Original article

Differential expression patterns of Arabinogalactan Proteins in *Arabidopsis thaliana* reproductive tissues

Running title: AGPs in Arabidopsis reproductive tissues

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Abstract

Arabinogalactan proteins (AGPs) are heavily glycosylated proteins existing in all plant kingdom and differentially distributed through distinctive developmental stages. Here we show the individual distribution of specific *Arabidopsis* AGPs: AGP1, AGP9, AGP12, AGP15 and AGP23, throughout reproductive tissues and point out their possible roles in several reproductive processes. AGP genes specifically expressed in the female tissues were identified using available microarray data. This selection was confirmed by promoter analysis using multiple GFP fusions to a nuclear localization signal (NLS), GUS fusions, and *in situ* hybridization as an approach to confirm the AGPs expression patterns. 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* is expressed in the stigma, the style, the transmitting tract, the funiculus, and in the chalazal and funiculus tissues of the ovules. *AGP9* is present along the vasculature of the reproductive tissues and *AGP12* is expressed in the stigmatic cells, the chalazal and funiculus cells of the ovules, and the septum. *AGP15* is expressed in all pistil tissues, except in the transmitting tract, while *AGP23* is pollen grain and pollen tube specific. The expression pattern of these AGPs brings new and significant evidences for the detection of a subset of specific AGPs involved in plant reproductive processes, being of great 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 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 (PT) (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 PT 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 funiculus, it makes a quick turn, and grows on the surface of the placenta and the funiculus until reaching the ovule opening, the micropyle (Hülskamp *et al.*, 1995). After growing through the micropyle, the PT 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). Until today, besides all the efforts carried out in this field of study, little information is available about which molecules function as signaling or as 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, also in the bryophyte *Physcomitrella patens* (Lee *et al.*, 2005; Fu *et al.*, 2007), and in all

hepatics (Basile *et al.*, 1989), including basal angiosperms (Costa *et al.*, 2013b) and many algae, indicating an ancient origin for these proteins (Popper *et al.*, 2011).

They are found in distinct developmental stages, 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 in four sub-groups according to their polypeptide core characteristics: the classical AGPs, that possess an N-terminal signal peptide, which is removed in the mature protein, a Pro/Hyp (Proline/ Hydroxiproline) 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 to 13 amino acid residues, 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, PT 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 glycosylphosphatidylinositol (GPI) anchor (Borner *et al.*, 2002; Schultz *et al.*, 2004), which provides a way for the AGPs to function as signaling molecules. After comparisons with GPI anchored proteins from animal cells, two mechanisms were proposed for AGP mediated signaling: the first consisted on the cleavage of the GPI anchor by specific phospholipases (C and D) that would release the glycoprotein to 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 signaling role for these proteins, the prominent carbohydrate content surrounding the core protein also led to some assumptions about their involvement in signaling mechanisms. The importance of sugars as signaling 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

signaling 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 is not yet demonstrated if 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 PT growth from the stigma to the ovules in *Amaranthus hypochondriacus, Actinidia deliciosa, Catharanthus roseus* and *Nicotiana tabacum* (Coimbra and Salema, 1997; Coimbra and Duarte, 2003; Cheung *et al.*, 1995). 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 which identify only the glycosidic epitopes of AGPs (Pennell *et al.*, 1989; Pennell *et al.*, 1991; Knox *et al.*, 1991). These two approaches have given us information about AGPs distribution and localization (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 AGPs oligosaccharides as signaling molecules. It will be interesting to check if this particular oligosaccharide is important for many of the physiological processes impaired when Yariv was used in different studies, or if Yariv only hampers AGPs ability to function by precipitating them.

Here we report the use of several constructs to explore the tissue and cell-specific promoter activity of specific *AGPs*. We have focused on those AGPs, which are particularly present along the PT pathway and other female reproductive tissues, according to the available microarray data. With this, we aim to complement work that has already been done by our group, describing the AGPs as molecular markers of different stages of the *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 (NASC). Plants were sown on soil, kept for two days at 4°C in the dark to induce stratification, and afterwards, they were grown at 22°C under a short day photoperiod (9h/15h light/dark cycles) for four weeks, followed by a long day photoperiod (16h/8h light/dark cycles) to induce flowering, with 60% relative humidity. For PAT (Phosphinotricin-Acetyltransferase) selection the seedlings were sprayed with 200 mg l^{-1} glufosinate ammonium (BASTA®; Bayer Crop Science) supplemented with 0.1% Tween-20 for three or four times, every two days, during a ten day period.

Constructs generation and plant transformation

Genomic regions corresponding to the promoters of five *AGPs*: *AGP1*, *AGP9*, *AGP12*, *AGP15*, *AGP23* were amplified using Phusion DNA polymerase (Thermo Scientific), with the primer pairs described in table 1 (supplemental material). The promoter regions were always amplified from the end of the UTR of the most proximal gene upstream of the respective *AGP* until its own start codon. For the genes with promoter regions with 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* 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 harboring the pGreenII helper plasmid, pSOUP. All the others expression vectors were delivered into *Agrobacterium tumefaciens* GV3101 (pMP90RK). All of them were then used to transform *Arabidopsis thaliana* (Col-0) by the floral dip method (Clough and Bent, 1998).

183
184 Preparation of plant material for microscopy
185 Pistils kept in 50 mM sodium phosphate buffer (pH 7.5) were dissected under a
186 stereomicroscope (Nikon, Model C-DSD230) by using hypodermic needles (0.4 x 20
187 mm, Braun). The opened carpels and the ovules that remained attached to the septum
188 were maintained in mounting medium and covered with a cover slip.
189
190 Confocal Laser Scanning Microscopy (CLSM)
191 A Zeiss Axiovert 200M inverted microscope equipped with a confocal laser scanning
192 module (LSM 510 META) was used for CLSM. GFP was excited by 488 nm and
193 detected with a BP 505-550 filter. Optical sections were generally between 0.40 and
194 0.50 μm each, observed at 20x, 40x or 63x magnifications. Histology mounting medium
195 Fluoroshield™ with 4',6-diamidino-2-phenylindole, DAPI (F6057 SIGMA) was used in
196 order to detect the nuclei in the pollen grains. Images were captured and processed using
197 the AxioCam HRc camera, the Zeiss LSM 510 META software and the Zeiss LSM
198 image browser version 3.5.0.359.
199
200 Detection of GUS activity
201 GUS assays were performed on inflorescences as described in Liljegren *et al.* (2000),
202 overnight. After chemical GUS detection, the samples were incubated in clearing
203 solution (160 g of chloral hydrate (C-8383; Sigma-Aldrich), 100 mL of water, and 50
204 mL of glycerol) and incubated at 4ºC overnight. The day after, inflorescences were
205 dissected under a stereomicroscope (Nikon, Model C-DSD230), to be observed at the
206 microscope. A Zeiss AxioImager AZ microscope equipped with Differential
207 Interference Contrast (DIC) optics was used. Images were captured with a
208 ZeissAxiocam MRc3 camera using the Zen Imaging Software.
209
210 Phylogenetic Analysis
211 To generate the phylogenetic tree for the AGP genes, the amino acid sequences of AGPs
212 coding sequences were aligned using Clustal W (Thompson *et al.*, 1994) and manually
213 edited using Jalview to reduce gaps (Clamp *et al.*, 2004). A neighbor-joining (NJ)

(Saitou *et al.*, 1987) tree was generated using the MEGA4 program (Tamura *et al.*, 2007). The bootstrap values were obtained by 10,000 repetitions.

Preparation of plant material for RNA extraction

Arabidopsis pistils from wild-type plants were emasculated one day before anthesis and collected two days after the emasculation procedure. Pollen from *Arabidopsis* wild-type recently opened flowers was collected according to Costa *et al.* (2013a). *Arabidopsis* seeds were sown in half strength Murashige and Skoog (MS) medium, complemented with 0.7% agar. Agar plates were kept for two days at 4°C in the dark, to induce stratification, and subsequently they 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 four to five days after germination.

RNA extraction, cDNA synthesis and Real Time RT-PCR

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

cDNA was amplified using the SSoFast™ SYBR® Green Supermix on the iQ5™ Real-Time PCR Detection System (Bio-Rad) using the primers listed in table 2 (supplemental material). Real-time RT-PCR reactions 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 °C 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 analyzed with the iQ5 2.0, Standard Edition Optical System Software v2.0.148.060623 (Bio-Rad).

Fluorescence *in situ* hybridization (FISH)

gDNA was obtained as described by Edwards *et al.* (1991) and used to amplify the *in situ* sense and anti-sense probes for *AGP1* and *AGP12* using the following primers: AGP1-F 5´-CAAAAACACTCCCAAACCAAA-3´, AGP1-R 5´-CTTCAGTCGGAGAATCGG-3', AGP12-F 5´-CACAACTCATCATTCGCACCAAAG-3´ and AGP12-R 5´-GCATCGGAAGTAGGACTTGG-3´. The amplified fragments were cloned in pGEMT-Easy (Promega). DIG-RNA probes were generated by *in vitro* transcription using the 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 where then treated with 2% cellulase (Onozuka R-10) for 1 h, afterwards washed and dried. RNA/RNA fluorescence *in situ* hybridization was performed as described in 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 (Saline-Sodium Citrate buffer), 2× SSC, and 0.1× SSC. Hybridization signal was detected by incubation with mouse anti-digoxigenin antibodies (1:5000 in 1% BSA, Sigma) for 90 min, followed by an incubation with Alexa-Fluor-488 anti-mouse antibody (1:25 in PBS for 45 min, Molecular Probes). 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 *AGPs* distribution across the genome

An alignment of full-length predicted AGP proteins was generated using Clustal W (Thompson *et al*., 1994) and afterwards manually refined (Fig. 1A). In this study 13 classical AGPs (AGP1, AGP2, AGP3, AGP4, AGP5, AGP6, AGP7, AGP9, AGP10, AGP11, AGP25, AGP26, AGP27), ten AG peptides (AGP12, AGP13, AGP14, AGP15, AGP16, AGP20, AGP21, AGP22, AGP23, AGP24) and three lysine-rich AGPs (AGP17, AGP18, AGP19) were considered. For this analysis only four Fasciclin-Like AGPs (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 chimeric AGPs (Showalter *et al.*, 2010). The phylogenetic distribution of the selected AGP sequences partially supports the four sub-groups 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 AGPs classification currently in use. As it was expected, the FLAs used in this study aligned together and independently from the other AGPs as a sub-group, but still related to the classical AGP25, AGP26 and AGP27. Looking at the AGP gene distribution along the different five *Arabidopsis* chromosomes (Fig.1 B), there is no evidence of clustering of any specific group of closely related AGP genes, or any specific class of AGPs. They seem to be randomly distributed across the different Arabidopsis chromosomes.

AGPs gene expression

As a first approach, data from microarray experiments available from on-line databases such as Genevestigator (http://genevestigator.ethz.ch; Zimmermann *et al.*, 2004) and the Arabidopsis eFPBrowser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Winter *et al.*, 2007) were used to evaluate the distinct levels of AGPs genes expression throughout the different plant tissues (Fig. 1C, only the Genevestigator data is shown). Eleven *AGPs* 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 does not show this pattern of expression, it was selected anyway, based on its predicted amino acid sequence high level of similarity with *AGP4*. *AGP23* was selected as a negative control, since eFP Browser and the literature data (Costa *et al.*, 2013c; Nguema-Ona *et al.*, 2013) indicates it is expressed only in pollen. However Genevestigator data indicates a poor expression also in the female tissues. To check the differences between AGP genes expression levels among this 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 seedlings cDNA. The results confirmed the microarray data initially considered (Fig. 2).

These analyses confirm the good quality of the microarray data. In this work the AGPs transcript levels were normalized to *ACT8* and *RUB1* reference gene levels, and are presented relative to the pollen transcript levels, since the main goal is to determine the AGPs genes that are more expressed in the female tissues than in the pollen. *AGP7* and *AGP23* are down-regulated in the pistil tissues when compared to their expression in pollen, while all the others *AGPs* are up-regulated. *AGP10, AGP12* and *AGP16* are the ones that revealed a higher level of over-expression when compared to their expression in pollen. *AGP1, AGP4, AGP15, AGP25* and *AGP26* revealed to be up-regulated in the pistils, comparing to pollen, but not in such high levels like the previous ones (*AGP10, AGP12* and *AGP16*). From this group of up-regulated *AGPs, 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 Jurgens, 2007). This consists of the SV40 nuclear localization signal (NLS) and three tandem enhanced green fluorescence protein (3xEGFP) sequences (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 has been limited to the nuclei like it was expected, as shown in Figs. 3B - M.

AGPs differential expression pattern 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 analyzed were in 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), the septum (Fig. 3C), the transmitting tract (Fig. 3D), the funiculus that attaches the ovules to the placenta (Figs. 3C, D) and in the chalazal region of the ovules (Fig. 3D). A weaker GFP expression was detected in the stigmatic cells (Fig. 3B) and in the integuments of the ovule (Fig. 3D). The *AGP12* promoter guided the expression of GFP strongly to the stigmatic cells (Fig. 3E) and to the chalazal pole of the ovules (Fig. 3F). A very weak

GFP expression was observed along the internal tissues of the funiculus and the 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 (Figs. 3G, H). The *AGP23* promoter drove the 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 solely DAPI stained and without any green signal. The *AGP9* promoter led to the expression of GFP in the vascular tissues of the: pistil transmitting tract, septum (Fig. 3K) and the funiculus (Fig. 3L), exhibiting a very weak expression in the chalazal pole of the ovules (Fig. 3M).

At the same time p*AGP*:GUS constructs were also analyzed for three *AGPs*: *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 almost through all the tissues of the pistil, except in the transmitting tract (Figs. 4C, D). As well as the plants expressing the three GFP molecules under the control of the *AGP23* promoter, the *Arabidopsis* plants bearing the GUS under the control of this same promoter showed a very specific and high GUS activity in the pollen (Figs. 4E, F). This activity was also observed in the PTs (Fig. 4G), and it was especially high when the PT 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, but only in embryo sacs fertilized with p*AGP23*:GUS pollen. This indicates that the GUS product present in the maternal embryo sac after fertilization is released by the burst of the PT.

FISH confirms the GFP reporter lines patterns of expression

Fluorescence *in situ* hybridization was used to verify if the GFP signals and GUS activity obtained with the p*AGP*:3GFP or p*AGP*:GUS fusions reflected in fact the real *AGP* gene expression. For this study FISH was analyzed for two AGP genes: *AGP1* and *AGP12*. Hybridization signals for the *AGP1* anti-sense probe were detected throughout

the septum, the transmitting tract and the 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 tissues (Fig. 5B). With the *AGP12* anti-sense 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 (Figs. 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 analyzing 26 of them, the ones with more information available. Even though, 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 pinpoint the artificial nature of the classification currently in use to organize this family of proteins. The availability of more data regarding *AGPs* expression patterns in different plant species, and more information regarding their functions may turn out possible to classify these proteins based 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 aminoacidic sequences, and, simultaneously, display a similar expression pattern in the reproductive tissues, suggesting that they might act redundantly, such as for the case of AGP16/AGP20, AGP1/AGP15, AGP5/AGP10 and AGP6/AGP11 pairs. AGP6/AGP11 is a pair of redundant AGPs involved in *Arabidopsis* pollen grain and PT 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 *in silico* search of AGP genes that could be transcribed preferentially in pistils rather than in the stamens or seedlings. This selection was based

406 on analyses of microarray data available for pistil and stamen tissues obtained from
407 Genevestigator, using the Anatomy tool provided by this service (Zimmermann *et al.*,
408 2004) and eFP Browser (Winter *et al.*, 2007). Although *AGP18* fits perfectly into this
409 category, it was not selected as it is already well described (Acosta-García and Vielle-
410 Calzada, 2004; Demesa-Arévalo and Vielle-Calzada, 2013). *AGP23* was chosen as a
411 control, since it is only transcribed during pollen development (Costa *et al.*, 2013c;
412 Nguema-Ona *et al.*, 2013). Although microarray data from Genevestigator also predicts
413 its expression in whole flowers and pistils, our qRT-PCR data confirmed that *AGP23* is
414 detected only in pollen, being highly down-regulated in pistils and seedlings.
415 The validation of this selection through qRT-PCR allowed us to limit the number of
416 *AGPs* selected for further analysis to four: *AGP1*, *AGP9*, *AGP12* and *AGP15*. *AGP9* and
417 *AGP15* are up-regulated in the pistil and down-regulated in the seedlings, being selected
418 for this reason. AGP1 was also selected, even if its transcripts show a higher up-
419 regulation in the seedlings than in the emasculated pistils, because it's phylogenetically
420 close to AGP15. *AGP12* was chosen as one of the most up-regulated AGPs in the pistil.
421 Regarding the *AGP* gene localization in the *Arabidopsis* chromosomes, it is clear that
422 AGPs are randomly distributed over the *Arabidopsis* genome. This is the case of *AGP16*
423 and *AGP20*, contained respectively on chromosome 2 and 3. Also for *AGP6*, located on
424 chromosome 5 and *AGP11*, on chromosome 3, two AGPs that were already shown to act
425 redundantly (Coimbra *et al.*, 2009). This is probably due to duplications in the genome,
426 since most of these genes are included in segments of the respective chromosomes that
427 were subject to large duplications events (Blanc *et al.*, 2000). This is consistent with the
428 prediction that genetic redundancy may occur as a consequence of gene duplication
429 (Kafri *et al.*, 2009). Only the pairs of most similar AGPs *AGP4*/*AGP7* and
430 *AGP1*/*AGP15* have their genes positioned in the same chromosome, but in opposed
431 regions. It is plausible that some of the AGP genes acquired a certain degree of
432 specialization, being now expressed in different tissues, under different conditions.
433
434 *AGPs* expression in the reproductive tissues
435 The results obtained in this work confirm the specific and differential pattern of
436 expression of AGPs previously predicted by immunolocalization studies, where several
437 monoclonal antibodies, which recognize distinctive AGPs 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 confirm and complement this older study but also improve the information already available about the AGPs distribution through the reproductive tissues by identifying specific AGPs present along these tissues. In Coimbra *et al.*, 2007, no antibody labelling was detected in the stigmatic cells, which, as it is shown here, are rich at least in AGP1 and AGP12. Neither it was detected any labelling in the funiculus of the ovules, whereas, in this study it is revealed the presence of several AGPs, such as AGP1, AGP12 and AGP15. This work reinforces the power of these techniques over the use of monoclonal antibodies to detect AGPs. As expected from qPCR data, *AGP23* is expressed only in pollen grains and PTs. Although microarray data available from Genevestigator expected *AGP23* to be present in pistils, this is 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 is 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, being easy to get pollen contamination in the stigma, misleading into some false positive expression. A summary of all the different approaches used to localize these *AGPs* and their differential pattern of expression along the reproductive tissues is shown in Fig. 6.

The FISH data obtained for *AGP1* and *AGP12* are partially consistent with the promoter analysis results shown for these two AGPs. The GFP expression driven by *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 maybe 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 thousands of base pairs away from the start site, in introns or even downstream 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 refers to a spatial-temporal analysis, much more detailed. The older immunolocalization studies (Junqueira, 2007) never detected the glycosidic AGP epitopes in these chalazal tissues. Even though we are aware that the antibodies used identify only sugar epitopes from all AGPs, we may conclude, with some cautions, that the accordance between the immunolocalization data and FISH results, fortifies the confidence in the use of antibodies to determine AGPs localization.

AGP1 and *AGP12* expression in the stigmatic cells suggests the possible involvement of this protein in pollen–stigmatic cells interactions, and acquisition of pollen grain competence to initiate PT growth. Losada and Herrero (2012) pointed out a role for AGPs in supporting PT 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 PTs 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 PT grows until it reaches the embryo sac – stigma, style, transmitting tract, septum, and funiculus – strengthens the putative role of AGPs in PT 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 PT 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 form an increasing gradient from the top to the bottom of the *Nicotiana* style, by the action of specific PT hydrolases, which may have a chemotropic effect on growing PTs. In *Arabidopsis*, the transmitting tract begins at the style between

the stigma-style boundaries, extending until 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 analyzed (data not shown) but revealed no visible phenotype. Most probably a double *agp1agp15* mutant is needed to access their precise function. These AGPs might act in these tissues in a similar manner as the TTS proteins in *Nicotiana*. The study of the *NTT* gene in *Arabidopsis* has indirectly implied the involvement of AGPs in PT 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 speculate 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 if there is a control of *AGPs* expression by this NTT zinc finger transcription factor.

AGP1, *AGP9* and more strongly *AGP12*, show 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, being this, 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 signaling between the vasculature and the embryo sac, endosperm or embryo, being quickly mobilized. The incomplete correlation between the GFP and GUS activity driven by *AGP12* and *AGP1* promoters in this region and their transcript expression reveals the importance of analyzing, hereafter, these AGPs at the protein level.

For double fertilization to take place, the PT must travel a long and challenging pathway, in order to reach its final destination: the micropylar entrance into 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 whole new generation (Márton and Dresselhaus, 2010). Along this narrow road the PT lengthens through a mucilage rich extracellular matrix (ECM) from the stigmatic cells, along the specialized transmitting tract cells, the funiculus and

534 the ovary integuments (Webb and Williams, 1988; Lennon *et al.*, 1998). Although most
535 of these studies showed that this ECM tract, through which the PT travel, is rich in
536 AGPs and pectins, to date, only some specific molecules have been shown to function as
537 PT growth enhancers such as GABA in *Arabidopsis* (Palanivelu *et al.*, 2003) and
538 chemocyanin in *Lilium longiflorum* (Kim *et al.*, 2003).
539 The results showed in this study, undoubtedly supports older works where AGPs were
540 proposed to be part of this pathway and to sustain PT growth (Clarke *et al.*, 1979;
541 Herrero and Dickinson, 1979; Gell *et al.*, 1986; Cheung *et al.*, 1995). *AGP1*, *AGP12* and
542 *AGP15* (Fig. 6) are located along all these tissues and might as well contribute to PT
543 growth from the top of the stigma to the base of the pistil, into the ovules, either by
544 nutritionally supporting their growth, facilitating their movement, guiding them to their
545 targets or even by making them competent for PT reception by the embryo sac. These
546 hypotheses needs further studies to fully assign AGPs functions in these tissues, much
547 probably involving the obtainment of double or triple null mutants. It is interesting to
548 note that we identified AGPs along the entire PT pathway (stigma, style and transmitting
549 tract) showing that AGPs are most probably essential for all the different steps of PT
550 growth through the pistil. The AGPs molecular mechanism of action and how they
551 interact with other cell wall and cell components is still elusive, although some
552 enlightening has been recently given to this matter (Costa *et al.*, 2013a). One possibility
553 may be related to the most recent finding that AGPs can act as calcium reservoirs,
554 making it available in a developmentally and temporarily way (Lamport and Várnai,
555 2012). It is well known the importance of calcium in sexual plant reproduction (Ge *et*
556 *al.*, 2007). One of the key characteristics of growing PTs is a tip-focused calcium
557 gradient maintained by the influx of extracellular calcium through calcium channels
558 active at the extreme end of the growing tip (Feijó *et al.*, 1995). AGPs may be regulating
559 in some way the release of calcium along the PT pathway making it available for the
560 PTs to grow. Most likely, different AGPs play several different roles during different
561 steps of the reproductive process, according to their localization and timing of
562 expression (Fig. 6). This work supports and improves the study of these enigmatic and
563 inscrutable glycoproteins in the sexual plant reproductive process, opening doors for
564 new pathways to the study of specific AGPs. Also, this type of analysis overcomes the
565 main difficulty regarding the older immunolocalization AGPs studies made by the use of

monoclonal antibodies that detect only the glycosidic epitope of the AGPs, allowing now the identification of a specific AGP in plant tissues.

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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 AGPs coding sequences were aligned using Clustal W and manually edited using Jalview to reduce gaps. Neighbor-joining (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 (10000 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. 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 violet square (AG peptides). (B) The 26 AGP and the four FLA genes were localized in 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 pattern for the 26 AGP and the four FLA genes was obtained using Genevestigator.

Fig. 2 qPCR relative expression levels of the selected AGPs 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). AGPs transcript levels were normalized to *ACT8* and *RUB1* reference genes levels, and are presented relative to the pollen transcript levels. In the panel, each bar represents an average of two independent reactions and technical replicates.

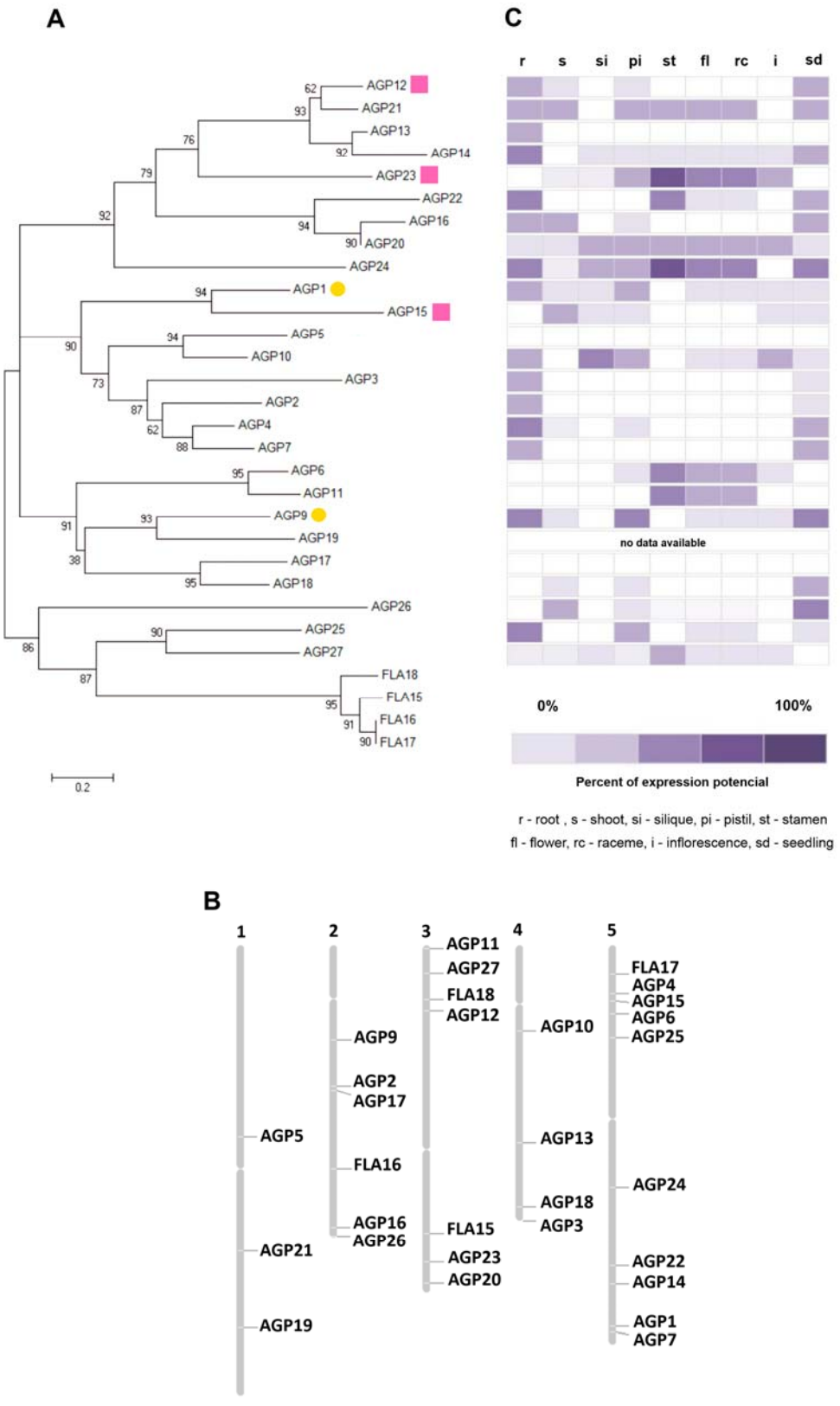
Fig. 3 Schematic representation of the expression cassette used in this study, and the resulting GFP signal shown in *Arabidopsis* reproductive tissues. (A) Expression cassette showing the relative position of promoter sequences (p*AGP*), nuclear localization signal (NLS), a fusion of three green fluorescent protein (3GFP) and the terminator Nos (NosT). (B) – (D) NLS-3GFP expression driven by the *AGP1* promoter in the style

612 tissues (B), in the opened pistil, in the funiculus and septum tissues (C), and seen in
613 more detail in the transmitting tissue, funiculus and the chalazal pole of the ovule (D).
614 (E) – (F) NLS-3GFP expression under the control of the AGP12 promoter is observed in
615 the stigmatic cells (E) and in the chalazal pole of the ovule (F). (G) – (H) NL-3GFP
616 expression driven by the *AGP15* promoter is detected in the ovule integuments, the
617 funiculus and the septum, but absent from the transmitting tissue (G). In (H) the GFP
618 signal is seen in more detail in the nuclei of the funiculus. (I) – (J) NLS-3GFP under the
619 control of the *AGP23* promoter is absent in all the sporophytic tissues (I) being its
620 expression restricted to the pollen grain, and, as can be seen in the detail in (J) DAPI
621 staining (here in magenta) revealed this expression to be limited to the vegetative cell of
622 the pollen grain; DAPI stained germinative nuclei are visible (white arrowheads). (K) –
623 (M) NLS-3GFP signals expressed by the *AGP9* promoter. Signals are observed in the
624 vascular bundle of the transmitting tract (K) and the funiculus (L) as well as in the
625 chalazal pole of the ovule (M). All the flowers used in these observations were at stage
626 12 and stage 13 according to Smyth *et al.* (1990). ch – chalaza; f – funiculus; m –
627 micropyle region of the ovule; ov – ovule; pg – pollen grain; s – stigma; sc – stigmatic
628 cell; sp – septum; st – style; v – vascultature; tt – transmitting tract. Bars: 100 μm in (B)
629 – (G) and (I), 50 μm in (H) and (K) – (M), 20 μm in (J).
630
631 Fig. 4 Histochemical localization of GUS activity in transgenic *Arabidopsis* reproductive
632 tissues expressing the p*AGP*:GUS fusion genes. (A) – (B) GUS activity driven by the
633 *AGP1* promoter is detected in the stigmatic cells (A) and the transmitting tract, the
634 funiculus and the integument cells (B). (C) – (D) GUS activity driven by the *AGP15*
635 promoter observed in the ovule integuments, funiculus and septum cells. (E) – (H) a
636 strong GUS activity driven by the *AGP23* promoter was identified inside the pollen
637 grains (E) and (F), the growing pollen tube (G). Upon fertilization, inside the embryo sac
638 a strong staining is observed at the local where the pollen tube bursts (H), followed by a
639 weak staining that spreads inside the whole embryo sac (H). Flowers of stage 12 and
640 stage 13 (Smyth *et al.*, 1990) were used in this study. ch – chalaza; es – embryo sac; f –
641 funiculus; ov – ovule; pg – pollen grain; pt – pollen tube; sc – stigmatic cell; sp –
642 septum; sy – synergid; tt – transmitting tract. Bars = 100 μm in (A) – (E) and (G) – (H),
643 50 μm in (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 transcript levels are detected in the funiculus, the transmitting tissue and the integuments. (C) and (E) AGP12 transcripts are localized in the stigmatic cells and along the septum tissues. (B), (D) and (F) FISH controls with the sense probe for *AGP1* in ovules (B), and *AGP12* in stigma (D), and ovules (F). All the flowers used in these observations were at stage 12 and stage 13 according to Smyth *et al.* (1990). f – funiculus; i – integuments; ov – ovule; s – stigma; sp – septum; st – style. Bars: 25μm in (A) – (B) and 75 μm in (C) – (F).

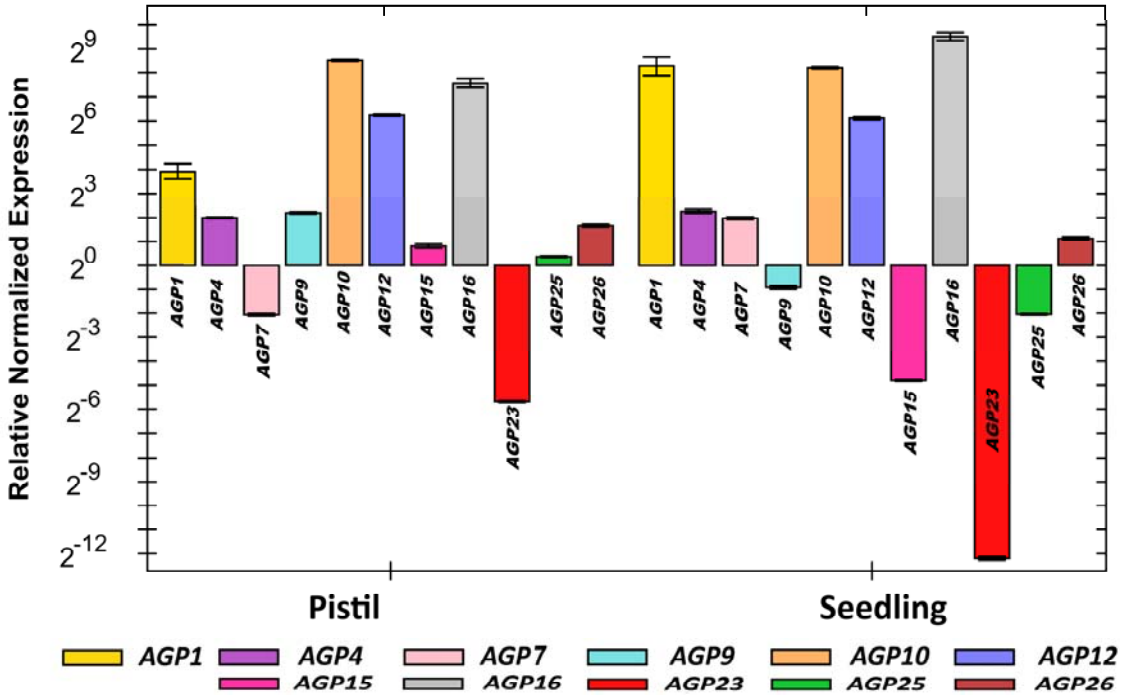
Fig. 6 A schematic representation of the reproductive structures and tissues of *Arabidopsis thaliana* and the distribution of the 5 AGPs analyzed in this study throughout the different tissues, regarding the different techniques used. GFP presence – green; GUS presence – blue; FISH positive – yellow; experiment not performed – red; absence of signal – grey. Sc – stigmatic cell; st – style; tt – transmitting tract; sp – septum; f – funiculus; ch – chalaza; i – integuments; pg – pollen grain; v – vasculature.

662 Figure 1



663

664 Figure 2



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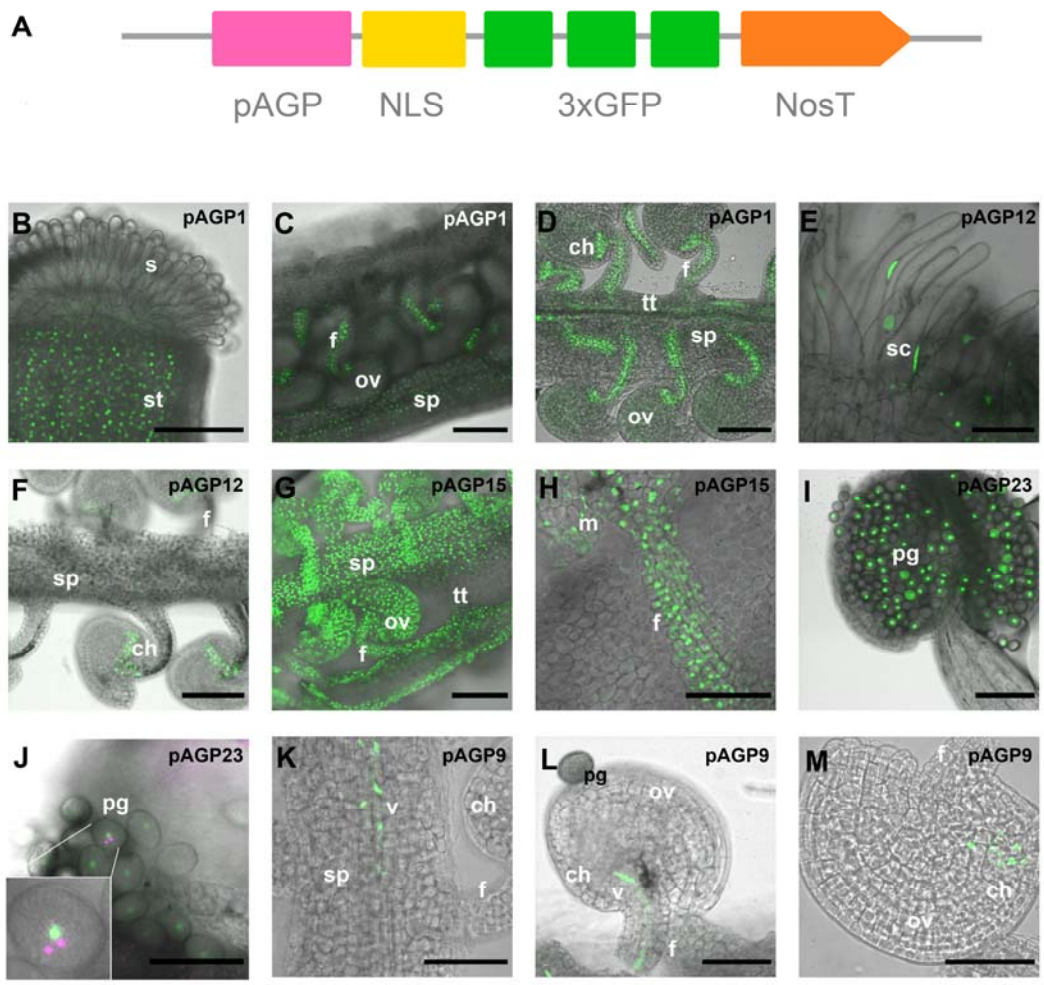
667 Figure 3



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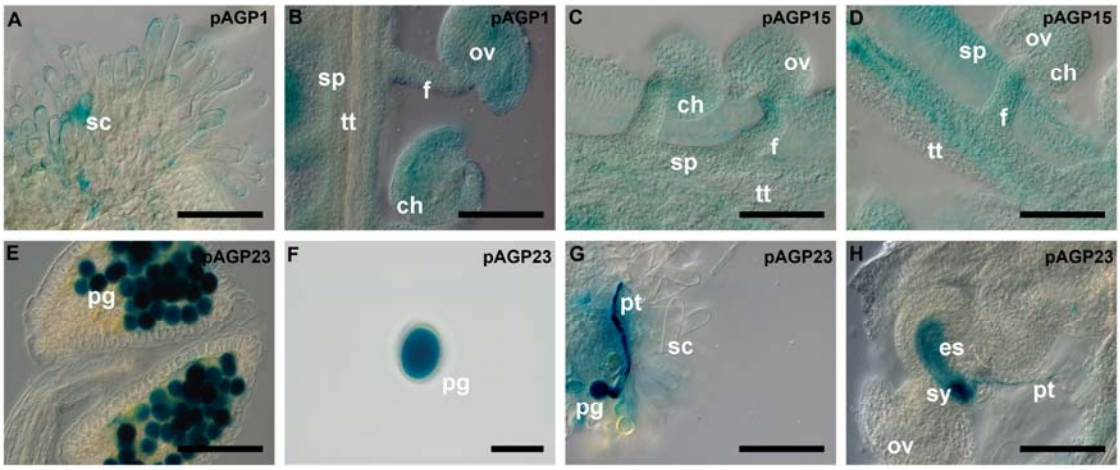
670 Figure 4



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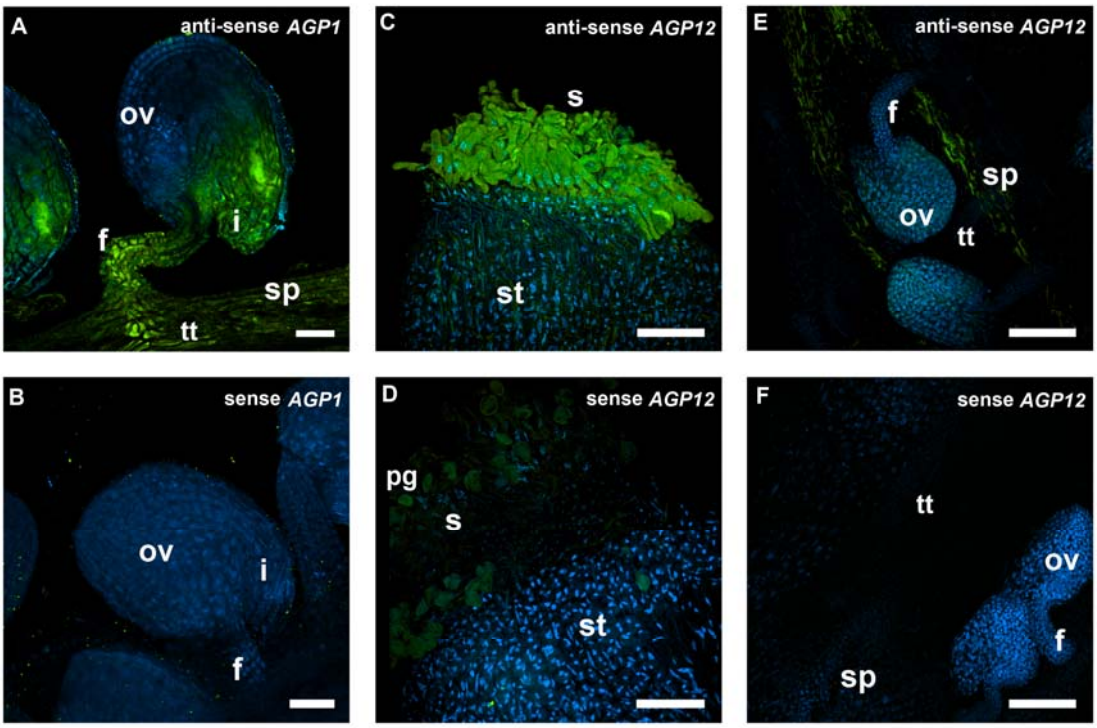
673 Figure 5



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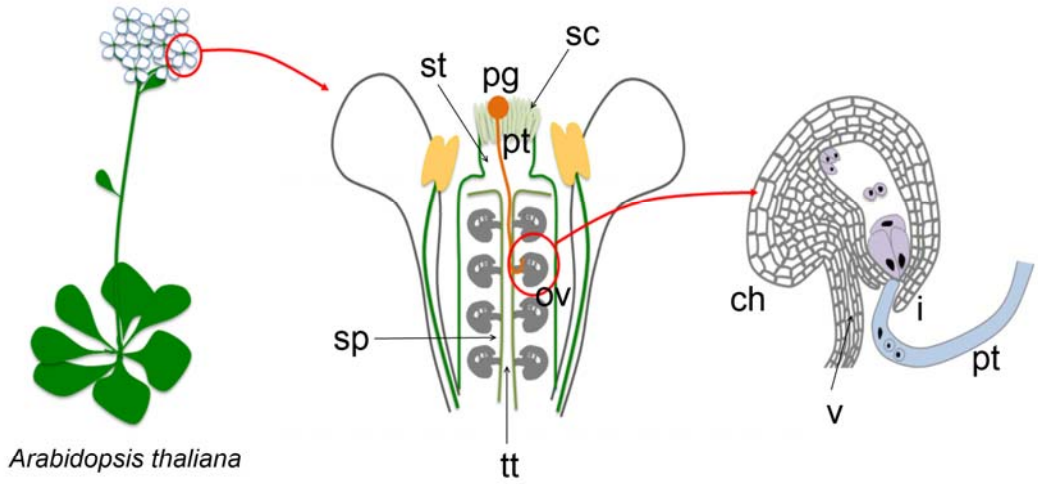
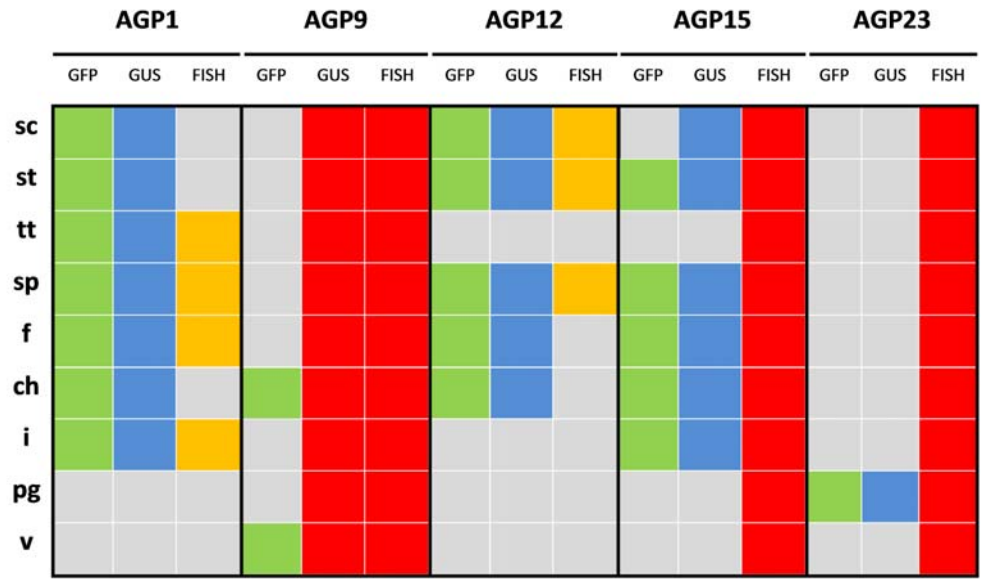
675

676 Figure 6





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