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RESEARCH PAPER

Differential expression patterns of arabinogalactan proteins in *Arabidopsis thaliana* reproductive tissues

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Abstract

Arabinogalactan proteins (AGPs) are heavily glycosylated proteins existing in all members of the plant kingdom and are differentially distributed through distinctive developmental stages. Here, we showed the individual distributions of specific *Arabidopsis* AGPs: AGP1, AGP9, AGP12, AGP15, and AGP23, throughout reproductive tissues and indicated their possible roles in several reproductive processes. AGP genes specifically expressed in female tissues were identified using available microarray data. This selection was confirmed by promoter analysis using multiple green fluorescent protein fusions to a nuclear localization signal, β-glucuronidase fusions, and *in situ* hybridization as approaches to confirm the expression patterns of the AGPs. Promoter analysis allowed the detection of a specific and differential presence of these proteins along the pathway followed by the pollen tube during its journey to reach the egg and the central cell inside the embryo sac. *AGP1* was expressed in the stigma, style, transmitting tract, and the chalazal and funiculus tissues of the ovules. *AGP9* was present along the vasculature of the reproductive tissues and *AGP12* was expressed in the stigmatic cells, chalazal and funiculus cells of the ovules, and in the septum. *AGP15* was expressed in all pistil tissues, except in the transmitting tract, while *AGP23* was specific to the pollen grain and pollen tube. The expression pattern of these AGPs provides new evidence for the detection of a subset of specific AGPs involved in plant reproductive processes, being of significance for this field of study. AGPs are prominent candidates for male–female communication during reproduction.

Key words: Arabinogalactan proteins, female gametophyte, funiculus, pistil, pollen tube guidance, transmitting tract.

Introduction

All flowering plants share a common characteristic that distinguishes them from all other organisms that reproduce sexually: double fertilization (Raghavan, 2003). During this process, two male sperm cells are delivered to the female gametophyte—the embryo sac—where one fuses with the egg and the other fuses with the central cell, giving rise to the embryo and the endosperm, respectively (Russell, 1992). In order for the sperm cells to be delivered into the embryo sac,

Abbreviations: AG, arabinogalactan; AGP, arabinogalactan protein; DAPI, 4′,6-diamidino-2-phenylindole; DIG, digoxygenin; FISH, fluorescence *in situ* hybridization; FLA, fasciclin-like AGPs; GPI, glycosylphophatidylinositol; GUS, β-glucuronidase; NJ, neighbour joining; NLS, nuclear localization signal; RT-PCR, reverse transcription-PCR.

several events need to occur, which implicates tightly regulated interactions between the female sporophytic tissues and the male gametophyte. Once the pollen grain is in contact with the stigmatic cells, it germinates, producing the pollen tube (Kandasamy *et al.*, 1994), which will deliver the two sperm cells to their final destination (Faure *et al.*, 2002; Dresselhaus and Franklin-Tong, 2013). In the majority of seed plants, the pollen tube grows through the stigmatic cells, into the style, and across the extracellular matrix of the transmitting tissue in a very precise way, never losing its focus, to reach the embryo sac. Once at the placenta, it makes a quick turn and grows on the surface of the funiculus until reaching the ovule opening, the micropyle (Hülskamp *et al.*, 1995). After growing through the micropyle, the pollen tube enters the female gametophyte, interacts with one of the two synergid cells and bursts, releasing the two sperm cells that will fuse with the central and the egg cell, ultimately giving rise to the seed and assuring the perpetuation of the next generation (Johnson and Preuss, 2002; Lord and Russell, 2002; Raghavan, 2003; Berger *et al.*, 2008; Sprunck, 2010; Palanivelu and Tsukamoto, 2012).

During the course of all these processes, numerous cell–cell communication events must take place between different cell types. Mainly, recognition signals and attracting signals have to be sent and perceived by the female tissues and the male tissues of the plant, and vice versa, in order for a successful fertilization to occur (Dresselhaus, 2006). To date, despite all the efforts carried out in this field of study, little information is available about which molecules function as signalling or receptor molecules.

Arabinogalactan proteins (AGPs) constitute a large family of hydroxyproline-rich proteins that are highly glycosylated and structurally complex (Showalter, 2001). AGPs are widely distributed in the plant kingdom, being ubiquitously present in land plants and also in the Bryophyta *Physcomitrella patens* (Lee *et al.*, 2005; Fu *et al.*, 2007) and in all Hepatophyta (Basile *et al.*, 1989), including basal angiosperms (Costa *et al.*, 2013*b*) and many algae, indicating an ancient origin for these proteins (Popper *et al.*, 2011).

They are found in distinct developmental stages and cell, tissue, and organ types, being mostly abundant in cell walls, plasma membranes, and extracellular secretions (Majewska-Sawka and Nothnagel, 2000). AGPs are typically divided into four subgroups according to their polypeptide core characteristics: the classical AGPs, which possess an N-terminal signal peptide that is removed in the mature protein, a proline/hydroxyproline-rich domain and a C-terminal signal for the addition of a glycosylphophatidylinositol (GPI) anchor; the arabinogalactan (AG) peptides, structurally similar to the classical AGPs but with a smaller protein backbone, consisting of 10–13 aa; the lysine-rich AGPs, with one or more lysine domains; and the fasciclin-like AGPs (FLAs) with one or more fasciclin-like domains in their polypeptide core (Schultz *et al.*, 2002; Johnson *et al.*, 2003).

AGPs have been implicated in many important processes for plant development and growth, such as cell expansion, proliferation and differentiation, cell–cell recognition, somatic embryogenesis, pollen tube growth, programmed cell death, seed germination, and resistance to infection (Majewska-Sawka and Nothnagel, 2000). Most AGPs are predicted to be anchored to the membrane by a GPI anchor (Borner *et al.*, 2002; Schultz *et al.*, 2004), which provides a way for the AGPs to function as signalling molecules. After comparisons with GPI -anchored proteins from animal cells, two mechanisms were proposed for AGP-mediated signalling: the first consisted of the cleavage of the GPI anchor by specific phospholipases (C and D) that would release the glycoprotein into the extracellular matrix, making it able to act as a signal itself or to be subject to further processing, generating different signals; the other mechanism proposed that AGPs could interact with other proteins and activate downstream signal transduction pathways (Gaspar *et al.*, 2001; Schultz *et al.*, 2004). Besides the hint given by the presence of the GPI anchor, implying a signalling role for these proteins, the prominent carbohydrate content surrounding the core protein also led to some assumptions about their involvement in signalling mechanisms. The importance of sugars as signalling molecules in plants is well known, and, according to some authors, the varied carbohydrate moieties of AGPs might be released via cleavage by specific enzymes (Showalter, 2001). The generated oligosaccharides might function as signalling molecules by binding to specific membrane receptors and activating specific signal transduction systems (Showalter, 2001). The fact that AGPs can act as chitinase substrates, being able to stimulate somatic embryogenesis, reinforces this hypothesis, although it has not yet been demonstrated whether this is an effect of the released oligosaccharides or the modified AGP (Van Hengel *et al.*, 2001).

AGPs have long been suggested to play important roles in sexual plant reproduction. Earlier studies have shown the developmentally regulated enrichment of AGPs in the extracellular matrix of the transmitting tract of several species such as *Gladiolus gandavensis*, *Lilium longiflorum*, *Nicotiana alata*, and *Lycopersicon peruvianum* (Hoggart and Clarke, 1984; Sedgley *et al.*, 1985; Webb and Williams, 1988; Gane *et al.*, 1995). AGPs have also been implicated in pollen tube growth from the stigma to the ovules in *Amaranthus hypochondriacus*, *Actinidia deliciosa*, *Catharanthus roseus*, and *Nicotiana tabacum* (Coimbra and Salema, 1997; Cheung *et al.*, 1995; Coimbra and Duarte, 2003). These studies were carried out using the β-glycosyl Yariv reagent that binds specifically to AGPs, precipitating them (Yariv *et al.*, 1967), or using monoclonal antibodies that identify only the glycosidic epitopes of AGPs (Pennell *et al.*, 1989, 1991; Knox *et al.*, 1991). These two approaches have given us information about the distribution and localization of AGPs (Coimbra *et al.*, 2007) and clues about their possible roles (Gao and Showalter, 2002; Sardar *et al.*, 2006), although they allow only the detection of general AGPs and not a specific AGP. The recent discovery that the Yariv reagent binds specifically to the β-1,3-galactooligosaccharides of AGPs (Kitazawa *et al.*, 2013) may bring new insights to the possible mode of action of AGP oligosaccharides as signalling molecules. It will be interesting to check whether this particular oligosaccharide is important for many of the physiological processes impaired when Yariv was used in different studies, or if Yariv only hampers the ability of AGPs to function by precipitating them.

Here, we report the use of several constructs to explore the tissue and cell-specific promoter activity of specific *AGP*s. We have focused on those AGPs that are particularly present along the pollen tube pathway and other female reproductive tissues, according to the available microarray data. With this, we aimed to complement work that has already been done by our group describing AGPs as molecular markers of different stages of *Arabidopsis* sexual reproductive processes (Coimbra *et al.*, 2007).

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh. seeds, ecotype Columbia were obtained from the Nottingham Arabidopsis Stock Centre, UK. Plants were sown on soil, kept for 2 d at 4 °C in the dark to induce stratification, and afterwards they were grown at 22 °C under a short-day photoperiod (9/15 h light/dark cycles) for 4 weeks, followed by a long-day photoperiod (16/8 h light/dark cycles) to induce flowering, with 60% relative humidity. For phosphinothricin acetyltransferase selection, the seedlings were sprayed with 200 mg l^{-1} of glufosinate ammonium (BASTA®; Bayer Crop Science) supplemented with 0.1% Tween 20 three or four times every 2 d, over a 10-day period.

Construct generation and plant transformation

Genomic regions corresponding to the promoters of five *AGP*s: *AGP1*, *AGP9*, *AGP12*, *AGP15*, and *AGP23*, were amplified using Phusion DNA polymerase (Thermo Scientific), with the primer pairs described in Supplementary Table S1 (at *JXB* online). The promoter regions were always amplified from the end of the untranslated region of the most proximal gene upstream of the respective *AGP* until its own start codon. For the genes with promoter regions of more than 3000 bp, genomic fragments of about 3000–3300 bp positioned upstream of the start codon of the *AGP* of interest were amplified. The PCR products were cloned into pENTR™/D-TOPO (Invitrogen). The resulting promoter fragments were subsequently transferred into a Gateway-compatible version (Zheng *et al.*, 2011) of the pGreenII-based vector NLS:3GFP:NOSt (Takada and Jürgens, 2007), termed pGII_GW:NLS:3GFP:NOSt. For *AGP1*, *AGP15*, and *AGP23* β-glucuronidase (GUS) constructs, the respective promoter fragments were cloned into the binary vector pBGWFS7 (Karimi *et al.*, 2002). All constructs were confirmed by DNA sequencing. The pGreenII-based expression vectors were introduced into *Agrobacterium tumefaciens* GV3101 harbouring the pGreenII helper plasmid pSOUP. All other expression vectors were delivered into *Agrobacterium tumefaciens* GV3101 (pMP90RK). They were all then used to transform *Arabidopsis thaliana* (Col-0) by the floral dip method (Clough and Bent, 1998).

Preparation of plant material for microscopy

Pistils kept in 50 mM sodium phosphate buffer (pH 7.5) were dissected under a stereomicroscope (model C-DSD230; Nikon) using hypodermic needles (0.4 × 20 mm; Braun). The opened carpels and the ovules that remained attached to the septum were maintained in mounting medium and covered with a cover slip.

Confocal laser-scanning microscopy

A Zeiss Axiovert 200M inverted microscope equipped with a confocal laser-scanning module (LSM 510 META) was used for confocal laser-scanning microscopy. Green fluorescent protein (GFP) was excited by 488 nm and detected with a BP 505–550 filter. Optical sections were generally between 0.40 and 0.50 µm each, observed at ×20, ×40 or ×63 magnification. Histology mounting medium Fluoroshield™ with 4′,6-diamidino-2-phenylindole (DAPI; Sigma) was used in order to detect the nuclei in the pollen grains. Images were captured and processed using an AxioCam HRc camera, Zeiss LSM 510 META software and a Zeiss LSM image browser version 3.5.0.359.

Detection of GUS activity

GUS assays were performed on inflorescences as described by Liljegren *et al.* (2000), overnight. After chemical GUS detection, the samples were incubated in clearing solution [160 g of chloral hydrate (Sigma-Aldrich), 100 ml of water, and 50 ml of glycerol] and incubated at 4 °C overnight. The next day, inflorescences were dissected under a stereomicroscope (model C-DSD230; Nikon) and observed under a microscope. A Zeiss AxioImager AZ microscope equipped with differential interference contrast optics was used. Images were captured with a ZeissAxiocam MRc3 camera using Zen Imaging Software.

Phylogenetic analysis

To generate a phylogenetic tree for the *AGP* genes, the amino acid sequences of AGP-coding sequences were aligned using Clustal W (Thompson *et al.*, 1994) and manually edited using Jalview to reduce gaps (Clamp *et al.*, 2004). A neighbour-joining (NJ) (Saitou and Nei, 1987) tree was generated using the MEGA4 program (Tamura *et al.*, 2007). The bootstrap values were obtained by 10 000 repetitions. Simultaneously, a maximum parsimony tree was generated using the same AGP amino acid sequences, and a NJ tree was also produced using only the three most conserved blocks of AGP amino acid sequences.

Preparation of plant material for RNA extraction

Arabidopsis pistils from wild-type plants were emasculated 1 d before anthesis and collected 2 d after the emasculation procedure. Pollen from wild-type *Arabidopsis* recently opened flowers was collected according to Costa *et al.* (2013*a*). *Arabidopsis* seeds were sown in half-strength Murashige and Skoog medium, complemented with 0.7% agar. Agar plates were kept for 2 d at 4 °C in the dark to induce stratification, and subsequently were transferred to a growth chamber at 22 °C under a long-day regime (16 h light/8 h dark), with irradiance of 130 µmol m^{-2} s^{-1} and 60% relative humidity. Seedlings were collected 4–5 d after germination.

RNA extraction, cDNA synthesis and real-time reverse transcription (RT)-PCR

Total RNA from emasculated pistils, pollen, and seedlings was extracted using PureZol™ RNA Isolation Reagent (Bio-Rad) following the manufacturer's instructions. DNA was removed by a DNase (Thermo Scientific) treatment. The isolated RNA samples were reverse transcribed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and oligo$(dT)_{18}$ primers to initiate the reactions, following the manufacturer's instructions.

cDNA was amplified using SSoFast™ SYBR® Green Supermix on an iQ5™ Real-Time PCR Detection System (Bio-Rad) using the primers listed in Supplementary Table S2 (at *JXB* online). Real-time RT-PCRs were run in duplicates. After 3 min at 95 °C followed by a 10 s denaturation step at 95 °C, samples were run for 40 cycles of 10 s at 95 °C and 30 s at 60 °C. After each run, a dissociation curve was acquired to check for amplification specificity by heating the samples from 60 to 95 °C. Serial dilutions of pure genomic DNA from *Arabidopsis* ecotype Columbia were used to set up a calibration curve, which was used to quantify plant DNA in each sample. At the end of the PCR cycles, data were analysed with iQ5 2.0, Standard Edition Optical System Software v2.0.148.060623 (Bio-Rad).

Fluorescence in situ *hybridization (FISH)*

Genomic DNA was obtained as described by Edwards *et al.* (1991) and used to amplify the *in situ* sense and antisense probes for *AGP1* and *AGP12* using the following primers: AGP1-F 5′-CAAAAACACTCCCAAACCAAA-3′, AGP1-R 5′-CTTCAGT CGGAGAATCGG-3′, AGP12-F 5′-CACAACTCATCATTCGCA CCAAAG-3′ and AGP12-R 5′-GCATCGGAAGTAGGACTT GG-3′. The amplified fragments were cloned in pGEMT-Easy (Promega). Digoxygenin (DIG)-RNA probes were generated by *in vitro* transcription using a DIG-RNA labelling kit (Roche). The dissected pistils were permeabilized by first dehydrating in a methanol series of increasing concentration and then rehydrating in a methanol series of decreasing concentration. The pistils were then treated with 2% cellulase (Onozuka R-10) for 1 h, and then washed and dried. RNA/RNA FISH was performed as described by Testillano and Risueño (2009) using DIG-RNA probes diluted 1:50 in hybridization buffer at 50 °C overnight. Post-hybridization washes were performed in 4× SSC, 2× SSC, and 0.1× SSC. The hybridization signal was detected by incubation with mouse anti-DIG antibodies (diluted 1:5000 in 1% BSA; Sigma) for 90 min, followed by incubation with Alexa Fluor 488-conjugated anti-mouse antibody (diluted 1:25 in PBS; Molecular Probes) for 45 min. After washing in PBS, sections were counterstained with DAPI, mounted in Mowiol, and observed by confocal microscopy. Controls were performed using the sense probes.

Results

Phylogenetic analysis and AGP *distribution across the genome*

An alignment of full-length predicted AGP proteins was generated using Clustal W (Thompson *et al.*, 1994) and then manually refined (Fig. 1A). In this study, 13 classical AGPs (AGP1, AGP2, AGP3, AGP4, AGP5, AGP6, AGP7, AGP9, AGP10, AGP11, AGP25, AGP26, and AGP27), 10 AG peptides (AGP12, AGP13, AGP14, AGP15, AGP16, AGP20, AGP21, AGP22, AGP23, and AGP24), and three lysine-rich AGPs (AGP17, AGP18, and AGP19) were considered. For this analysis, only four FLAs were used: FLA18, FLA16, FLA17, and FLA15. These FLAs were chosen randomly and included in the analyses only as outgroup, since they are particularly different from the rest of the family and considered to be chimaeric AGPs (Showalter *et al.*, 2010). The phylogenetic distribution of the selected AGP sequences partially supported the four subgroups of AGPs proposed by previous studies (Schultz *et al.*, 2002; Johnson *et al.*, 2003). The alignments showed a high level of similarity between the predicted amino acid sequences of AGP15, an AG peptide, and AGP1, a classical AGP, as well as the inclusion of the three lysine-rich AGPs in the same branch as the classical AGPs, not supporting the AGP classification currently in use. As expected, the FLAs used in this study aligned together and independently from the other AGPs as a subgroup but were still related to the classical AGP25, AGP26, and AGP27. Comparing the NJ tree generated using the AGPs complete amino acid sequences with the maximum parsimony tree and the NJ tree generated using only the three conserved blocks of AGP amino acid sequences (Supplementary Figs 1 and 2, at *JXB* online), we could see almost no difference among them. All the main blocks of closely related AGPs remained grouped together in the three different trees, reinforcing the strength of this analysis. These two trees were generated using the three most conserved regions revealed by the AGP multiple alignments (Supplementary Fig. S3, at *JXB* online). Looking at the *AGP* gene distribution along the different five *Arabidopsis* chromosomes (Fig. 1B), there was no evidence of clustering of any specific group of closely related *AGP* genes or any specific class of AGPs. They seemed to be randomly distributed across the different *Arabidopsis* chromosomes.

AGP *gene expression*

As a first approach, data from microarray experiments available from online databases such as Genevestigator (https://www.genevestigator.com/gv/; Zimmermann *et al.*, 2004) and the Arabidopsis eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Winter *et al.*, 2007) were used to evaluate the distinct levels of *AGP* gene expression throughout the different plant tissues (Fig. 1C; only Genevestigator data are shown). Eleven *AGP*s were selected for further analysis: *AGP1*, *AGP4*, *AGP7*, *AGP9*, *AGP10*, *AGP12*, *AGP15*, *AGP16*, *AGP23*, *AGP25*, and *AGP26*, most of them based on the presence of their transcripts in pistil tissues and their absence in stamen tissues. In the case of *AGP7*, although it did not show this pattern of expression, it was selected anyway, based on its predicted high level of amino acid sequence similarity with *AGP4*. *AGP23* was selected as a negative control, since the eFP Browser and the literature data (Costa *et al.*, 2013*c*; Nguema-Ona *et al.*, 2013) indicated that it is expressed only in pollen. However, the Genevestigator data also indicated poor expression in female tissues. To check the differences between *AGP* gene expression levels in these tissues and to validate the microarray-based information, a real-time RT-PCR was performed using emasculated pistils, pollen from flowers at anthesis (stage 13 according to Smyth *et al.*, 1990), and seedling cDNA. The results confirmed the microarray data initially considered (Fig. 2). These analyses confirmed the good quality of the microarray data. In this work, the *AGP* transcript levels were normalized to *ACT8* and *RUB1* reference gene levels, and are presented relative to the pollen transcript levels, since the main goal was to determine the *AGP* genes that are expressed more in the female tissues than in the pollen. *AGP7* and *AGP23* were downregulated in the pistil tissues when compared with their expression in pollen, while all the other *AGP*s were upregulated. *AGP10*, *AGP12*, and *AGP16* were the genes that revealed a higher level of overexpression when compared with their expression in pollen. *AGP1*, *AGP4*, *AGP15*, *AGP25*, and *AGP26* were revealed to be upregulated in the pistils, compared with pollen, but not at such high levels as the genes *AGP10*, *AGP12*, and *AGP16*. From this group of upregulated *AGP*s, *AGP1*, *AGP9*, *AGP12*, and *AGP15* were selected for further analyses.

Plasmid construction and expression in A. thaliana

To improve the visualization and to avoid diffuse fluorescent signals in the detection of the promoter activities, the reporter gene *NLS:3GFP* was used (Takada and Jürgens, 2007). This consists of the simian virus 40 nuclear localization signal (NLS) and three tandem enhanced GFP (3×EGFP) sequences

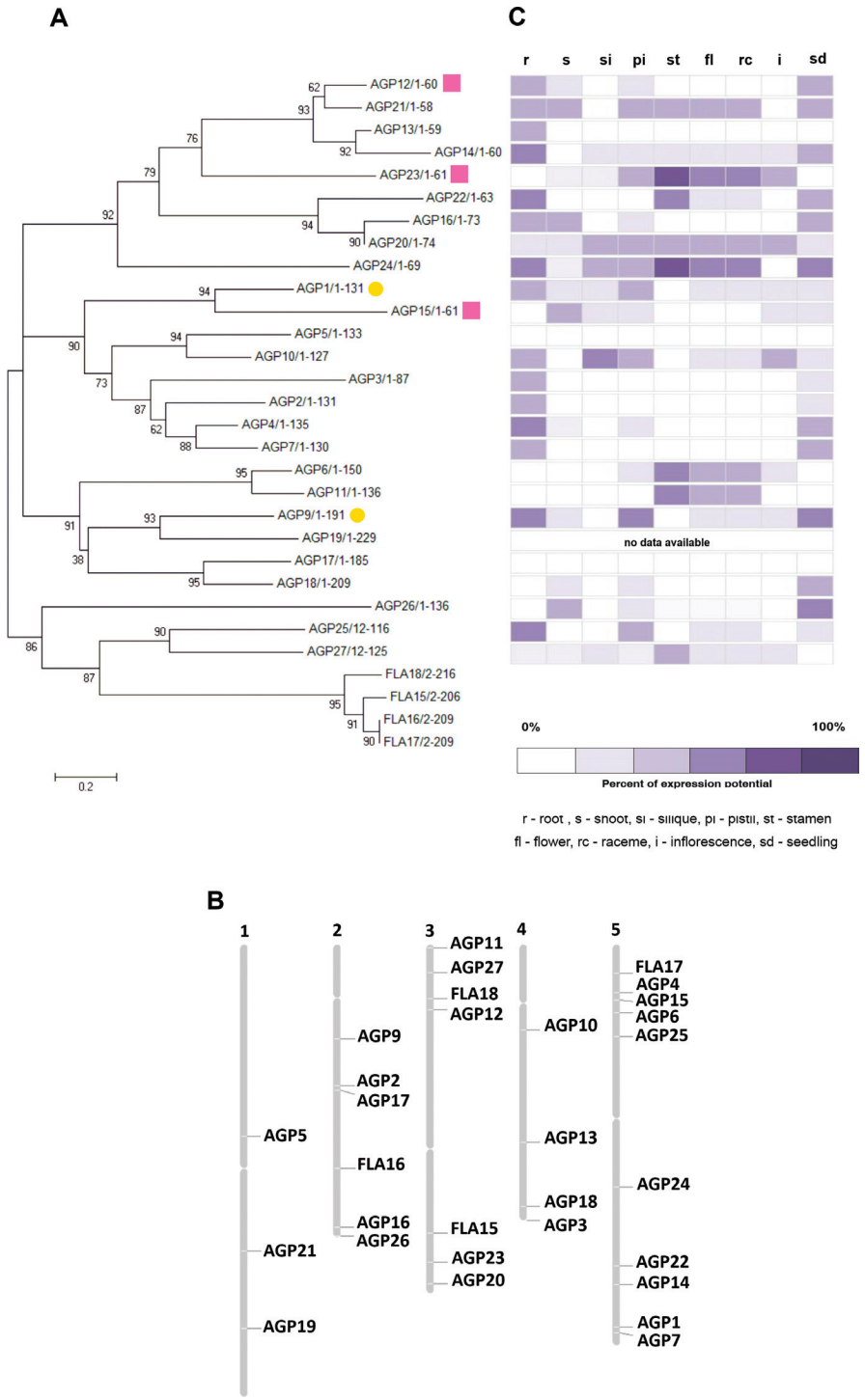

Fig. 1. The AGP protein family, gene expression, and *AGP* gene localization in *A. thaliana.* (A) Phylogenetic analysis of the AGP family in *A. thaliana*. To generate the phylogenetic tree for AGPs, all the amino acid sequences of AGP-coding sequences were aligned using Clustal W and manually edited using Jalview to reduce gaps. A NJ tree was generated using the MEGA4 program. The optimal tree with the sum of branch length=14.47033254 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (10 000 replicates) is shown next to the branches.The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site (bar). The analysis involved 30 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 241 positions in the final dataset. AGPs selected for further analysis are indicated by a yellow circle (classical AGPs) and a pink square (AG peptides). (B) The 26 *AGP* and four *FLA* genes were localized on the *Arabidospis* chromosomes using the Chromosome Map Tool available at The Arabidopsis Information Resource (TAIR: http://www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp). (C) Gene expression patterns for the 26 AGP and four *FLA* genes obtained using Genevestigator.

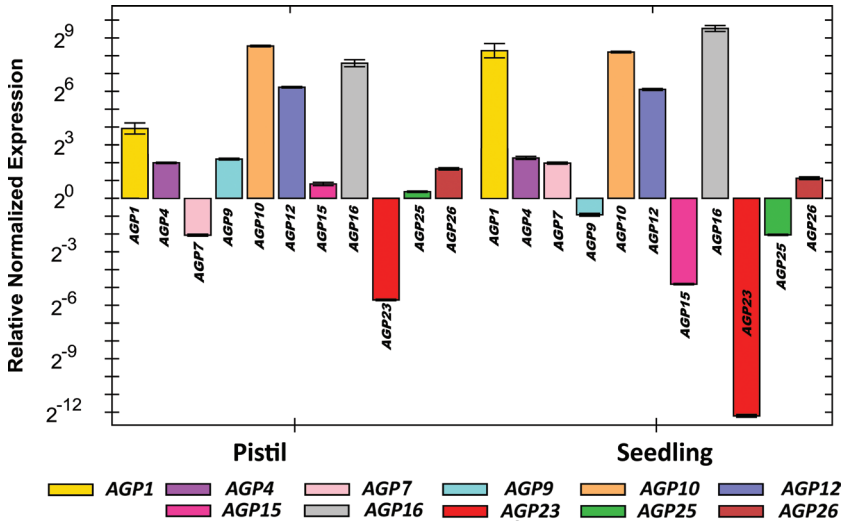

Fig. 2. Quantitative PCR relative expression levels of the selected *AGP* mRNA transcripts in emasculated pistils, pollen, and seedlings of wild-type *Arabidopsis* plants. The pollen was collected from anthers at stage 12 of flower development according to Smyth *et al.* (1990). *AGP* transcript levels were normalized to *ACT8* and *RUB1* reference gene levels, and are presented relative to the pollen transcript levels. Each bar represents an average of two independent reactions and technical replicates.

(Fig. 3A). The fluorescent signal should be then targeted to the nuclei, thereby enhancing the sensitivity of the GFP signal. In all the transgenic *A. thaliana* plants bearing the different p*AGP*:NLS:3GFP constructs, the GFP reporter expression was limited to the nuclei as expected, as shown in Fig. 3B–M.

AGP *differential expression patterns in* A. thaliana *reproductive tissues*

The *AGP* promoters selected for this study allowed us to detect the different patterns of expression of these proteins in the female reproductive tissues. All the flowers analysed were between stages 12 and 13 according to Smyth *et al.* (1990). GFP expression driven by the *AGP1* promoter was strong in the style tissues (Fig. 3B), septum (Fig. 3C), transmitting tract (Fig. 3D), funiculus that attaches the ovules to the placenta (Fig. 3C, D), and chalazal region of the ovules (Fig. 3D). Weaker GFP expression was detected in the stigmatic cells (Fig. 3B) and the integuments of the ovule (Fig. 3D). The *AGP12* promoter guided the expression of GFP strongly to the stigmatic cells (Fig. 3E) and the chalazal pole of the ovules (Fig. 3F). Very weak GFP expression was observed along the internal tissues of the funiculus and septum (Fig. 3F). Plants transgenic for the p*AGP15*:NLS:3GFP expression cassette exhibited GFP expression in all the female reproductive tissues, except in the transmitting tract cells (Fig. 3G, H). The *AGP23* promoter drove GFP expression specifically into the vegetative cell of the pollen grains (Fig. 3I, J). This was clarified by the DAPI staining of the pollen grains, showing that the GFP signal was present only in the nucleus of the vegetative cell and not in the generative cell nuclei, where there was only DAPI staining without any green signal. The *AGP9* promoter led to the expression of GFP in the vascular tissues of the pistil transmitting tract, the septum (Fig. 3K), and the funiculus (Fig. 3L), exhibiting very weak expression in the chalazal pole of the ovules (Fig. 3M).

At the same time, p*AGP*:GUS constructs were also analysed for three *AGP*s: *AGP1*, *AGP15*, and *AGP23*. For the p*AGP1*:GUS fusion-expressing plants, a low GUS activity was observed in the stigmatic cells, while a higher GUS activity was detected in the septum, transmitting tract, funiculus, chalaza, and ovule integument cells (Figs. 4A, B). Regarding the plants expressing GUS under the control of the *AGP15* promoter, a high GUS activity was detected in almost all the tissues of the pistil, except in the transmitting tract (Fig. 4C, D). As well as the plants expressing the three GFP molecules under the control of the *AGP23* promoter, the *Arabidopsis* plants bearing GUS under the control of this same promoter showed a very specific and high GUS activity in the pollen (Fig. 4E, F). This activity was also observed in the pollen tubes (Fig. 4G), and it was especially high when the pollen tube burst occurred inside the embryo sac (Fig. 4H), staining almost all the embryo sac with a weaker GUS signal. This GUS expression in the embryo sac was never observed when p*AGP23*:GUS pistils were pollinated with wild-type pollen, only in embryo sacs fertilized with p*AGP23*:GUS pollen. This indicated that the GUS product present in the maternal embryo sac after fertilization was released by the burst of the pollen tube.

FISH confirms the GFP reporter line patterns of expression

FISH was used to verify whether the GFP signals and GUS activity obtained with the p*AGP*:3GFP and p*AGP*:GUS fusions in fact reflected the real *AGP* gene expression. For this study, FISH was used to analyse two *AGP* genes: *AGP1* and *AGP12*. Hybridization signals for the *AGP1* antisense probe were detected throughout the septum, transmitting tract, and funiculus cells, as well as in the integuments surrounding the micropylar region of the embryo sac (Fig. 5A). The same experiment using the *AGP1* sense probe revealed the absence of hybridization signal along all the reproductive

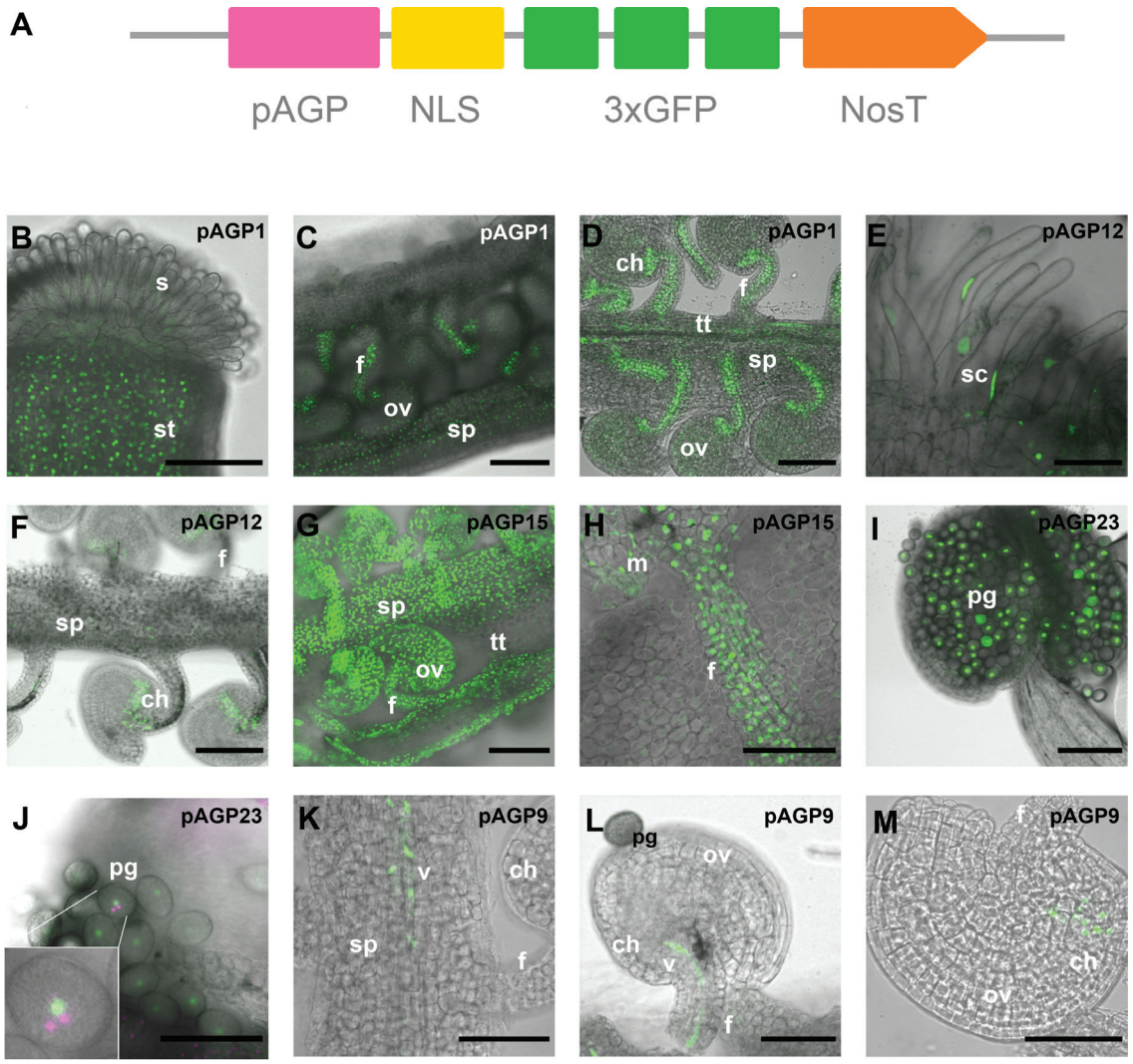

Fig. 3. Schematic representation of the expression cassette used in this study, and the resulting GFP signal shown in *Arabidopsis* reproductive tissues. (A) Expression cassette showing the relative position of the promoter sequence (p*AGP*), NLS, a fusion of three GFPs (3×GFP), and the terminator Nos (NosT). (B–D) NLS:3GFP expression driven by the *AGP1* promoter in the style tissues (B), the opened pistil, and the funiculus and septum tissues (C), and seen in more detail in the transmitting tissue, funiculus, and the chalazal pole of the ovule (D). (E–F) NLS:3GFP expression under the control of the *AGP12* promoter was observed in the stigmatic cells (E) and in the chalazal pole of the ovule (F). (G–H) NLS:3GFP expression driven by the *AGP15* promoter was detected in the ovule integuments, funiculus, and septum, but absent from the transmitting tissue (G). In (H) the GFP signal is seen in more detail in the nuclei of the funiculus. (I, J) NLS:3GFP under the control of the *AGP23* promoter is absent in all the sporophytic tissues (I), with its expression restricted to the pollen grain, and, as can be seen in the insert in (J), DAPI staining (here in magenta) revealed this expression to be limited to the vegetative cell of the pollen grain; DAPI-stained germinative nuclei are visible (white arrowheads). (K–M) NLS:3GFP signals expressed by the *AGP9* promoter. Signals were observed in the vascular bundle of the transmitting tract (K) and funiculus (L) as well as in the chalazal pole of the ovule (M). All the flowers used in these observations were at stages 12 and 13 according to Smyth *et al.* (1990). ch, Chalaza; f, funiculus; m, micropyle region of the ovule; ov, ovule; pg, pollen grain; s, stigma; sc, stigmatic cell; sp, septum; st, style; tt, transmitting tract; v, vascultature. Bars, 100 μm (B–G, I); 50 μm (H, K–M); 20 μm (J).

tissues (Fig. 5B). With the *AGP12* antisense probe, strong hybridization signals were detected in the stigmatic cells (Fig. 5C) and a weaker signal was observed across the style and the septum (Fig. 5E). The corresponding *AGP12* sense probe did not show any hybridization signals along the reproductive tissues (Fig. 5D, F).

Discussion

AGPs selection

Bioinformatics analyses recently allowed the identification of 64 potential AGPs in *Arabidopsis* (Showalter *et al.*, 2010). The present work started by analysing 26 of these: those with more information available. However, it is important to keep in mind that for all individual AGPs almost no information is available at the structural level. Sequence comparisons revealed a high level of similarity between amino acid sequences of AGP15, an AG peptide, and AGP1, a classical AGP, as well as the inclusion of the three lysine-rich AGPs in the same branch as the classical AGPs. These results pinpointed the artificial nature of the classification currently in use to organize this family of proteins. The availability of more data regarding *AGP* expression patterns in different plant species and more information regarding their functions may allow the classification of these proteins based

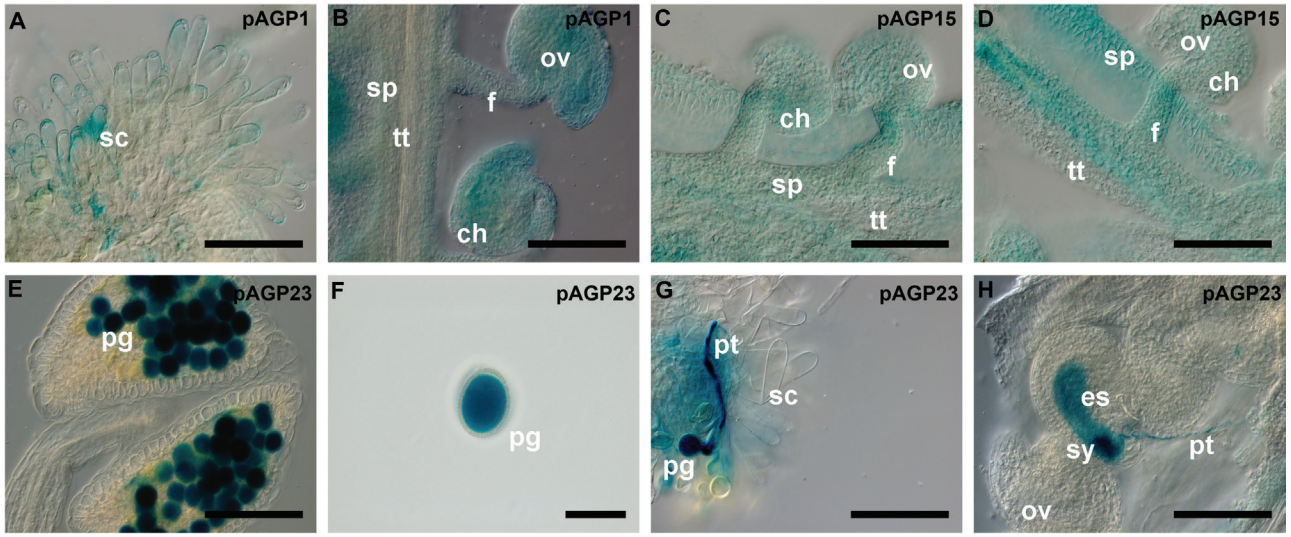

Fig. 4. Histochemical localization of GUS activity in transgenic *Arabidopsis* reproductive tissues expressing the p*AGP*:GUS fusion genes. (A, B) GUS activity driven by the *AGP1* promoter is detected in the stigmatic cells (A) and the transmitting tract, funiculus, and integument cells (B). (C, D) GUS activity driven by the *AGP15* promoter observed in the ovule integuments, funiculus, and septum cells. (E–H) Strong GUS activity driven by the *AGP23* promoter was identified inside pollen grains (E) and (F) and the growing pollen tube (G). Upon fertilization, inside the embryo sac, a strong staining is observed at the local where the pollen tube bursts (H), followed by a weak staining that spread inside the whole embryo sac (H). Flowers of stages 12 and 13 (Smyth *et al.*, 1990) were used in this study. ch, Chalaza; es, embryo sac; f, funiculus; ov, ovule; pg, pollen grain; pt, pollen tube; sc, stigmatic cell; sp, septum; sy, synergid; tt, transmitting tract. Bars, 100 μm (A–E, G, H); 50 μm (F).

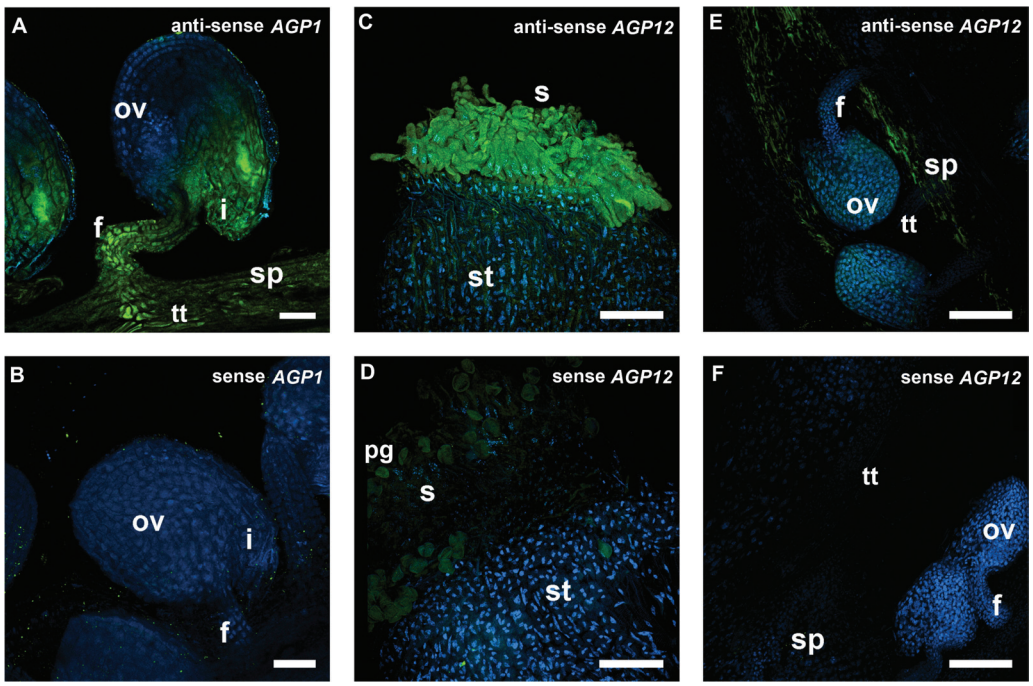

Fig. 5. FISH localization of *AGP1* and *AGP12* transcripts in *Arabidopsis* pistil tissues. Merged images of FISH signals (green) and DAPI staining of nuclei (blue) are shown. (A) *AGP1* transcripts were detected in the funiculus, transmitting tissue, and integuments. (C, E) *AGP12* transcripts were localized in the stigmatic cells and along the septum tissues. (B, D, F) FISH controls with the sense probe for *AGP1* in ovules (B) and for *AGP12* in stigma (D) and ovules (F). All the flowers used in these observations were at stages 12 and 13 according to Smyth *et al.* (1990). f, Funiculus; i, integuments; pg, pollen grain; ov, ovule; s, stigma; sp, septum; st, style. Bars, 25 μm (A, B); 75 μm (C–F).

on their functions and localization, rather than on their amino acid sequences similarities. However, there are still some pairs of AGPs that share a high degree of similarity between their amino acid sequences, and, simultaneously, display a similar expression pattern in the reproductive tissues, suggesting that they might act redundantly, such as the AGP16/AGP20, AGP1/AGP15, AGP5/AGP10, and AGP6/AGP11 pairs. AGP6/AGP11 is a pair of redundant AGPs involved in *Arabidopsis* pollen grain and pollen tube growth and development (Coimbra *et al.*, 2009). A total of 11 AGPs were picked for further analysis: AGP1, AGP4, AGP7, AGP9, AGP10, AGP12, AGP15, AGP16, AGP23, AGP25,

and AGP26. This group was selected by an *in silico* search of *AGP* genes that could be transcribed preferentially in pistils rather than in the stamens or seedlings. This selection was based on analyses of microarray data available for pistil and stamen tissues obtained from Genevestigator, using the Anatomy tool provided by this service (Zimmermann *et al.*, 2004) and the eFP Browser (Winter *et al.*, 2007). Although *AGP18* fits perfectly into this category, it was not selected as it is already well described (Acosta-García and Vielle-Calzada, 2004; Demesa-Arévalo and Vielle-Calzada, 2013). *AGP23* was chosen as a control, since it is only transcribed during pollen development (Costa *et al.*, 2013*c*; Nguema-Ona *et al.*, 2013). Although microarray data from Genevestigator also predicted its expression in whole flowers and pistils, our real-time RT-PCR data confirmed that *AGP23* was detected only in pollen, being highly downregulated in pistils and seedlings.

The validation of this selection through real-time RT-PCR allowed us to limit the number of *AGP*s selected for further analysis to four: *AGP1*, *AGP9*, *AGP12*, and *AGP15*. *AGP9* and *AGP15* are upregulated in the pistil and downregulated in the seedlings, and were selected for this reason. *AGP1* was also selected, although its transcripts show a higher upregulation in seedlings than in emasculated pistils, because it is phylogenetically close to *AGP15*. *AGP12* was chosen as one of the most upregulated *AGP*s in the pistil.

Regarding *AGP* gene localization in the *Arabidopsis* chromosomes, it was clear that the *AGP*s were randomly distributed over the *Arabidopsis* genome. This was the case for *AGP16* and *AGP20*, located, respectively, on chromosomes 2 and 3, and also for *AGP6*, located on chromosome 5, and *AGP11*, on chromosome 3, two AGPs that have already been shown to act redundantly (Coimbra *et al.*, 2009). This is probably due to duplications in the genome, since most of these genes are included in segments of the respective chromosomes that have been subject to large duplications events (Blanc *et al.*, 2000). This is consistent with the prediction that genetic redundancy may occur as a consequence of gene duplication (Kafri *et al.*, 2009). Only the pairs of the most similar *AGP*s, *AGP4*/*AGP7* and *AGP1*/*AGP15*, have their genes positioned in the same chromosome but in opposed regions. It is plausible that some of the *AGP* genes acquired a certain degree of specialization, and are now expressed in different tissues under different conditions.

AGP *expression in the reproductive tissues*

The results obtained in this work confirmed the specific and differential pattern of expression of AGPs predicted previously by immunolocalization studies, where several monoclonal antibodies, recognizing distinctive AGP glycosidic epitopes revealed the presence of these proteins throughout diverse tissues in different developmental stages in *Arabidopsis* (Coimbra *et al.*, 2007). These results not only confirmed and complemented this previous study but also improved the information already available about AGP distribution throughout the reproductive tissues by identifying specific AGPs present in these tissues. In the study by Coimbra *et al.* (2007), no antibody labelling was detected in the stigmatic cells, which, as was shown here, are rich at least in AGP1 and AGP12. Also, in the same study, no antibody labelling was detected in the funiculus of the ovules, whereas, in this study, the presence of several AGPs, such as AGP1, AGP12, and AGP15, was revealed in this tissue. This work illustrates the usefulness of these techniques in contrast to the use of monoclonal antibodies to detect AGPs. As expected from quantitative PCR data, *AGP23* was expressed only in pollen grains and pollen tubes. Although the microarray data available from Genevestigator indicated that *AGP23* should be present in pistils, this was not observed here. The analysis of transgenic *Arabidopsis* plants carrying the p*AG23*:GUS and the p*AGP23*:NLS:3GFP constructs revealed that both reporters were detected in pollen, proving that AGP23 is specific to the pollen vegetative cell. The prediction of potential of expression of *AGP23* in flowers and pistils was most probably due to the high levels of *AGP23* expression in pollen grains contained in the samples used for those studies. Concerning the pistil, the manipulation of these tissues is complicated if the flowers are not in the correct stage of development, as it is easy to get pollen contamination in the stigma, misleading to some false-positive expression. A summary of the different approaches used to localize these *AGP*s and their differential patterns of expression in the reproductive tissues is shown in Fig. 6.

The FISH data obtained for *AGP1* and *AGP12* were partially consistent with the promoter analysis results shown for these two AGPs. The GFP expression driven by the *AGP1* and *AGP12* promoters revealed the presence of GFP signal in the chalazal tissues of the ovule, and, surprisingly, this was not observed in FISH results. This technique implies the analysis of whole-ovule amounts, making the tissue permeabilization more difficult in order for the probe to reach the most internal cell layers of the ovules, as is the case of the chalazal region (García-Aguilar *et al.*, 2005; Hejátko *et al.*, 2006). Still, we are aware that some regulatory elements of these two promoters might be missing, thus leading to the AGP misexpression in the chalazal tissues. Besides having regulatory sequences within the promoter itself, in eukaryotes there may be regulatory elements located tens of thousands of base pairs away from the start site, in introns, or even downstream from the coding sequence of the gene (Korkuć *et al.*, 2014). Also, *AGP1* transcripts were not detected in the stigmatic cells or in the style by FISH analysis. It is important to underline the fact that the microarray data used and the FISH technique were performed with whole organs, while the promoter analysis referred to a spatial–temporal analysis, which was much more detailed. The older immunolocalization studies (Junqueira, 2007) did not detect the glycosidic AGP epitopes in the chalazal tissues. Although we are aware that the antibodies used identify only sugar epitopes from all AGPs, we may conclude, with some caution, that the accordance between the immunolocalization data and FISH results fortifies confidence in the use of antibodies to determine AGP localization.

AGP1 and *AGP12* expression in the stigmatic cells suggested the possible involvement of this protein in pollen–stigmatic cells interactions, and acquisition of pollen grain

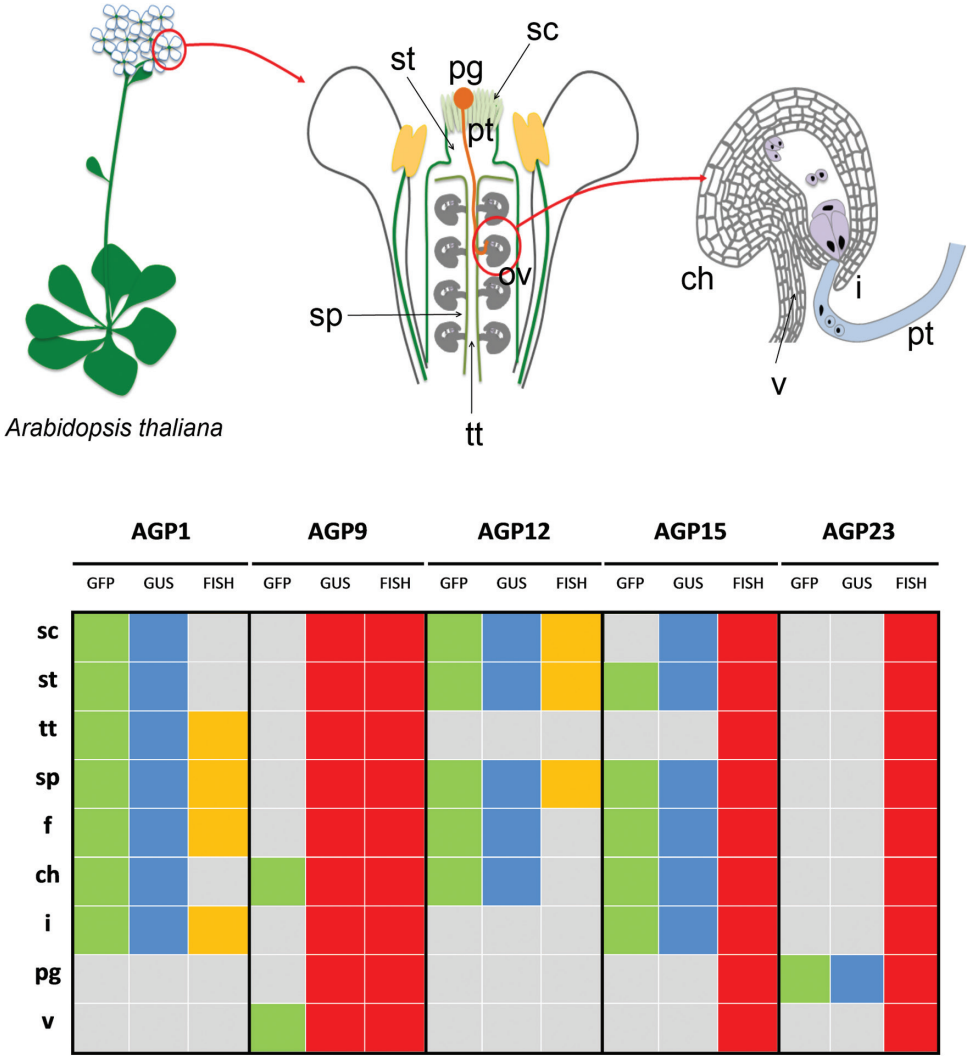

Fig. 6. A schematic representation of the reproductive structures and tissues of *A. thaliana* and the distribution of the five AGPs analysed in this study throughout the different tissues, regarding the different techniques used. GFP presence, green; GUS presence, blue; FISH positive, yellow; experiment not performed, red; absence of signal, grey. ch, Chalaza; f, funiculus; i, integuments; pg, pollen grain; sc, stigmatic cell; sp, septum; st, style; tt, transmitting tract; v, vasculature.

competence to initiate pollen tube growth. Losada and Herrero (2012) indicated a role for AGPs in supporting pollen tube germination, suggesting that the secretion of AGPs can be associated with the acquisition of stigma receptivity in apple flower. The same mechanism may occur with *AGP12* and *AGP1* in *Arabidopsis*. Also, in the early divergent angiosperm *Trithuria*, immunocytochemistry results suggest AGPs to be involved in attracting the pollen tubes through the stigmatic cuticle, as in most evolved angiosperms (Prychid *et al.*, 2011), reinforcing our hypothesis.

The presence of *AGP1* and *AGP15* in the main female reproductive tissues through which the pollen tube grows until it reaches the embryo sac – the stigma, style, transmitting tract, septum, and funiculus – strengthens the putative role of AGPs in pollen tube growth and fertilization. Many early studies implied AGPs from the female tissues as playing major roles in reproductive processes (Du *et al.*, 1994; Cheung *et al.*, 1995; Cheung and Wu, 1999; Wu *et al.*, 2000; Coimbra *et al.*, 2007). For example, TTS proteins (AGPs from *Nicotiana tabacum*) were shown to attract and promote pollen tube growth either *in vivo* or *in vitro*, nutritionally supporting its growth and providing it with guidance cues (Cheung *et al.*, 1995; Wu *et al.*, 2000). Wu *et al.* (1995) also revealed that the carbohydrate part of these TTS proteins forms an increasing gradient from the top to the bottom of the *Nicotiana* style, by the action of specific pollen tube hydrolases, which may have a chemotropic effect on growing pollen tubes. In *Arabidopsis*, the transmitting tract begins at the style between the stigma–style boundaries, extending to the base of the ovary (Crawford and Yanofsky, 2008). *AGP1* is mainly present along this transmitting tract, while *AGP15* is mostly present at the septum surrounding the transmitting tract. Since these two proteins are closely related to each other, this fortifies their possible redundant function in these tissues. *agp1* null mutants were analysed (data not shown) but revealed no visible phenotype. Most probably a double *agp1 agp15* mutant is needed to access their precise function. These AGPs might act in these tissues in a similar manner to the TTS proteins in *Nicotiana*. A study of the *NTT* gene in *Arabidopsis* has indirectly implied the involvement of

AGPs in pollen tube guidance through the transmitting tract (Crawford *et al.*, 2007). The *ntt* mutants lacked a functional transmitting tract and exhibited a reduced staining for acidic polysaccharides. Crawford *et al.* (2007) speculated that AGPs, acidic glycoproteins that are a main component of the transmitting tract, might be reduced in these mutants. It will be extremely interesting to check whether there is a control of *AGP* expression by this NTT zinc-finger transcription factor.

AGP1, *AGP9*, and more strongly *AGP12*, showed expression at the chalazal tissues of *Arabidopsis* ovules and at the cells located on the top of the vascular supply coming from the funiculus, as well as along this tissue. It is known that the main nutrient uptake into the endosperm occurs via the chalazal pole, with this being important for nutrient transfer from the maternal parent to the developing embryo (Debeaujon *et al.*, 2003; Ingram, 2010). This may indicate the possible participation of these glycoproteins in nutrition or signalling between the vasculature and the embryo sac, endosperm, or embryo, being quickly mobilized. The incomplete correlation between GFP and GUS activity driven by the *AGP12* and *AGP1* promoters in this region and their transcript expression revealed the importance of analysing these AGPs at the protein level in future studies.

For double fertilization to take place, the pollen tube must travel a long and challenging pathway in order to reach its final destination: the micropylar entrance to the embryo sac, where it will discharge, through one of the two synergids, two immotile sperm cells to fertilize the egg cell and the central cell, giving rise to the embryo and the endosperm, respectively, initiating a new generation (Márton and Dresselhaus, 2010). Along this narrow road, the pollen tube lengthens through a mucilage-rich extracellular matrix from the stigmatic cells, along the specialized transmitting tract cells, funiculus, and ovary integuments (Webb and Williams, 1988; Lennon *et al.*, 1998). Although most of these studies showed that this extracellular matrix tract, through which the pollen tube travels, is rich in AGPs and pectins, to date only some specific molecules have been shown to function as pollen tube growth enhancers such as GABA in *Arabidopsis* (Palanivelu *et al.*, 2003) and chemocyanin in *Lilium longiflorum* (Kim *et al.*, 2003).

The results shown in this study support previous work where AGPs were proposed to be part of this pathway and to sustain pollen tube growth (Clarke *et al.*, 1979; Herrero and Dickinson, 1979; Gell *et al.*, 1986; Cheung *et al.*, 1995). *AGP1*, *AGP12*, and *AGP15* (Fig. 6) are located along all these tissues and might well contribute to pollen tube growth from the top of the stigma to the base of the pistil, into the ovules, either by nutritionally supporting their growth, facilitating their movement, guiding them to their targets, or even by making them competent for pollen tube reception by the embryo sac. These hypotheses need further studies to fully assign AGP functions in these tissues, most probably involving studies with double or triple null mutants. It is interesting to note that we identified AGPs along the entire pollen tube pathway (stigma, style, and transmitting tract), showing that AGPs are most probably essential for all the different steps of pollen tube growth through the pistil. The molecular mechanism of action of AGPs and how they interact with other cell-wall and cell components is still elusive, although some enlightenment has recently been given to this matter (Costa *et al.*, 2013*a*). One possibility may be related to the most recent finding that AGPs can act as calcium reservoirs, making calcium available temporarily in a developmental way (Lamport and Várnai, 2012). The importance of calcium in sexual plant reproduction is well known (Ge *et al.*, 2007). One of the key characteristics of growing pollen tubes is a tip-focused calcium gradient maintained by the influx of extracellular calcium through calcium channels active at the extreme end of the growing tip (Feijó *et al.*, 1995). AGPs may be regulating in some way the release of calcium along the pollen tube pathway, making calcium available for the pollen tubes to grow. Most likely, different AGPs play several different roles during different steps of the reproductive process, according to their localization and timing of expression (Fig. 6). Our results support and improve the study of these enigmatic and inscrutable glycoproteins in the sexual plant reproductive process, opening doors for new pathways for the study of specific AGPs. Also, this type of analysis overcomes the main difficulty regarding the older immunolocalization AGP studies made by the use of monoclonal antibodies that detected only the glycosidic epitope of the AGPs, instead allowing the identification of a specific AGP in plant tissues.

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