the role of diversity in pollination. *The Plant Cell* **16**: S84–S97.

Ellis M, Egelund J, Shultz CJ, Bacic A. 2010. Arabinogalactan-proteins: key regulators at the cell surface? *Plant Physiology* **153**: 403–419.

Estévez JM, Kieliszewski MJ, Khitrov N, Somerville C. 2006. Characterization of synthetic hydroxyproline-rich proteoglycans with arabinogalactan protein and extensin motifs in Arabidopsis. *Plant Physiology* **142**: 458–470.

Feder N, O'Brien TP. 1968. Plant microtechnique: some principles and new methods. *American Journal of Botany* **55**: 123–142.

Gell AC, Bacic A, Clarke AE. 1986. Arabinogalactan-proteins of the female sexual tissue of *Nicotiana alata. Plant Physiology* **82**: 885–889.

González MV, Coque M, Herrero M. 1995a. Papillar integrity as an indicator of stigmatic receptivity in kiwifruit (*Actinidia deliciosa*). *Journal of Experimental Botany* **46**: 263–269.

González MV, Coque M, Herrero M. 1995b. Stigmatic receptivity limits the effective pollination period in kiwifruit. *Journal of the American Society of Horticultural Science* **120**: 199–202.

de Graaf BHJ, Knuiman BA, Derksen J, Mariani C. 2003. Characterization and localization of the transmitting tissue-specific PELP III proteins of *Nicotiana tabacum*. *Journal of Experimental Botany* **54**: 55–63.

Hancock CN, Kent L, McClure BA. 2005. The stylar 120kDa glycoprotein is required for S-specific pollen rejection in *Nicotiana*. *The Plant Journal* **43**: 716–723.

Hedhly A. 2011. Sensitivity of flowering plant gametophytes to temperature fluctuations. *Environmental and Experimental Botany* **74**: 9–16.

Hedhly A, Hormaza JI, Herrero M. 2003. The effect of temperature on stigmatic receptivity in sweet cherry (*Prunus avium* L.). *Plant, Cell & Environment* **26**: 1673–1680.

Hedhly A, Hormaza JI, Herrero M. 2005. The effect of temperature on pollen germination, pollen tube growth, and stigmatic receptivity in peach. *Plant Biology* **7**: 476–483.

Hedhly A, Hormaza JI, Herrero M. 2009. Global warming and sexual plant reproduction. *Trends in Plant Science* **14**: 30–36.

van Hengel AJ, van Kammen A, de Vries SC. 2002. A relationship between seed development, arabinogalactan-proteins (AGPs) and the AGP mediated promotion of somatic embryogenesis. *Physiologia Plantarum* **114**: 637–644.

Herrero M, Arbeloa A. 1989. Influence of the pistil on pollen tube kinetics in peach (*Prunus persica*). *American Journal of Botany* **76**: 1441–1447.

Herrero M, Dickinson HG. 1979. Pollen–pistil incompatibility in *Petunia hybrida*: changes in the pistil following compatible and incompatible intraspecific crosses. *Journal of Cell Science* **36**: 1–18.

Heslop-Harrison Y. 1977. The pollen–stigma interaction: pollen tube penetration in crocus. *Annals of Botany* **41**: 913–922.

Heslop-Harrison Y. 2000. Control gates and micro-ecology: the pollen–stigma interaction in perspective. *Annals of Botany* **85**: 5–13.

Heslop-Harrison Y, Shivanna KR. 1977. The receptive surface of the angiosperm stigma. *Annals of Botany* **41**: 1233–1258.

Higashiyama T. 2010. Peptide signalling in pollen–pistil interactions. *Plant Cell Physiology* **51**: 177–189.

Hiscock SJ, Allen AM. 2008. Diverse cell signalling pathways regulate pollen–stigma interactions: the search for consensus. *New Phytologist* **179**: 286–317.

Hiscock SJ, Coleman JOD, Dewey FM, Dickinson HG. 1994. Identification and localization of an active cutinase in the pollen of *Brassica napus* L. *Planta* **193**: 377–384.

Hiscock SJ, Bown D, Gurr SJ, Dickinson HG. 2002. Serine esterases are required for pollen tube penetration of the stigma in *Brassica*. *Sexual Plant Reproduction* **15**: 65–74.

Hormaza JI, Herrero M. 1992. Pollen selection. *Theoretical and Applied Genetics* **83**: 663–672.

Hughes J, McCully ME. 1975. The use of an optical brightener in the study of plant structure. *Stain Technology* **50**: 319–329.

Inamura T, Kusano H, Kajigaya Y, Ichikawa M, Shimada H. 2007. A rice dihydrosphingosine C4 hydrolase (*DSH1*) gene, which is abundantly expressed in the stigmas, vascular cells and apical meristem, may be involved in fertility. *Plant Cell Physiology* **48**: 1108–1120.

Jefferies CJ, Belcher AR. 1974. A fluorescent brightener used for pollen tube identification *in vivo*. *Stain Technology* **49**: 199–202.

Johansen DA. 1940. *Plant microtechnique.* New York, NY: McGraw-Hill Books.

Juah GY, Lord EM. 1995. Localization of pectins and arabinogalactan-proteins in lily (*Lilium longiflorum* L.) pollen tube and style, and their possible roles in pollination. *Planta* **2**: 251–261.

Kim ST, Mollet JC, Dong J, Zhang K, Park SY, Lord EM. 2003. Chemocyanin, a small basic protein from the lily stigma, induces pollen tube chemotropism. *Proceedings of the National Academy of Sciences of the USA* **100**: 16125–16130.

Kim ST, Zhang K, Dong J, Lord EM. 2006. Exogenous free ubiquitin enhances lily pollen tube adhesion to an *in vitro* stylar matrix and may facilitate endocytosis of SCA. *Plant Physiology* **142**: 1397–1411.

Knox JP, Linstead PJ, Peart J, Cooper C, Roberts K. 1991. Developmentally-regulated epitopes of cell surface arabinogalactan-proteins and their relations to root tissue pattern formation. *The Plant Journal* **1**: 317–326.

Konar RN, Linskens HF. 1966. Physiology and biochemistry of the stigma fluid of *Petunia hybrida*. *Planta* **71**: 327–387.

Lee CB, Kim S, McClure B. 2009. A pollen protein, NaPCCP, that binds pistil arabinogalactan proteins also binds phosphatidylinositol 3-phosphate and associates with the pollen tube endomembrane system. *Plant Physiology* **149**: 791–802.

Linskens HF, Esser KL. 1957. Über eine spezifische Anfärbung der Pollenschläuche im Griffel und die Zahl der Kallosepfropfen nach Selbstung und Fremdung. *Die Naturwissenschaften* **44**: 16–16.

Lora J, Hormaza JI, Herrero M. 2011. Stigmatic receptivity in a dichogamous early-divergent angiosperm species, *Annona cherimola* (Annonaceae): influence of temperature and humidity. *American Journal of Botany* **98**: 1–10.

Lord EM, Russell S. 2002. The mechanisms of pollination and fertilisation in plants. *Annual Review of Cell and Developmental Biology* **18**: 81–105.

Ma H, Zhao J. 2010. Genome-wide identification, classification, and expression analysis of the arabinogalactan protein gene family in rice (*Oryza sativa* L.). *Journal of Experimental Botany* **61**: 2647–2668.

Majewska-Sawka A, Nothnagel EA. 2000. The multiple roles of arabinogalactan proteins in plant development. *Plant Physiology* **122**: 3–9.

McInnis SM, Desikan R, Hancock JT, Hiscock SJ. 2006a. Production of reactive oxygen species and reactive nitrogen species by angiosperm stigmas and pollen: potential signalling crosstalk? *New Phytologist* **172**: 221–228.

McInnis SM, Emery DC, Porter R, Desikan R, Hancock JT, Hiscock SJ. 2006b. The role of stigma peroxidases in flowering plants: insights from further characterization of a stigma-specific peroxidase (SSP) from *Senecio squalidus* (Asteraceae). *Journal of Experimental Botany* **57**: 1–12.

Motose H, Sugiyama M, Fukuda H. 2004. A proteoglycan mediates inductive interaction during plant vascular development. *Nature* **429**: 873–878.

Nicholas JR, Gates PJ, Grierson P. 1987. The use of fluorescence microscopy to monitor root development in micropropagated explants. *Journal of Horticultural Science* **61**: 417–421.

Park SY, Jauh GY, Mollet JC, *et al*. 2000. A lipid transfer-like protein is necessary for lily pollen tube adhesion to an *in vitro* stylar matrix. *The Plant Cell* **12**: 151–163.

Pennell RI, Janniche L, Kjellbom PP, Scofield GN, Peart J, Roberts K. 1991. Developmental regulation of a plasma membrane arabinogalactan protein epitope in oilseed rape flowers. *The Plant Cell* **3**: 1317–1326.

Pereira LG, Coimbra S, Oliveira H, Monteiro L, Sotomayor M. 2006. Expression of arabinogalactan protein genes in pollen tubes of *Arabidopsis thaliana*. *Planta* **223**: 374–380.

Prychid CJ, Skoloff DD, Remizowa MV, Tuckett RE, Yadav SR, Rudall PJ. 2011. Unique stigmatic hairs and pollen-tube growth within the stigmatic cell wall in the early-divergent angiosperm family Hydatellaceae. *Annals of Botany* **108**: 599–608.

Qin Y, Zhao J. 2007. Localization of arabinogalactan-proteins in different stages of embryos and their role in cotyledon formation of *Nicotiana tabacum* L. *Sexual Plant Reproduction* **20**: 213–224.

Quiapim AC, Brito MS, Bernardes LAS, *et al*. 2009. Analysis of the *Nicotiana tabacum* stigma/style transcriptome reveals gene expression differences between wet and dry stigma species. *Plant Physiology* **149**: 1211–1230.

Rogers HJ. 2006. Programmed cell death in floral organs: how and why do flowers die? *Annals of Botany* **97**: 309–315.

Sabatini DD, Bench K, Barrnett RJ. 1963. Cytochemistry and electron microscopy: the preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *Journal of Cell Biology* **17**: 19–58.

Sanzol J, Rallo P, Herrero M. 2003. Asynchronous development of stigmatic receptivity in the pear (*Pyrus comunis*; Rosaceae) flower. *American Journal of Botany* **90**: 78–84.

Seifert GJ, Roberts K. 2007. The biology of arabinogalactan proteins. *Annual Review of Plant Biology* **58**: 137–161.

Sheffield CS, Smith RF, Kevan PG. 2005. Perfect syncarpy in apple (*Malus×domestic*a 'Summerland McIntosh') and its implications for pollination, seed distribution and fruit production (Rosaceae: Maloideae). *Annals of Botany* **95**: 583–591.

Shivanna KR, Sastri DC. 1981. Stigma-surface esterase activity and stigma receptivity in some taxa characterized by wet stigmas. *Annals of Botany* **47**: 53–64.

Showalter AM. 2001. Arabinogalactan proteins: structure, expression and function. *Cell and Molecular Life Sciences* **58**: 1399–1417.

Solís MT, Pintos T, Prado MJ, *et al*. 2008. Early markers of *in vitro* microspore reprogramming to embryogenesis in olive (*Olea europaea* L.). *Plant Science* **174**: 597–605.

Šukalović VHT, Veljović-Jovanović S, Maksimović JD, Maksimović V, Pajić Z. 2010. Characterisation of phenol oxidase and peroxidase from maize silk. *Plant Biology* **12**: 406–413.

Vaughn KC, Talbot MJ, Offler CE, McCurdy DW. 2007. Wall ingrowths in epidermal transfer cells of *Vicia faba* cotyledons are modified primary walls marked by localized accumulations of arabinogalactan proteins. *Plant Cell Physiology* **48**: 159–168.

Verhoeven T, Feron R, Wolters-Arts M, *et al*. 2005. STIG1 controls exudate secretion in the pistil of *Petunia* and tobacco. *Plant Physiology* **138**: 153–160.

Williams RR, Brain P, Curch RM, Flook VA. 1984. Flower receptivity, pollen transfer and fruit set variations during a single flowering period of Cox's Orange Pippin apple. *Journal of the Horticultural Science* **59**: 337–347.

Wolters-Arts M, Lush WM, Mariani C. 1998. Lipids are required for directional pollen-tube growth. *Nature* **392**: 818–821.

Wu H, Cheung AY. 2000. Programmed cell death in plant reproduction. *Plant Molecular Biology* **44**: 1573–5028.

Wu HM, Wong E, Ogdahi J, Cheung AY. 2000. A pollen tube growth-promoting arabinogalactan protein from *Nicotiana alata* is similar to the tobacco TTS protein. *The Plant Journal* **22**: 165–176.

Wu H, de Graaf B, Mariani C, Cheung AY. 2001. Hydroxyproline-rich glycoproteins in plant reproductive tissues: structure, functions and regulation. *Cell and Molecular Life Sciences* **58**: 1418–1429.

Yang J, Sardar HS, McGovern KR, Zhang Y, Showalter AM. 2007. A lysine-rich arabinogalactan protein in *Arabidopsis* is essential for plant growth and development, including cell division and expansion. *The Plant Journal* **49**: 629–640.

Yariv J, Lis H, Katchalsi E. 1967. Precipitation of arabic acid and some seed polysaccharides by glycosylphenylazo dyes. *Biochemical Journal* **105**: p1c–2c.

Zhang Y, Brown G, Whetten R, *et al*. 2003. An arabinogalactan protein associated with secondary cell wall formation in differentiating xylem of loblolly pine. *Plant Molecular Biology* **52**: 91–102.