Journal of
**Experimental
Botany**
www.jxb.oxfordjournals.org

RESEARCH PAPER

Proteomics profiling reveals novel proteins and functions of the plant stigma exudate

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Abstract

Proteomic analysis of the stigmatic exudate of *Lilium longiflorum* and *Olea europaea* led to the identification of 51 and 57 proteins, respectively, most of which are described for the first time in this secreted fluid. These results indicate that the stigmatic exudate is an extracellular environment metabolically active, participating in at least 80 different biological processes and 97 molecular functions. The stigma exudate showed a markedly catabolic profile and appeared to possess the enzyme machinery necessary to degrade large polysaccharides and lipids secreted by papillae to smaller units, allowing their incorporation into the pollen tube during pollination. It may also regulate pollen-tube growth in the pistil through the selective degradation of tube-wall components. Furthermore, some secreted proteins were involved in pollen-tube adhesion and orientation, as well as in programmed cell death of the papillae cells in response to either compatible pollination or incompatible pollen rejection. Finally, the results also revealed a putative cross-talk between genetic programmes regulating stress/defence and pollination responses in the stigma.

Key words: Eastern lily, exudate, olive, proteomics, stigma, secretome.

Introduction

In angiosperms, pollination begins when pollen comes into contact with the stigma surface and hydrates. Pollen behaviour on the stigma differs among species. In plants with a wet stigma, the surface cells release a viscous secretion in which pollen grains are embedded without any species selectivity (Sanchez *et al.*, 2004). Pollen capture and adhesion is mediated mainly by the stickiness and surface tension of the stigma exudate. This secretion also provides a water source

for rapid hydration of pollen and a protective environment for fragile pollen tubes during the first steps of germination. Pollen grains and pollen tubes also uptake nutrients and other compounds from this fluid such as pectic wall precursors (Labarca and Loewus, 1972).

The stigmatic exudate (SE) is synthesized and secreted by differentiated and specialized cells of the stigma surface, which usually protrude as papillae. A Cys-rich protein called

Abbreviations: AGP, arabinogalactan protein; GH, *O*-glycosyl hydrolase; HSP, heat-shock protein; LTP, lipid transfer protein; nanoLC-MS/MS, nanoflow liquid chromatography coupled to tandem mass spectrometry; PLC, phospholipase C; PME, pectin methylesterase; PMEI, PME inhibitor; RLK, receptor-like protein kinase; SE, stigmatic exudate; SEM, scanning electron microscopy; TEM, transmission electron microscopy

stigma-specific protein 1 (STIG1) has been proposed to play a function in the temporal regulation of SE deposition on the stigma surface in tobacco and petunia (Verhoeven *et al.*, 2005). The SE mainly consists of water, polysaccharides, lipids, and proteins (Labarca *et al.*, 1970; Suárez *et al.*, 2012). It has been shown that the tobacco SE has cell-wall-loosening activity, which relies on the action of a lipid transfer protein (LTP) (Nieuwland *et al.*, 2005). Another LTP, named stigma/stylar cysteine-rich adhesin (SCA), is involved in pollen-tube adhesion in lily (Park *et al.*, 2000). In combination with a small stigma protein called chemocyanin, SCA protein also induces chemotropic activity (Kim *et al.*, 2003). Pathogenesis-related proteins also accumulate in the SE (Busot *et al.*, 2008; Wakelin and Leung, 2009), and a function in plant reproduction cannot be ruled out. Despite all these data, the qualitative composition of the SE protein pool and its involvement in pollen–stigma interaction is poorly known to date. In this work, we have carried out the first large-scale analysis of the SE proteins of two species using a proteomic approach in order to find new proteins and provide novel data about the biological and molecular processes that occur in this extracellular fluid. As result, we have provided a comprehensive list of the proteins, and we discuss their physiological relevance in the framework of pollination.

Materials and methods

Plant material and sampling

Flowering cuttings of Eastern lily (*Lilium longiflorum* Thunb. cv. 'White Heaven') were purchased at a local market. Cuttings were kept in a preservative solution of VIVEFLOR® Complet (Agrosport SL, Barcelona, Spain) and flower buds were left to open at 25 °C, 60% humidity, and a 12 h photoperiod under natural irradiation. Flowers were emasculated just before anthesis to keep pollen away from the stigma surface. The stigmatic secretion was collected from flowers by pipetting the drops formed on the stigma surface at 1 day intervals during the 7 days following the anthesis. Three independent samples (100 flowers each) were collected and stored at –20 °C until use. Orange [*Citrus×sinensis* (L.) Osbeck], pomegranate (*Punica granatum* L.), and olive (*Olea europaea* L. cv. 'Picual') flowers at anthesis were collected from individual trees at the germplasm collection of the Estación Experimental del Zaidín in Granada (Spain). Early flowering branches were put inside paper bags to avoid pollen contamination, cut just before anthesis, and left to open on the bench in the laboratory. Pistils were dissected using a stereomicroscope (M165FC; Leica, Germany) and pictures were taken with a high-resolution digital camera. For each species, three independent samples of SE were collected from 250 pistils each using a very fine synthetic hair paintbrush soaked in 0.1 M phosphate buffer (pH 7.0) containing 0.1% (v/v) Tween 20 and 20 mM dithiothreitol and stored at −20 °C until use. After brushing, ten olive stigmas were chosen randomly, and fixed and embedded for transmission electron microscopy (TEM) as described below. We found that papillae cells remained intact after exudate collection and there were no changes at the ultrastructural level compared with untreated stigmas (Supplementary Fig. S1 at *JXB* online). The protein content of each sample was measured with a 2-D Quant kit (Amersham Biosciences, Piscataway, NJ, USA) following the manufacturer's instructions.

SDS-PAGE

Exudate proteins were precipitated in 9 vols of 20% (w/v) trichloroacetic acid and 0.2% (w/v) dithiothreitol in acetone at −20 °C for 6 h.

After centrifugation at 10 000*g*, pellets were washed twice in acetone and finally resuspended in 0.5 ml of sample buffer [10 mM Tris/HCl (pH 8.0), 1 mM EDTA, 5% (v/v) β-mercaptoethanol, 5% (w/v) SDS, 10% (v/v) glycerol and 0.01% (w/v) bromophenol blue]. Exudate protein samples (15 µg of total protein each) were boiled for 5 min and then separated by SDS-PAGE according to standard procedures (Laemmli, 1970). Briefly, proteins were separated on 1 mm thick gels using a 4% acrylamide stacking gel and a 5–15% acrylamide linear gradient as the resolving gel. Gels were run for 1 h at 5 mA per gel and then overnight at a constant current (7 mA per gel). Gels were fixed for 2 h in 50% (v/v) ethanol, 3% (v/v) phosphoric acid, followed by three washes in water for 1 min each. Gels were stained for 24 h with colloidal Coomassie blue solution [0.12 % (w/v) Coomassie Brilliant Blue G250, 10% (w/v) ammonium sulphate, 10% (v/v) phosphoric acid and 20% (v/v) methanol]. Two gels were run for each biological sample (*n*=6 gels). Protein band intensities were profiled from one-dimensional gels using Quantity One v4.6.2 software (Bio-Rad).

For each species, two gels corresponding to two independent samples were systematically cut into slices, and *in gel* digestion was performed with an automated protein digestion system (MassPREP Station; Waters, Manchester, UK). The gel slices were washed three times in a mixture containing 25 mM NH_4HCO_3:CH_3CN (1:1, v/v). The cysteine residues were reduced by 50 µl of 10 mM dithiothreitol at 57 °C and alkylated with 50 µl of 55 mM iodoacetamide. After dehydration with acetonitrile, proteins were cleaved *in gel* with 40 µl of 12.5 ng µl^{-1} of modified porcine trypsin (Promega, Madison, WI, USA) in 25 mM NH_4HCO_3 at 37 °C for 4 h. Tryptic peptides were extracted using 60% (v/v) acetonitrile prepared in 0.5% (v/v) formic acid, followed by a second extraction with 100% acetonitrile before nanoflow liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS) analysis.

LC-MS/MS and data analysis

NanoLC-MS/MS analyses were performed on a nanoACQUITY UltraPerformance LC® System (UPLC®) coupled to a quadrupole time-of-flight mass spectrometer (maXis; Bruker Daltonics, Bremen, Germany) equipped with a nano-electrospray source. The UPLC® was equipped with a Symmetry C18 pre-column (20 × 0.18 mm, 5 µm particle size; Waters, Milford, USA) and an ACQUITY UPLC® BEH130 C18 separation column (75 *µm* × 250 mm, 1.7 µm particle size, Waters, Milford, USA). Five microlitres of each sample was loaded. The solvent system consisted of 0.1% (v/v) formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Peptides were trapped for 3 min at a flow rate of 5 µl min^{-1} with 99% solvent A and 1% solvent B. Elution was performed at 45 °C at a flow rate of 400 nl min^{-1}, using a linear gradient of 1–40% solvent B over 35 min. The mass spectrometer was operating in positive mode, with the following settings: source temperature was set to 200 °C, while dry gas flow was at 4 l min^{-1}. The nano-electrospray voltage was optimized to −4500 V. External mass calibration of the time of flight was achieved before each set of analyses using Tuning Mix (Agilent Technologies, Palo Alto, USA) in the mass range of 322–2722 *m/z*. Mass correction was achieved by recalibration of the acquired spectra to the applied lock masses [methylstearate ([M+H]$^+$ 299.2945 *m/z*) and hexakis(2,2,3,3,-tetrafluoropropoxy) phosphazine ([M+H]$^+$ 922.0098 *m/z*)].

For tandem MS experiments, the system was operated with automatic switching between MS and MS/MS modes in the range of 50–2200 *m/z*. The four most abundant peptides (absolute intensity threshold of 1500), preferably doubly, triply, and quadruply charged ions, were selected from each MS spectrum for further isolation and collision induced dissociation fragmentation using argon as the collision gas. Ions were excluded after the acquisition of one MS/MS spectrum and the exclusion was released after 0.6 min. The complete system was fully controlled by Hystar 3.2 (Bruker Daltonics).

Raw data (Supplementary Table S1 at *JXB* online) collected during nanoLC-MS/MS analyses were processed and converted into mgf files with DataAnalysis 4.0 (Bruker Daltonics), and all mgf files

for a given gel lane were merged using a tool developed in house (available at https://msda.unistra.fr). MS/MS spectra were smoothed using the Savitzky–Golay algorithm with a smoothing width of 0.2 *m/z* in one cycle. A charge deconvolution was applied to the MS full scan and the MS/MS spectra.

Protein identification

Mass data collected during nanoLC-MS/MS were searched using a local Mascot server (v2.2.0; MatrixScience, London, UK) against an in-house-generated protein database composed of protein sequences of Viridiplantae (Taxonomy ID 33090) and known contaminant proteins such as human keratins and trypsin, extracted from the NCBInr database (version of 19 March 2013) and combined with reverse sequences for all entries using an in-house database generation toolbox (https://msda.unistra.fr; total 2 692 822 entries). Searches were performed without any molecular weight or isoelectric point restrictions, trypsin was selected as the enzyme, carbamidomethylation of cysteine (+57 Da) and oxidation of methionine (+16 Da) were set as variable modifications and mass tolerances on precursor and fragment ions of 20 ppm and 0.07 Da were used, respectively. Mascot (.dat) results were loaded into the Scaffold software (v2.2.0; Proteome Software Inc., Portland, USA) and filtered to evaluate the false discovery rate (Elias and Gygi, 2007). Protein identification was confirmed when at least two peptides with high-quality MS/MS spectra (less than 10 points below Mascot's threshold score of identity at a 95% confidence level) were identified. A more stringent filter was applied for single peptide identifications, the score of the unique peptide had to be higher than 20 points above the Mascot's threshold score of identity at the 95% confidence level. These thresholds led to protein identification with a false discovery rate of less than 1.5%.

Bioinformatic analysis

Unknown protein sequences were analysed to find conserved domains/regions/motifs using Pfam (http://pfam.sanger.ac.uk) and CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) databases. These sequences were also blasted using the DELTA-BLAST algorithm (http://blast.ncbi.nlm.nih.gov/) to search for homology with homologous proteins present in the databases. The SignalP algorithm was used to search for putative signal peptides (http://www.cbs.dtu.dk/services/SignalP/). Functional categorization of proteins was carried out according to both the Fun-CatDB functional catalogue (http://mips.helmholtz-muenchen.de/proj/funcatDB) and the Gene Ontology database (http://www.ebi.ac.uk/QuickGO).

Electron microscopy

For TEM analysis, unpollinated olive pistils were processed as described previously (Suárez *et al.*, 2012). For scanning electron microscopy (SEM) analysis, unpollinated olive pistils were fixed as described above and post-fixed in 1% (w/v) osmium tetroxide in 100 mM sodium cacodylate buffer (pH 7.2) for 2 h at 4 °C and dehydrated in a graded ethanol series. The samples were then subjected to critical point drying in a CPD-7501 Drier (Polaron Equipment Ltd, UK), mounted on SEM stubs with double-sided tape, and coated with carbon and gold in an E5000 Sputter Coater (Polaron Equipment Ltd). Samples were observed and photographed in an FEI Quanta 400 FEG ESEM/EDAX Pegasus X4 microscope (Zeiss, Germany), operating at 15 kV.

Results and discussion

Proteome wide-analysis reveals novel proteins and functions of the plant SE

Lily and olive were chosen for this study on the basis of their divergent taxonomical position, pistil anatomy,

exudate composition, geographical distribution, cultivation purposes, and sampling procedure (Table 1). In lily, the SE was deposited as macroscopic drops on the stigma surface (Fig. 1A). In the olive, this secretion appeared as a thin layer of vesicular aspect that coated the stigma surface (Fig. 1B), which was only visible under the electron microscope (Fig. 1C–E). The protein profiles of the SE in several angiosperms showed quantitative and qualitative differences (Fig. 2). Because Eastern lily and olive genomes are not yet fully annotated, the MS/MS-based identification strategy was not trivial. We evaluated in parallel a classical approach using a database search algorithm (Mascot) and a *de novo* sequencing approach, best adapted for organisms not well represented in available protein databases. The latter strategy implies the individual treatment of the peptide fragmentation spectra to deduce a complete or partial peptide sequence tag and a subsequent sequence homology search using BLAST alignments (Castro *et al.* 2005; Catusse *et al.* 2008). The results of the two approaches were compared and the *de novo* sequencing approach did not allow identification of additional proteins when compared with classical database searches. It only allowed the identification of a few additional peptides and minor increases in sequence coverage of several proteins already identified using database search algorithms. Additionally, the statistical methods used to validate identifications by classical database search algorithms (target/decoy approaches) are not applicable when *de novo* sequencing is required. Merging identification results obtained with those two approaches is a difficult task. Therefore, only classical database search results are presented in this paper.

Our data illustrate the fact that plant proteomes are starting to be very well represented in available protein databases, which makes classical database search algorithms applicable, even for not yet fully sequenced and annotated plant species. Thus, using this approach, we identified 51 different proteins in lily and 57 in olive, of which only 13 were present in both SEs (Supplementary Table S2 at *JXB* online). Lily and olive proteins were classified into 64 and 81 families, respectively, according to the Pfam database (Bateman *et al.*, 2004), of which only 19 were identified in both exudates (Supplementary Table S2). Four proteins identified in lily (chitinase, cysteine proteinase, α-galactosidase, and α-mannosidase), another four in olive (invertase, fructose bi-P aldolase and ATPase α- and β-subunits), and six in both

Table 1. *Main features of plants chosen for this study*

| Feature	Lily	Olive
Taxonomy	Monocots	Dicots
Type of stigma	Wet/Papillary	Wet/Papillary
Exudate composition	Aqueous	Lipidic
Stigma/style anatomy	Hollow	Solid
Pollination	Entomophilous	Anemophilous
Geographical distribution	Japan to Philippines	Mediterranean
Cultivation purpose	Ornamental	Agricultural
Sampling method	Pipetting	Brushing
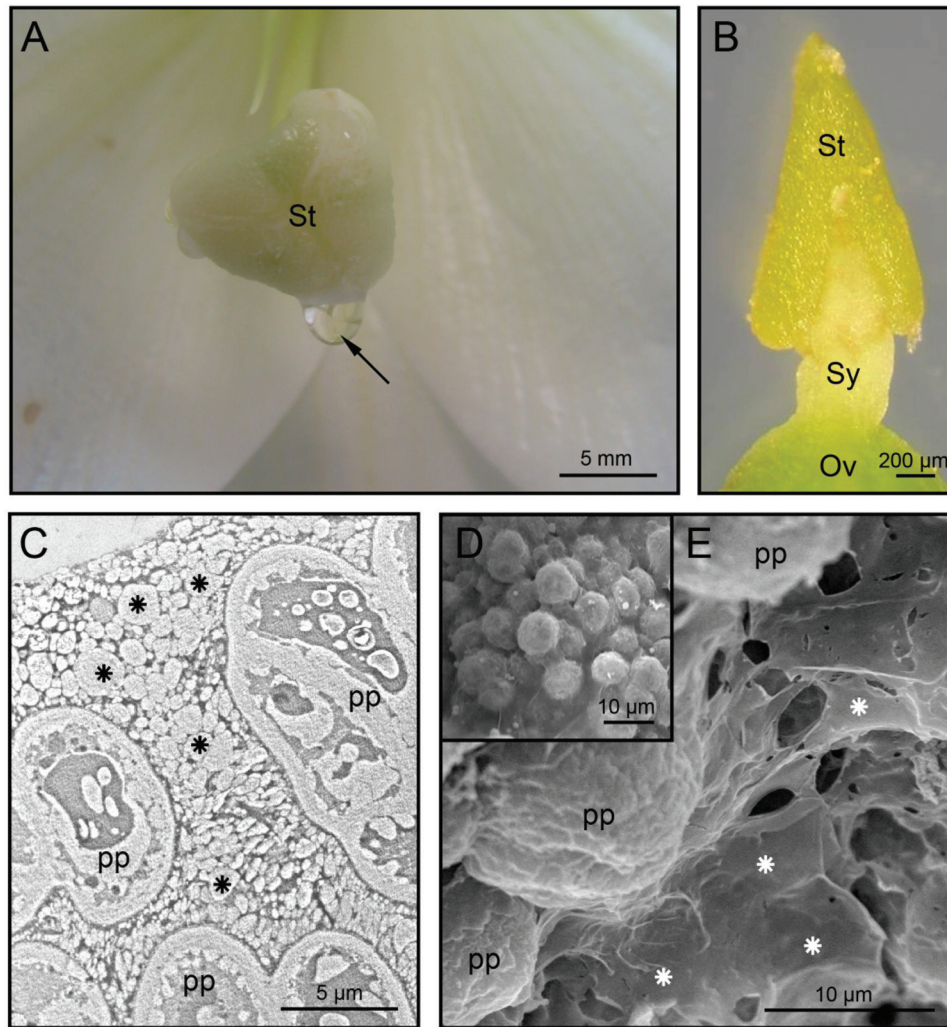
Fig. 1. The lily and olive SE. (A) Macroscopic drops of exudate (arrow) formed on the lily stigma surface. (B) Morphology of the olive stigma. (C) TEM photomicrograph showing the olive SE (asterisks) at anthesis. (D, E) SEM photomicrographs of the olive SE (asterisks) as above. Ov, ovary; pp, papillae; St, stigma; Sy, style. (This figure is available in colour at *JXB* online.)

species (malate dehydrogenase, triose-phosphate isomerase, endo-β-1,3-glucanase, Hsp70, LRR receptor kinase and thaumatin-like protein) were recently reported in tobacco stigma surface eluates (Sang *et al.*, 2012) (Fig. 3). Despite our data suggesting functional divergences between lily and olive SEs, a laborious process of experimental validation would be necessary to probe such differences. Instead, proteomics profiling discrepancies might rely on the fact that both species are not fully annotated in databases; hence, some peptides could not be identified. Moreover, sampling methods also differed between the two species studied. Thus, several proteins identified in the olive SE (e.g. apoplastic invertase, accession no. 359431001) might instead be associated with the cell wall/apoplast and have been collected during sampling.

About 70% of lily and 35% of olive proteins (Supplementary Table S2) were predicted to contain a signal peptide (SignalP v4.0 Server; Petersen *et al.*, 2011). Secretion of signal peptide-containing proteins might occur by the canonical endoplasmic reticulum–Golgi exocytic pathway. Indeed, both the lily and the olive stigmatic papillae possess the ultrastructural features typical of secretory cells (Miki-Hiroshige *et al.*, 1987; Suárez *et al.*, 2012). However, about 30% of matched proteins in Eastern lily and up to 65% in olive lacked a signal peptide. Similarly, about 45 and 80% of proteins present in *Arabidopsis* cell culture and pea root cap secretomes, respectively, lack an identifiable signal sequence (Oh *et al.*, 2005; Wen *et al.*, 2007). Most of the SE proteins were actually identified in other plant species. Moreover, signal peptide-predicting algorithms are subject to failure. Therefore, contrary to predictions, these lily and olive proteins may indeed contain signal sequences.

Based on ontological classifications (Ruepp *et al.*, 2004; The Gene Ontology Consortium, 2010), we found that the SE might be involved in at least 80 different biological processes and 97 molecular functions (Supplementary Table S2). Major functional categories included one-carbon and carbohydrate metabolism, protein fate, cell-wall organization, cell signalling, and response to biotic and abiotic stresses (Fig. 4). Below, we discuss the most outstanding findings revealed by the present study in the context of sexual reproduction in angiosperms.

A

B
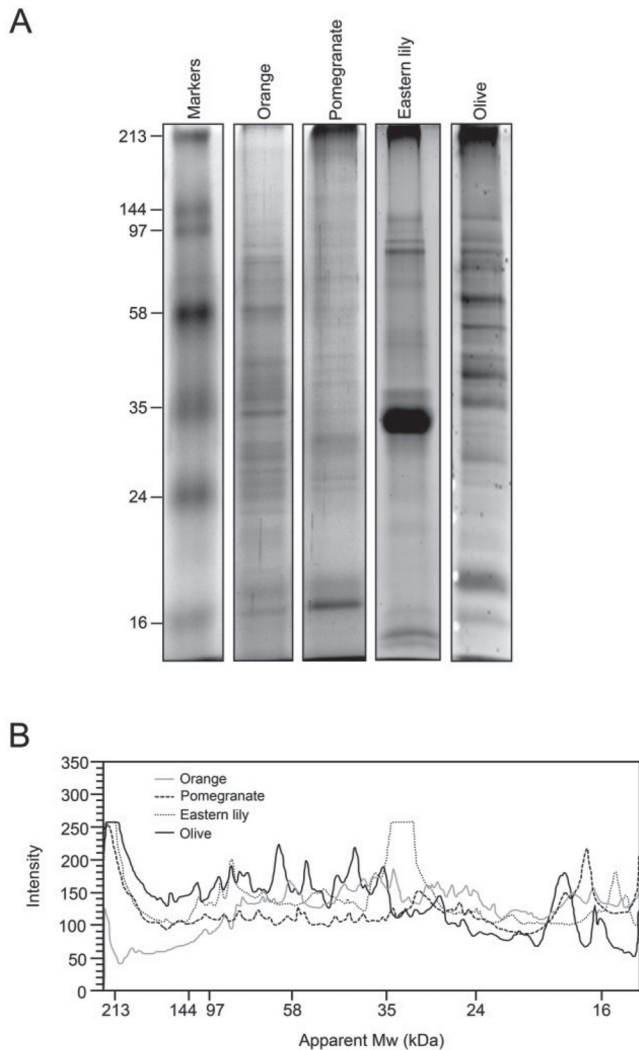
Fig. 2. Protein profile of the SE in several plant species. (A) Exudate protein samples (~15 µg per lane) were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Protein markers are displayed on the left. (B) The gel band intensities were profiled for each species.

The SE proteome shows a markedly catabolic profile

O-Glycosyl hydrolases (GHs) were by far the most abundant group of proteins in the lily SE. This indicated the importance of polysaccharide catabolism. Indeed, arabinogalactan proteins (AGPs) and pectins are the major components of lily SE (Labarca *et al.*, 1970). In a pioneer study, Labarca and Loewus (1972) demonstrated that lily pollen tubes uptake carbohydrate wall precursors from the SE. In this context, a plausible scenario would be one in which some GH enzymes such as β-galactosidases, β-xylosidases, and α-arabinofuranosidases would contribute to degrade large polysaccharides secreted by papillae to smaller units, allowing their incorporation into the pollen tube. We also identified an endo-β-mannosidase, which hydrolyses the Manβ1-4GlcNAc linkage in the trimannosyl core structure of *N*-glycans (Ishimizu *et al.*, 2004), as well as a putative α-mannosidase involved in the hydrolysis of Manα1-3Man
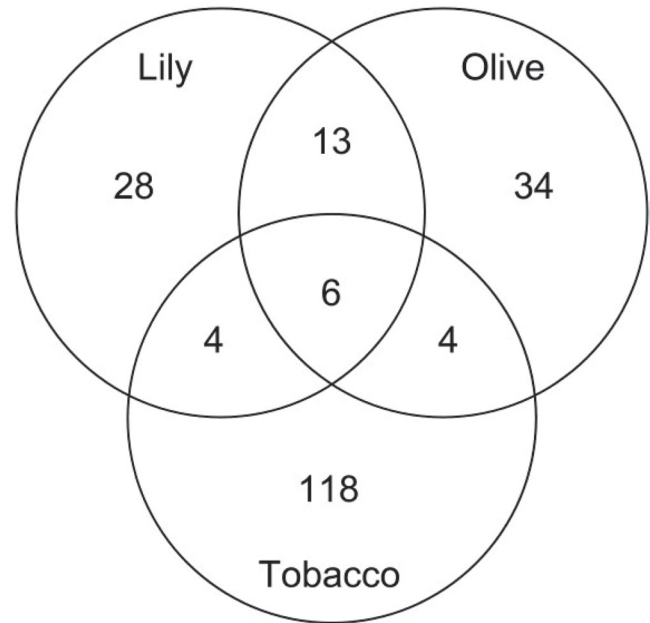

Fig. 3. Venn diagram comparing the total number of proteins identified in Eastern lily, tobacco, and olive SEs. Tobacco data from Sang *et al.* (2012).

and Manα1-6Man linkages (Hossain *et al.*, 2009). Based on our findings, it is tempting to suggest that these two mannosidases might cooperate to degrade high-mannose-type *N*-glycans to GlcNAcβ1-4GlcNAc on glycoproteins secreted by the stigma. For instance, *N,N´*-diacetyl chitobiose (GlcNAcβ1-4GlcNAc) is a major *N*-glycan of S-RNase, a glycoprotein associated with gametophytic self-incompatibility (Woodward *et al.*, 1992).

A second group of hydrolases in the lily SE consisted of two classes of proteolytic enzymes, namely cysteine and aspartic proteases. Proteases are normally found at different intracellular locations, but various ones accumulate extracellularly in the transmitting tissue (Vieira *et al.*, 2001). Accordingly, they might participate in limited proteolysis reactions important for pollen–pistil interaction events including pollen adhesion and recognition. Alternatively, these proteases might generate or modify precursors or signals for directional tube growth.

Finally, a third group of hydrolases included GDSL-like lipases, which were identified in the SE of both species. Extracellular GDSL lipases have been identified in several plant secretions including nectar (Kram *et al.*, 2008). Pollen coat lipids modulate water transfer to desiccated pollen in dry-type stigmas (Wolters-Arts *et al.*, 2002). The lipid-rich exudate of wet stigmas is thought to be functionally analogous to the pollen coat. Interestingly, a pollen coat GDSL-like lipase is necessary for efficient hydration of *Arabidopsis* pollen (Updegraff *et al.*, 2009). In this context, GDSL-like lipases might promote pollen hydration by modifying lipid composition at the pollen–SE interface. As suggested previously for nectar GDSL-like lipases, it is also plausible that these enzymes might function in preventing microbial growth (Kram *et al.*, 2008).
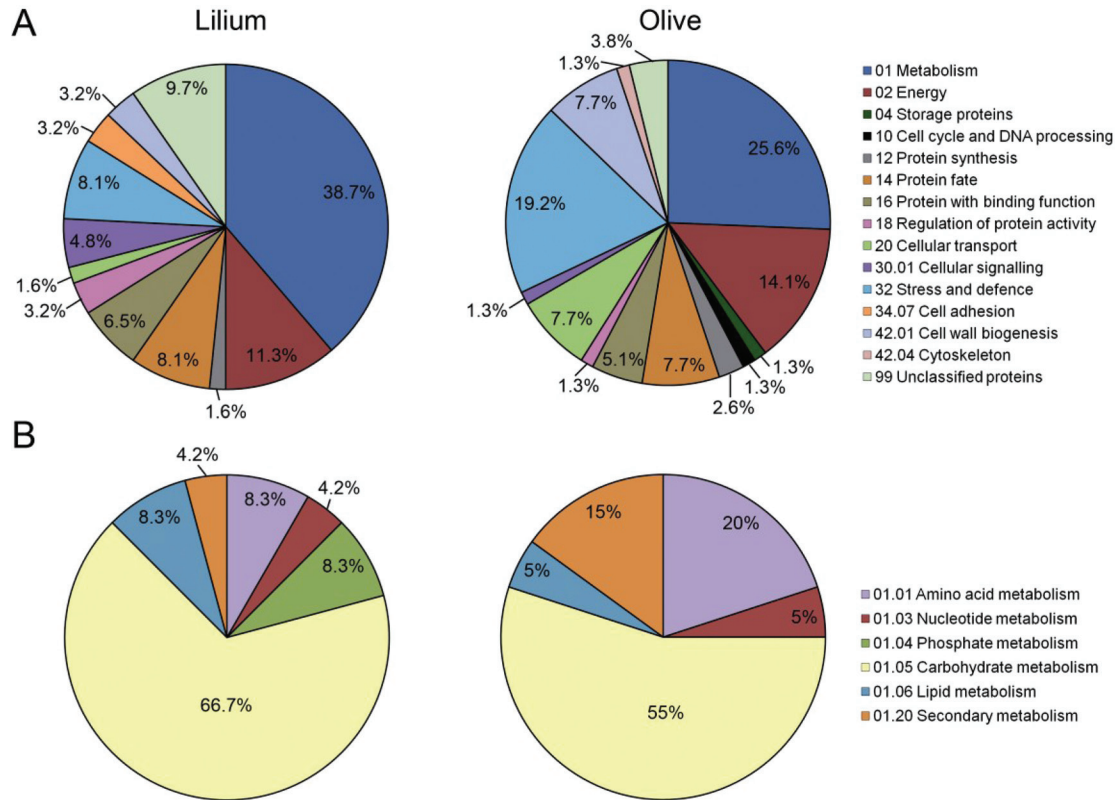
Fig. 4. Distribution of functional classes of lily and olive stigma exudate proteins. (A) Pie chart of main functional categories (Ruepp *et al.*, 2004). (B) Pie chart of secondary functional categories referred to metabolism (Ruepp *et al.*, 2004). The percentage of proteins in each category is shown.

Is there cross-talk between genetic programmes regulating stress/defence and pollination responses in the stigma?

The sugar-enriched SE is particularly susceptible to infection by fungi and bacteria. This fact may explain the presence of several pathogenesis-related protein families and other defence-related proteins. However, these defence-related genes might also function in response to pollination. For instance, chitinases might be involved in the degradation of AGPs, and the released carbohydrates might function as signalling molecules (Van Hengel *et al.*, 2002). Moreover, the stigmas of healthy mature petunia flowers show β-1,3-glucanase activity, but this enzyme has no antifungal activity (Wakelin and Leung, 2009). Papain (C1)- and subtilisin-like proteases are also involved in local and systemic defence responses, including that against incompatible pollen (van der Hoorn, 2008). The pollen of lily and other plants releases cyclophilins to the extracellular medium under unfavourable conditions (Yokota *et al.*, 2004), and pollen germination is inhibited in the medium into which cyclophilins are released. Some other identified proteins including lectins, protein inhibitors of cell-wall degrading enzymes [e.g. polygalacturonase-inhibiting proteins (PGIPs) and pectin methylesterases (PME) inhibitors (PMEIs)], and reactive oxygen species detoxifying enzymes might also function in response to pollination (Wilkins *et al.*, 2011).

Interestingly, several aspects of sexual reproduction in the olive, such as stigmatic receptivity, pollen performance, and self-incompatibility relationships, are notably affected by temperature (Koubouris *et al.*, 2009). In response to heat stress, plants synthesize a group of proteins called heat-shock proteins (HSPs), which act as molecular chaperones for other proteins. Interestingly, the olive stigma secretes HSP60, HSP70, and HSP90 proteins. Extracellular HSPs are emerging as important mediators in cell signalling in response to stress (Calderwood *et al.*, 2007). These proteins might also modulate temperature-dependent reproductive processes in the olive.

The SE may regulate pollen-tube growth by selective degradation of cell-wall polysaccharides

Several enzymes secreted into the SE might regulate pollen tube growth in the stigma through selective degradation of tube wall pectins (Table 2). Polygalacturonases hydrolyse homogalacturonans to oligogalacturonides, which can act as signalling molecules (Ridley *et al.*, 2001). The exogenous addition of polygalacturonase to the culture medium promotes early pollen-tube wall extension, probably as result of an increase in plasticity in the tip region (Roggen and Stanley, 1969). PMEs found in the SE of olive and lily contain N-terminal pro-regions with homology to PMEIs. Interestingly, a recent study suggested that PMEIs might be internalized by endocytosis at the flanks of the pollen tube tip, regulating pollen-tube wall stability by locally inhibiting pollen PME activity (Röckel *et al.*, 2008).

Table 2. *List of cell-wall-modifying enzymes identified in the stigma exudates of lily and olive*

Olive[a]	Lily[a]	Cell-wall process
Dhurrinase (*Sorghum bicolor*) [1127575]	–	Cellulose, xyloglucan catabolism
β-Glucosidase (*Glycine max*) [356565758]	β-glucosidase (*Picea sitchensis*) [116786797]	Cellulose, xyloglucan catabolism
–	β-Xylosidase/α-arabinofuranosidase (*Medicago sativa*) [292630922]	Arabinan catabolism
–	Exo-1,3-β-glucanase (*Lilium longiflorum*) [46091271]	1,3-β-glucan (callose) catabolism
UDP-glucose 6-dehydrogenase (*Arabidopsis lyrata*) [297805822]	–	Unknown
Xyloglucan endotransglucosylase (*Actinidia deliciosa*) [187372962]	–	Xyloglucan catabolism
Endo-1,3-β-glucanase (*Olea europaea*) [75165700]	Endo-1,3-β-glucanase (*Zea mays*) [223945355]	1,3-β-glucan (callose) catabolism
–	α-Galactosidase (*Selaginella moellendorffii*) [302753418]	Galactomannan catabolism
Polygalacturonase (*Ricinus communis*) [255553564]	Polygalacturonase (*Vitis vinifera*) [359480238]	Homogalacturonan catabolism
–	β-Galactosidase, GH family 35 (*Sorghum bicolor*) [242090613]	Galactan catabolism
–	β-Galactosidase, GH family 42 (*Cucumis sativus*) [449489943]	Galactan catabolism
Pectinesterase (*Olea europaea*) [449061779]	Pectinesterase (*Citrus sinensis*) [2098713]	Homogalacturonan catabolism
Pectin acetylesterase (*Ricinus communis*) [255557763]	–	Homogalacturonan catabolism
Lipid transfer protein (*Olea europaea*) [154257299]	–	Cellulose, xyloglucan catabolism
–	Glycerophosphodiester Pdiesterase (*Ricinus communis*) [255547185]	Unknown
α-1,4-Glucan-protein synthase (*Picea sitchensis*) [116779321]	–	Cellulose biosynthesis

[a]The accession no. in Supplementary Table S2 is given in parentheses.

According to our findings, it is tempting to suggest that these PMEI isoforms might be of stigmatic origin. A PGIP was identified in the olive SE, but whether this protein regulates polygalacturonase activity in the stigma is unknown. Finally, a pectin acetylesterase was also identified in the olive SE. This enzyme has been suggested to function as an important structural regulator by modulating the status of pectin acetylation, thereby affecting cell extensibility (Gou *et al.*, 2012). In addition to pectinases, bifunctional α-arabinosidases/β-xylosidases and β-galactosidases might be also involved in degradation of arabinan and galactan side chains of pectins, respectively.

Two classes of enzyme activities associated with callose degradation were present in the SE (Table 2). Thus, endo-1,3-β-glucanases are involved in cleavage of glucan polymers to glucooligosaccharides, which, in turn, are degraded to glucose units by exo-1,3-β-glucanases. Application of endo-1,3-β-glucanase to the culture medium at the beginning of germination increases pollen-tube growth rate, probably as result of callose degradation inside the apertural intine (Roggen and Stanley, 1969). On the other hand, exo-β-1,3-glucanases might be involved in controlled degradation of callose just behind the pollen tube tip to facilitate tip expansion, to prevent the tip from clogging and/or to increase the porosity of the callosic wall for pollen tube–stigma communication (Takeda *et al.*, 2004).

A recent study showed that cellulose has a role in the mechanical stabilization of the pollen tube subapical transition region (Aouar *et al.*, 2010). Addition of cellulase at low concentration to the germination medium has similar effects to pectinase (Roggen and Stanley, 1969). Accordingly, we found putative cellulose degrading β-glucosidases in the SE of both species, as well as a dhurrinase and an LTP in the olive SE (Table 2). Controlled digestion of cellulose might release trapped xyloglucans, resulting in enhanced wall extensibility (Ohmiya *et al.*, 2000). Cellulose microfibrils form a stable three-dimensional network by interacting with hemicelluloses. Therefore, the dynamic nature of this cross-linking might also be a major factor modulating the tube wall expansion rate. The SE also contains a set of enzymes involved in hemicellulose restructuration. Thus, the action of xyloglucan endotransglucosylase and β-glucosidase might release xyloglucan oligosaccharides and cause the cell-wall loosening (Fry *et al.*, 1992). In addition, α-xylosidase and β-galactosidase might cleave xylosylated and galactosylated xyloglucan oligosaccharides. Studies on *Arabidopsis* pollen have shown that xylosidases are involved in cell expansion during pollen germination (Hrubá *et al.*, 2005). Other putative cell-wall-loosening enzymes identified in the SE included several LTPs in olive. These proteins are purportedly involved in pollen-tube growth regulation (Nieuwland *et al.*, 2005). We also identified a glycerophosphoryl diester phosphodiesterase (GDP) in the lily SE. Interestingly, SHV3, a GDP-like protein required for root hair tip growth, was described as a novel factor involved in cell-wall organization (Parker *et al.*, 2000).

Pollen-tube adhesion and guidance is mediated by SE proteins

Cell–cell adhesion is one of the key features for proper pollen germination and pollen-tube growth through the pistil tissues. Several proteins identified in the lily SE are involved in cell–cell adhesion and chemotropic phenomena, including two SCA isoforms, a chemocyanin protein, and a fasciclin-like domain (FAS) protein. The SCA protein is a small cysteine-rich LTP necessary for pollen-tube adhesion to the stylar matrix (Park *et al.*, 2000). Chemocyanin is a small basic protein that induces pollen chemotropism and its activity is potentiated by SCA (Kim *et al.*, 2003). Two LTPs were also identified in the olive SE. The fasciclin-like domain protein identified in lily shows homology with a group of AGPs, called fasciclin-like AGPs (FLAs), which have been shown to function as adhesion molecules in cell walls (Shi *et al.*, 2003). Plant lectins sited at the cell surface or extracellularly are rare, but a function in cell adhesion cannot be ruled out. Finally, polygalacturonases and other cell-wall-remodelling enzymes may also regulate pollen-tube adhesion by selective degradation of cell-wall pectins (Atkinson *et al.*, 2002).

Cell–cell recognition and signalling in the SE

Some of the proteins identified in the SE are typically involved in cell–cell recognition and signalling. Thus, we identified receptor-like protein kinases in the SE of both lily and olive. Interestingly, the first signal transduction mechanism described in pollen comprised two receptor-like protein kinases (RLKs), namely LePRK1 and LePRK2 (Muschietti *et al.*, 1998). In tomato, the extracellular domain of LePRK2 interacts with a pollen cysteine-rich extracellular protein called LAT52 (Tang *et al.*, 2002). LePRK1 and LePRK2 associate in mature pollen, but dissociate in the presence of the stigmatic factor LeSTIG1, which could displace binding of LAT52, thus triggering an autocrine signalling cascade in the pollen tube (Tang *et al.*, 2004).

The lily SE also contained a phospholipase C (PLC). There is evidence suggesting that PLCs are involved in lipid-mediated modulation of reproductive processes such as pollen-tube growth in petunia (Dowd *et al.*, 2006) and self-incompatibility in poppy (Franklin-Tong *et al.*, 1996). However, no extracellular PLCs have been described in plants to date. Interestingly, *Pseudomonas aeruginosa* secretes an extracellular PLC that is required for phospholipid chemotaxis (Barker *et al.*, 2004).

The olive SE also contained a 14-3-3 protein. These proteins function as cellular integrators of different signalling pathways (DeLille *et al.*, 2001). The presence of secreted 14-3-3 proteins has been reported in the extracellular matrix of the green alga *Chlamydomonas* (Voigt and Frank, 2003) and in pea root tip exudate (Wen *et al.*, 2007). In *Chlamydomonas*, secreted 14-3-3 proteins appear to play a role in cross-linking of hydroxproline-rich glycoproteins in the cell wall (Voigt and Frank, 2003). The presence of a 14-3-3 protein in the olive SE suggests interactions with different targets. The search for protein partners will help to elucidate the function of this protein in the SE. A PGIP was also identified in the olive SE. Because olive pollen secretes a polygalaturonase (unpublished data), its interaction with the stigma PGIP may constitute a recognition signal between the pollen and the stigma in this species.

Regulation of reactive oxygen species is key for plant reproduction (McInnis *et al.*, 2006; Wilkins *et al.*, 2011). Several antioxidant enzymes were identified in the SE, including a Cu/Zn-superoxide dismutase and a putative bifunctional acid phosphatase/peroxidase enzyme in lily, and an annexin in olive. Stigmatic peroxidases might participate indirectly through H_2O_2 catabolism in signalling networks that modulate, for instance, species–species pollen recognition (McInnis *et al.*, 2006). Interestingly, some plant annexins exhibit peroxidase activity (Laohavisit *et al.*, 2009). However, other functions cannot be ruled out. For instance, annexins might modulate Ca^{2+} influx, as shown recently in maize (Laohavisit *et al.*, 2009).

Programmed cell death at the stigma: a putative role for SE proteins

Senescence is the final stage of stigma development in unpollinated pistils (Suárez *et al.*, 2012). Moreover, in pollinated pistils, stigmatic cells undergo programmed cell death after both compatible pollination and incompatible pollen rejection (Wu and Cheung, 2000). The lily SE contained several proteins potentially involved in programmed cell death including several proteases and their inhibitors (Panavas *et al.*, 1999), an S1-type nuclease homologous to SAP6 (senescence-associated protein 6) from *Hemerocallis* (Solomon *et al.*, 1999), and a trehalase (Fernandez *et al.*, 2010).

Unexpected proteins and functions in the SE

Among the unexpected proteins, an *N*-acetylglucosaminidase involved in the remodelling of *N*-acetylglucosamine (GlcNAc) residues of extracellular proteoglycans and a DUF642 protein were identified in the lily SE. The heparan sulphate polysaccharide has not been found in plants so far, but some AGPs present in the extracellular matrix contain GlcNAc residues (van Hengel *et al.*, 2001). Therefore, it is likely that the substrate of this enzyme possesses signalling/regulatory properties. DUF642 proteins interact with cell-wall polysaccharides *in vitro* (Vázquez-Lobo *et al.*, 2012). We also identified actin, a few enzymes of the methylation cycle, calnexin and calreticulin proteins, and several membrane-bound ATPases in the olive SE. All these proteins were identified previously in the pea root cap secretome (Wen *et al.*, 2007). Actin is associated with plasmodesmata within the cell wall (White *et al.*, 1994). Both SEs also contained translational elongation factors and several intracellular glycolytic enzymes. Interestingly, the plant extracellular adhesion vitronectrin-like 1 protein (PVN1) is nearly identical to the translational elongation factor 1α (Zhu *et al.*, 1994). Moreover, some glycolytic enzymes have been found in apoplastic (Chivasa *et al.*, 2005) and extracellular (Oh *et al.*, 2005; Wen *et al.*, 2007) fluids, but their biological relevance is unknown.

Supplementary data

Supplementary data is available at *JXB* online.

Supplementary Fig. S1. (A) Transmission electron microscopy (TEM) photomicrograph of an olive stigma before pollination showing papillae ultrastructure and the presence of a copious exudate covering its surface. (B) TEM photomicrograph as above showing intact papillae after exudate collection by brushing.

Supplementary Table S1. Raw data files from nanoLC-MS/MS analysis of Eastern lily and olive stigma exudates.

Supplementary Table S2. List of *Lilium* and olive stigmatic exudate proteins identified by nanoLC-MS/MS analysis.

Acknowledgements

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