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Arabinogalactan Proteins in *Arabidopsis thaliana* Pollen Development

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1. Introduction

Pollen ontogeny is an attractive model to study cell division and differentiation. The progression from proliferating microspores to terminally differentiated pollen is characterized by large-scale repression of early program genes and the activation of a unique late gene-expression program in mature pollen.

Among the genes, or gene families that conform to the transition from a sporophytic type of development to a gametophytic program are the arabinogalactan protein (AGP) genes. AGPs are a class of plant proteoglycans, virtually present in all plant cells and in all plant species, from Algae to Angiosperms. They are predominantly located at the periphery of cells, i.e. on the plasma membrane and in the apoplast. Such ubiquitous presence insinuates that AGPs are vital components of the plant cell. Indeed, many studies have implicated AGPs in important biological phenomena, such as cell expansion, cell division, cell death, seed germination, pollen tube growth and guidance, resistance to infection, etc. (Seifert & Roberts, 2007). Evidences implicating AGPs in sexual reproduction have been obtained in our group, for several plant species (Coimbra & Duarte, 2003; Coimbra & Salema 1997; Coimbra et al., 2005) but how these molecules exert their function or how they interact with other cell components is yet to be defined. Only then the fragmentary knowledge that we have today about the function of these proteins may become pieces of a puzzle.

Following studies of pollen and pistil development in *Arabidopsis* with anti-AGP monoclonal antibodies (Coimbra et al., 2007; Pereira et al., 2006), it became clear that some AGPs may be suitable as molecular markers for gametophytic cell differentiation. Despite the tissue-specific carbohydrate epitopes of AGPs, these investigations do not allow the study of single AGP gene products. Therefore, a reverse genetics approach was undertaken to try to identify particular phenotypic traits attributable to certain AGPs, namely AGP6 and AGP11 that we had shown earlier to be pollen-specific (Pereira et al., 2006).

2. Arabinogalactan protein structure

2.1 Protein core

AGPs are complex macromolecules composed of a highly glycosylated protein core whose total mass may amount to only 5% or less of the total mass of the molecule (Fig. 1; Bacic et al., 2000; Nothnagel, 1997; Serpe & Nothnagel, 1999).

The nascent polypeptide chains of AGPs follow the cell's secretory pathway, and therefore contain an N-terminal signal sequence which targets the protein to the endoplasmic reticulum (ER). Most AGPs either contain or are predicted to contain, a C-terminal glycosylphosphatidylinositol (GPI) anchor addition sequence that is excised upon transfer of the protein to a pre-formed anchor present in the ER membrane. The mature protein core is rich in Pro/Hyp, Ala, Ser and Thr, which compose specific repetitive sequence modules. The pattern of these modules presumably constitutes the code that accounts for the characteristic glycan chains present in AGPs (Kieliszewski & Shpak, 2001; Schultz et al., 2004; Showalter et al., 2010). The repetitive amino acid modules (such as AP, TP, SP, or combinations of these) are typically scattered throughout the sequence of the mature protein. It is thus not likely that point mutations should affect the function of AGPs. Even if a mutation disrupts a glycosylation site, a number of others remain.

The properties of the protein backbone define subclasses of AGPs (Schultz et al., 2004). "Classical" AGPs typically contain the central Pro/Hyp-rich domain sandwiched between the N-terminal signal peptide and the C-terminal GPI addition sequence; the lysine-rich AGPs are like "classical" AGPs but contain a Lys-rich module, arabinogalactan (AG) peptides contain small mature protein cores, typically under 30 amino acid residues, the fasciclin-like AGPs (FLAs) may be considered chimeric AGPs because they contain typical glycosylation AGP modules and fasciclin-like domains (Johnson et al., 2003; Schultz et al., 2004; Showalter et al., 2010). Other atypical or chimeric AGPs, such as AGP31, have also been described (Showalter et al., 2010).

Fig. 1. Highly schematic representation of the molecular structure of a "classical" AGP. (A) Immature polypeptide, and (B) Mature proteoglycan with attached GPI anchor. SP, N-terminal signal peptide; C-ter, C-terminal GPI anchor addition sequence.

2.2 Glycan

AGPs belong to a wider superfamily of plant proteins, the hydroxyproline-rich glycoproteins (HRGPs) which also comprise the extensins and the proline-rich proteins. This classification is mainly based on the sugar content and glycosylation patterns (Kieliszewski

& Shpak, 2001; Shpak et al., 1999) of the molecules, the AGPs being the most heavily glycosylated of all.

AGP glycans are polyssacharide chains O-glycosidically linked to Hyp residues. Polysacharide chains are type II arabinogalactan chains, which consist of a main chain of β -(1 $\rightarrow$ 3)-galactopyranose units variously substituted at C6 with oligosacharide or polysacharide chains rich in Gal and Ara but may also contain other monosacharide residues such as Rha and GlcA (see Ellis et al., 2010 for a recent review).

One of the main tools to study the biology of AGPs has been the use of monoclonal antibodies that bind to AGP-specific sugar epitopes. From those studies we know that different AGP epitopes are only present in specific cell types or tissues, or in particular developmental stages. The genetic regulation of this diversity, both in space and in time, is further complicated by the regulation of the necessary battery of glycosyl transferase activities needed to the synthesis of complex sugar chains.

2.3 GPI anchor

The GPI anchor is a post-translational modification of eukaryotic proteins. Newly synthesized proteins that contain a C-terminal GPI-addition signal sequence become attached to a preassembled GPI anchor present in the membrane of the ER, concomitant with the cleavage of the signal sequence. As a result of this modification, GPI-anchored proteins (GAPs) become tethered to the outer layer of the plasma membrane facing the extracellular environment.

Many AGPs were either experimentally shown to be GPI-anchored (about twenty AGPs in *Arabidopsis*; Borner et al., 2003; Elortza et al., 2003, 2006; Lalanne et al., 2004; Schultz et al., 2004) or, based on amino acid sequence analysis, predicted to be GPI-anchored. In *Arabidopsis*, only a few AGPs are not predicted to contain a GPI anchor.

GPI anchors are themselves complex structures, having a highly conserved core composed of ethanolamine-PO₄-6Man α 1-2Man α 1-6Man α 1-4GlcN α 1-6*myo*-inositol-1-PO₄-lipid. As opposed to the majority of known GPI anchors, whose lipid part is diacylglycerol-based, the inositolphospholipid part of the only two plant GPI anchors characterized to date is an inositolphosphoceramide (Oxley & Bacic, 1999; Svetek et al., 1999).

Despite the high biological investment in such structures, involving more than twenty dedicated gene products for its biosynthesis, the role of the GPI anchor, or its contribution to the protein biological function is not evident, and remains unsolved for most, if not all, studied examples of GAPs. Moreover, during transit through the secretory system, GPI anchors may be subjected to lipid and/or carbohydrate side-chain remodeling, resulting in a number of GPI structural variants, which may be present in the same organism (Ferguson et al., 2008), and which undoubtedly raises the level of complexity of the biology of GAPs.

Despite all the unanswered questions, specific and commonly referred properties of GAPs mediated by the GPI are their likely association with lipid rafts (Borner et al., 2005), cellular polar sorting (particularly in animal and yeast cells; Legler et al., 2005), and controlled release to the extracellular matrix through the specific action of phospholipases. Indeed, stress conditions such as salinity, cold, drought, heat, wounding, and pathogen attack, are

known to activate phospholipase D (PLD), or phospholipase C (PLC) pathways (Testerink & Munnik, 2011), but the actual release of GAPs as a direct consequence of environmental or physiological stimuli is yet to be demonstrated.

Nevertheless, the combination of AGP characteristics, namely cell and/or tissue localization, possible release from the plasma membrane upon stimuli, and complex sugar content that makes AGPs, or specific fragments of AGPs, as likely candidates to perform signaling functions in plants.

3. Pollen development

In flowering plants, development of the haploid male gametophytes (pollen grains) occurs inside a specialized structure called the anther. Successful pollen development, and thus sexual reproduction, requires the correct development of the anther wall layers, and an increased growth of the four locules with fusion into two pollen sacs, and relies on the provision of nutrients and other materials from a specialized secretory tissue, the tapetum. The importance of the tapetum for pollen development is highlighted by findings that the majority of male-sterility mutants involve injuries that affect the expression of tapetum-specific genes. The gene products are released from the tapetal cells and transferred to the pollen surface in wild-type plants. Although there have been many excellent ultrastructural studies of tapetal and pollen development, the underlying biochemical processes have, until recently, remained unclear. This situation is now changing rapidly with the advent of the genome sequence and tools to allow the analysis of gene function (Wilson & Zang, 2009).

3.1 Microsporogenesis

In *Arabidopsis*, at the beginning of anther development, under the protoderm layer, groups of cells develop to give rise to a primary parietal layer and to a sporogenous layer (Owen & Makaroff, 1995). The first will divide to originate the different anther wall layers, and the sporogenous tissue will give rise to the microsporocyte cells. At the pre-meiotic stage of microsporogenesis, the five wall layers of the anther are well differentiated; the microsporocytes have thin cell walls and are successively surrounded by the tapetum, median layer, endothecium and epidermis (Fig. 2).

At the beginning of meiosis, microsporocytes are connected with the tapetal cells by plasmodesmata and it is at this moment that callose deposition begins. During meiosis, the microsporocytes are interconnected by cytomictic channels and their cytoplasm starts to dedifferentiate, which is probably related with the transition from a type of sporophytic gene expression to one of gametophytic gene expression. This transition relates to the change from a diploid to a haploid generation. At this time callose deposition continues, resulting in thick callose walls surrounding the microsporocytes. It has already been assumed that this callose wall can be the trigger to initiate the gametophytic type of development (McCormick, 1993). This physical isolation is important to activate such dramatic changes in development. Following meiosis and cytokinesis, the four haploid microspores are arranged in tetrads encased by a thick callose wall, within the callose wall a microspore-produced cell wall, the primexine is present (Coimbra et al., 2007).

At the end of meiosis, the external walls of the tetrads are dissolved to release individual microspores, by a mixture of enzymes containing endoglucanases and exoglucanases

secreted by the tapetum (callase). Alterations in the timing of this event, or failure to express β -1,3-glucanases, leads to abnormal disruption of the callose walls, which has been shown to be a primary cause of male sterility in cytoplasmic male-sterile lines of several species, including *Petunia* (Izhar & Frankel, 1971).

Fig. 2. Light microscopy image of *Amaranthus hypochondriacus* anthers, showing well differentiated microsporocytes (m) and the undifferentiated anther wall layers; from the outside the epidermis (ep), the endothecium (arrow), the middle layer (arrow head) and the tapetum (t) surrounding the microsporocyte cells. Bar 10 μ m.

The coordinated development of the microsporocytes and the surrounding cell layers might involve cell-to-cell interactions and localized signaling. From studies with the *SPOROCTELESS* (*SPL*) mutants, it was suggested that microsporocyte formation and anther wall development might be coupled. In these mutants, neither microsporocytes nor anther walls are formed and because the microsporocytes and the haploid spores of the tetrads become isolated by callose deposition it is likely that the signaling occurs early during the formation of microsporocytes. The expression of the *SPL* gene in the microsporocytes and its absence from the parietal cells suggests that *SPL* functions within the microsporocytes to regulate expression of a subset of genes required for microsporocyte formation. Therefore, the authors suggested that the microsporocytes promote, through signal exchange or cell-to-cell interactions, the differentiation and growth of the parietal cell layers, and consequently anther wall development (Yang et al., 1999).

3.2 Microgametogenesis

Microgametogenesis starts with the release of the microspores from the tetrads and leads through a simple cell lineage, to the formation of the mature pollen grains. The ultrastructural description of this developmental stage shows high exocytic activity in tapetum cells, the elaboration of the intine wall by microspores and the deposition of sporopollenin, one of the most complex polymers of plants, which is the outer pollen wall constituent. This outer wall, the exine, is initiated with the formation of the primexine layer at the tetrad stage of development (Owen & Makaroff, 1995).

Microspores start to build a roundish shape due to the formation of a central vacuole, which originates from the fusion and enlargement of small vacuoles. In response to this, the microspore nucleus shifts to an eccentric position against the microspore wall. The first mitosis of the microspore is asymmetric and originates a large and transcriptionally active vegetative cell, and a small generative cell with condensed chromatin that will divide again and originate the two male gametes (Tanaka, 1997) (Fig. 3).

Fig. 3. Anther of *Amaranthus hypochondriacus* showing bicellular pollen grains with a large vegetative cell (CV) and a small and generative cell (CG) in an asymmetric position. The exine wall (arrow head) is being built by the pollen grain and by the sporopollenin depositions from tapetal cells (arrow). Bar 5 μ m

The vegetative cell cytoplasm shows strong metabolic alterations related to the asymmetric cell division, one of the most striking events of cell differentiation occurring during the plant life cycle. A single mitotic division will give rise to two completely different cells in size, function and gene activity (Fig. 4). This asymmetric division is vital for generative cell differentiation. Microgametogenesis is quite regular in flowering plants, being the

distinction between the timing of the second mitosis the biggest difference; in the majority of Angiosperms, pollen is shed in a bicellular condition, and the generative cell divides inside the pollen tube; whereas in tricellular pollen species, like *Arabidopsis*, the generative cell divides inside the anther. Among the first gametophytic cell markers to be identified is the tomato pollen-specific LAT52 promoter that drives expression specifically in the vegetative cell after pollen mitosis I (Eady et al., 1995), and the lily generative cell-specific H3 histones (gcH2A, gcH3) (Xu et al., 1999). The cytoskeleton plays a central role in determining both nuclear migration and the eccentric division plane. After mitosis, the generative cell migration is a uniquely specialized cell-cell support, which creates a “cell within a cell”. The generative cell then assumes a spindle shape and DNA replication takes place, whereas the vegetative nucleus remains arrested in G1 (Fig. 5E). At the end of microgametogenesis, the exine wall will be completely formed, with depositions secreted by tapetal cell (Fig. 3) and the tapetal cells will finally degenerate, depositing their contents in the exine sculptures of the pollen grains, forming the pollen coat.

Fig. 4. Electron micrograph of a young bicellular pollen grain of *Amaranthus hypochondriacus*. The generative cell (CG) just formed is still in a spindle shape adjacent to the pollen wall. E- exine (b- baculae, v- vacuole, m- mitochondria, n- nucleus).

All the steps of this complex process rely on a complex network of signaling events, which probably involve molecules of different kinds (Preuss, 2002). Pollen ontogeny is an attractive model to study cell division and differentiation. In this genomic era, new technologies begin to unravel the roles of specific genes involved in male gametogenesis (Honys et al., 2006; Quan et al., 2008; Toller et al., 2008; Twell 2011).

3.3 Pollen tube growth

Pollen tube growth begins with its emergence from the vegetative cell of the pollen grain after adhesion to the stigma surface. This process depends on the hydration of the pollen

grain which is accomplished after recognition. A strictly apical cell growth process maintains the pollen tube cytoplasm and its cargo, the sperm cells, in the most proximal region of the tube as it elongates through the female sporophytic tissues. Callose plugs are laid down at regular intervals behind the growing tip, and the region adjacent to the plug becomes vacuolated. The extreme end of the growing tip consists of a highly dynamic clear zone that contains vesicles and cell wall precursors. Activity in this zone involves continual biosynthesis of cell wall and plasma membrane and turnover of cytoskeletal components as the tube elongates.

In recent years, substantial progress has been made toward understanding tip growth mechanisms (Feijó et al., 1995). Pollen tube reorientation can occur in several minutes, so this might exclude the involvement of newly synthesized gene products. The existence of a tip-focused gradient of cytosolic free calcium has been shown to be maintained by an asymmetric activity of calcium channels and to be essential for growth (Malhó et al., 1995). Polymerization of the actin cytoskeleton is also essential, and tip localized F-actin is thought to mediate the membrane trafficking of secretory vesicles to the apex.

Mutants have been described in which pollen tubes are unable to locate the ovules, as a result of altered interactions with these structures (Higashiyama et al., 2003; Hülskamp et al., 1995). There has been controversy over whether a diffusible signal attracts the pollen tube or whether the female tissues define its path, but recent genetic and physiological data for several plant species, has showed that the female gametophyte produces at least one diffusible signal, which is derived from the two synergids cells (Higashiyama et al., 2003).

4. Arabinogalactan proteins in *Arabidopsis* pollen development

4.1 Immunolocalization of glycan epitopes

Specific monoclonal antibodies (mAbs) that bind to structurally complex carbohydrate epitopes of AGPs have been very useful in revealing the developmental dynamics of the AGP glycan moiety and represent a diagnostic tool for AGPs. Frequently used anti-AGP mAbs are JIM8, JIM13, JIM14, LM2, and MAC207. Accumulated information, obtained by the extensive use of anti-AGP mAbs by the scientific community, shows that AGPs are finely regulated and differentially expressed during pollen development, namely during sexual plant reproduction.

Given the importance of *Arabidopsis* as a model plant, a detailed map of AGP sugar epitopes in different flower parts and at different stages of development was obtained and clearly showed differences in the pattern of distribution of specific AGP sugar epitopes, during *Arabidopsis* anther development. These differences are apparent both in sporophytic and in gametophytic tissues, and it became evident that AGP-specific epitopes can work as markers for certain cell or tissue types, in very precise stages of sporogenesis and gametogenesis.

In the premeiotic stage of microsporogenesis, the epitopes recognized by the mAbs JIM8 and JIM13 are specifically and intensely localized in the tapetum cells and in microsporocytes (Fig. 5A). The selective labelling of the microsporocyte walls is quite

important at this stage of development when microsporocytes start a dedifferentiation program related with the transition from a sporophytic gene expression to a type of gametophytic gene expression. This transition relates to the change of generations, from diploid to haploid. As soon as prophase starts, the callose deposition also starts, resulting in thick callose walls surrounding the microsporocytes (Fig. 5C). After meiosis and cytokinesis of the four haploid microspores, the tetrads are completely encased by a thick callose wall. Within the callose wall a microspore-produced cell wall, the primexine, is present. It is interesting to notice that the first existent separation wall in microsporocytes is still present at this stage and still labelled by mAbs JIM8 and JIM13. This labelling pattern can be associated to the signals that must be produced for the efficient release of callase, by the tapetum cells, or a type of developmental time specificity related to the gametophytic development (Fig. 5D).

The presence of AGPs in the tapetum clearly shows that this tissue synthesizes and secretes these molecules (Fig. 5D). The interaction of the sporophytic tapetal cells and the gametophytic differentiation of meiocytes into microspores, are present in the synchronism of callase release from the tapetum ER, as well as from the sporopollenin precursors released into the anther locule. AGPs are strong candidates for cell differentiation signals at this stage of development. It can also be correlated the presence of AGPs with tissues that are set out to programmed cell death. In this study it was observed that the stage at which programmed cell death is triggered, the end of the tetrad stage, is associated with the stronger presence of AGPs recognized by mAbs JIM8 and JIM13.

The labelling with mAbs JIM8 and JIM13 is also strong in the microspores outer surface, which is the site where the intine wall will be built, indicating some association of this important developmental stage with AGP synthesis (Fig. 5D).

During pollen development, it is well documented the strong metabolic alterations in the vegetative cell cytoplasm related to the asymmetric cell division, one of the most striking events of cell differentiation occurring during the plant life cycle. JIM8 and JIM13 specifically label the generative and gametic cells, but not the gametophytic cell. This labelling may function as a molecular marker for cell development and may also be related to the signals necessary to direct these cells inside the pollen tube into their targets, in the embryo sac (Fig. 5E). Moreover, after the second pollen mitosis, the two resulting sperm cells that are inside the pollen grain or inside the pollen tube are still strongly labelled by these two mAbs. The specific labelling of the generative cell was also reported for oilseed rape (Pennell et al., 1991), for *Nicotiana tabacum* (Li et al., 1995) and for *Brassica campestris* male gametes (Southworth & Kwiatkowski, 1996). JIM8 and JIM13 do not label the pollen tube wall, which instead is labelled by MAC207 and LM2 (Pereira et al., 2006). These two mAbs are probably related to epitopes in structural AGPs present in several types of plant cell walls.

Antibodies MAC207 and LM2 showed similar binding patterns, both defining extended cell populations in different tissues (Figs. 5B and 5F), as opposed to JIM8 and JIM13 which seemed to define single tissues or single cell types and that can be suitable as molecular markers for pollen development in *Arabidopsis*.

Fig. 5. Fluorescence microscopy of *Arabidopsis* anthers at different stages of pollen development labelled with monoclonal antibodies specific for arabinogalactan proteins (JIM8, JIM13 and MAC207) with FITC-conjugated secondary antibody. A) JIM8 labelling at the stage before meiosis. Tapetal cells and microsporocytes are labelled but there is a remarkable labelling of the wall that surrounds the microsporocytes that are dedifferentiating. B) Same stage of development labelled by MAC207 showing a diffuse labelling in the endothecium and epidermis cells. C) Tetrads of haploid microspores encased by a thick callose wall, the mAb JIM8 is labelling the initial wall of the microsporocytes. D) Microspores just released from tetrads. AGP epitopes recognized by JIM13 are present in tapetal cells and also in the endothecium, which is now developing. This mAb also binds to the microspores cytoplasm and outer surface where the intine wall will eventually develop. E) Tricellular pollen showing specifically the labelling of the two male gametes with JIM8. F) Same stage as in E showing a unspecific labelling with MAC207. T - Tapetum; mi - microsporocytes, E - endothecium.

In order to study the adhesion of pollen tubes via cell wall molecules, the cell wall characteristics of in vitro-grown *Arabidopsis* pollen tubes were investigated using a combination of immunocytochemical and biochemical techniques. Results showed a well-defined localization of cell wall epitopes. Low esterified homogalacturonan epitopes were found mostly in the pollen tube wall back from the tip. Xyloglucan and arabinan from rhamnogalacturonan I epitopes were detected along the entire tube within the two wall layers and the outer wall layer, respectively. In contrast, highly esterified homogalacturonan and arabinogalactan protein epitopes were found associated predominantly with the tip region. This work demonstrated that the *Arabidopsis* pollen tube wall has its own characteristics compared with other cell types in the *Arabidopsis* sporophyte which are probably related to the pollen tube specific growth dynamics (Dardelle et al., 2010).

4.2 AGP tagging

The use of tags engineered into specific AGP gene products is a particularly pertinent approach to study individual AGPs since alternative approaches, such as the use of specific anti-peptide antibodies or even basic analytical tools such as SDS-PAGE may convey challenging difficulties imposed by the massive presence of glycan chains of indeterminate composition and size. Several studies have been published during recent years that include experiments with AGP tagging (Levitin et al., 2008; Li et al., 2010; Sun et al., 2004a, 2004b, 2005; Yang & Showalter, 2007; Van Hengel & Roberts 2003; Zhao et al., 2002). The DNA constructs that underlie those experiments have been used for intracellular localization, expression characterization or purification.

For intracellular localization studies, DNA constructions should contain: i) a functional signal peptide sequence to direct the fusion protein to the ER; ii) the reporter gene, in general green fluorescent protein (GFP) gene has been used; and iii) the mature AGP sequence together with the GPI addition sequence for proper anchoring to the plasma membrane. Constructions such as these have been placed under the influence of the 35S Cauliflower Mosaic Virus (CaMV) promoter for constitutive overexpression studies (phenotype analysis) and to study the consequences of deleting the GPI addition sequence or the Lys-rich domain in Lys-rich AGPs (Li et al., 2010; Sun et al., 2004a, 2004b, 2005; Yang & Showalter, 2007; Zhao et al., 2002). DNA constructions placed under the control of AGP endogenous promoters instead of the 35S CaMV promoter are presently under analysis in our laboratory (unpublished results).

Van Hengel & Roberts (2003) used a *c-myc* tag placed at the C-terminus of a DNA construction containing the complete coding sequence of AGP30 (35S::AGP30-*c-myc*) and used it for electroblot analysis of plant extracts. Indeed, for those few *Arabidopsis* AGPs that may not have a GPI anchor, the tag may be placed at the C-terminus with reduced risk of becoming separated from the remaining polypeptide.

For cell or tissue expression studies, DNA constructions are technically simpler, and may consist of endogenous AGP promoter sequence plus reporter gene, with or without AGP-specific sequences. This approach has been used to confirm and characterize the developmental expression of two pollen-specific *Arabidopsis* classical AGPs (AGP6 and AGP11), and one pollen-specific FLA (FLA3), using either GFP, GUS, or the red fluorescent

protein RFP as reporter genes (Coimbra et al., 2008, 2009; Levitin et al., 2008; Li et al., 2010). The temporal expression of AGP6 was determined with great accuracy in *Arabidopsis* plants that were transformed with a ProAGP6::GFP gene construct. GFP fluorescence was absent in all vegetative plant parts, but became clearly visible just after the appearance of the locules in anthers. This stage was identified as corresponding to stage 9 (as described by Smyth et al., 1990). GFP fluorescence was limited to pollen and pollen tubes and could be clearly differentiated from the green yellow autofluorescence characteristic of the exine and of the endothecium lignin thickenings of the anther wall. GFP fluorescence persisted through to the mature pollen grains and was observed in growing pollen tubes (Coimbra et al., 2008; 2009).

4.3 Reverse genetics

The distribution and expression patterns of pollen-specific AGPs have been examined closely in our laboratory. So we tried to identify particular phenotypic traits attributable to either AGP6 or AGP11, or both, in a reverse genetics approach. *AGP6* and *AGP11* are closely related genes, sharing 68% of the amino acid sequence, and therefore seemingly constituting a pair of paralog genes, the function of which may be mutually overlapping. Ds transposon insertion mutant lines for these two genes were available from RIKEN BioResource Center. However, as with so many other plant single gene null mutants, these did not produce recognizable phenotypes (Coimbra et al., 2009). At least in the case of *AGP6* and *AGP11*, a double *agp6 agp11* null mutant did produce identifiable phenotypic traits which could be ascribed to the simultaneous lack of both gene products. In *agp6 agp11* double homozygous mutant lines, but also in plants homozygous for one of the insertions and heterozygous for the other, many of the pollen grains failed to develop normally and collapsed, indicating that the genes are important gametophytically for pollen development (Fig. 6). The collapsed pollen phenotype of *agp6 agp11* was characterized both by scanning electron microscopy and by transmission electron microscopy of pollen grains that clearly showed the degeneration of pollen contents (Coimbra et al., 2009).

Despite the collapsed pollen phenotype typical of the homozygous *agp6 agp11* mutants, a percentage of the pollen grains were able to develop and germinate into functional pollen tubes, as assessed by the presence of seeds in self-pollinated plants. Thus it can be assumed that *AGP6* and *AGP11* are non-essential for stabilizing pollen grain development, or else that an alternative pathway, potentially involving the ectopic or up-regulation of the expression of other AGP family members, is able partially to compensate for the loss of the two proteins.

In other studies aiming at a more inclusive phenotypic characterization of the *agp6 agp11* double null mutants, it was detected that a number of pollen grains germinated precociously inside the anthers and this phenomenon was dependent upon the relative humidity of the growth chamber (Coimbra et al., 2010). Pollen germination inside anthers was not observed in single *agp6* or *agp11* mutants, and therefore those observations indicated that the double mutation was needed to induce the precocious germination character. Precocious germination of pollen was never found in wild-type plants, even in conditions of high relative humidity, a factor that increased the presence of the phenotypic

trait in the double mutant. AGP6 and AGP11 thus seem to have a role in preventing an early and wasteful germination of pollen inside the anthers. As a rule, untimely germination inside anthers does not occur, so some factor or factors must be preventing it from happening. It is also noteworthy that the minimal conditions for pollen germination and tube growth are reproduced inside the anthers. The fact that pollen tubes can germinate and elongate inside the anther locules poses interesting questions regarding germination control and nutritional requirements necessary to support the high respiration rates generally believed to occur for rapid tube growth. It is indeed interesting and challenging to appreciate that arabinogalactan proteoglycans may be interfering with the timing of pollen germination, maybe by a relatively simple process of modulating access of water for hydration (or for the earlier dehydration process), or by interfering with some kind of signaling pathway.

Fig. 6. Light micrograph of an *agp6 agp11* anther showing collapsed pollen grains and some roundish normal pollen grains.

4.4 Microarrays

A series of studies have revealed distinctive transcriptome profiles in microspores, mature pollen and germline cells. The most complete datasets have been generated in the genetic model *Arabidopsis*, where transcriptome analyses have revealed developmental expression profiles from microspores to mature pollen (Honys & Twell, 2004; Pina et al., 2005), the sperm cell transcriptome (Borges et al., 2008) and transcript changes associated with pollen germination and tube growth (Quin et al., 2009; Wang et al., 2008). Sequentially, in our work with the aim of dissecting the biological function of AGPs, we decided to evaluate the whole set of pollen tube expressed genes. For this purpose, we performed microarrays, using the Affymetrix ATH1 genome arrays in the *agp6 agp11* double null mutant pollen tube. We believe that this work is of great general interest for the field of plant science, not only because it highlights interactions between AGPs and other specific gene products but also

because of the surprisingly high number of genes whose expression is altered in the pollen tubes of the mutant line, revealing that these molecules can only be involved in very complex phenomena.

This work identified 1300 genes which have either reduced or elevated expression in the *agp6 agp11* pollen tube as compared to wild-type pollen tube, being some of these genes completely inactivated. These genes can be used as starting points to dissect the gene regulatory networks where AGPs are involved during pollen tube growth.

5. Arabinogalactan proteins in other species pollen development

5.1 Rice

Working with *Oryza sativa indica*, Anand & Tyagi (2010) reported the molecular characterization and the promoter activity of a rice pollen-preferential gene, OSIAGP. The authors isolated this gene and identified it as an arabinogalactan protein gene, during a differential screening of inflorescence-specific cDNA libraries. OSIAGP protein has a secretory domain at its N-terminus and *in silico* analysis revealed it to be a secretory and transmembrane protein. When databases were searched using OSIAGP nucleotide and protein sequences, they showed significant homology with a pollen-preferential gene, AGP23 from *Arabidopsis* (AT3G57690). The eight amino acids of the secretory domain at the N-terminus are 100% conserved among OSIAGP and AGP23. The AGP23 gene is 369 bp long and encodes a 61-amino acid protein with homology with other arabinogalactan proteins from *Arabidopsis*. OSIAGP is 59 amino acids and a pollen-preferential gene falling in the category of late pollen genes and is speculated to play important role in pollen tube growth. Its promoter harboring regulatory elements for pollen expression and light regulation could be of interest to the plant community.

Ma & Zhao (2010) identified 69 AGPs from the rice genome, including 13 classical AGPs, 15 AG peptides, 3 non-classical AGPs, 3 early nodulin-like AGPs (eNod-like AGPs), 8 non-specific lipid transfer protein-like AGPs (nsLTP-like AGPs), and 27 FLAs. The results from expressed sequence tags, microarrays, and massively parallel signature sequencing tags revealed that several rice AGP-encoding genes are predominantly expressed in anthers and display differential expression patterns in response to abscisic acid, gibberellic acid, and abiotic stresses, which is highly in agreement with the microarray results obtained in our group for *Arabidopsis* (manuscript in preparation). The two classical AGP-encoding genes, OsAGP7 and OsAGP10, are highly expressed in pollen, similar to AtAGP6 and AtAGP11. The phylogenetically closest rice gene is OsAGP6, but it has a different expression pattern from that of AtAGP6 and AtAGP11. In this case, it is possible that the genes sharing the same function are not those with higher identity. Therefore, OsAGP7 and OsAGP10 may play a conserved role in pollen development, like AtAGP6 and AtAGP11 which redundantly control pollen development and fertility (Coimbra et al., 2009, 2010; Levitin et al., 2008).

5.2 Brassica

The firstly characterized pollen-specific putative AGP genes, Sta 39-4 and Sta 39-3 were isolated from *Brassica napus* flowers in 1996 (Gerster et al., 1996). These two genes are highly

homologous; they are 95% identical at the nucleic acid level and 98% identical at the amino acid level. Park et al. (2005) isolated and characterized a pollen preferential gene, *BAN102*, from the Chinese cabbage (*B. campestris*). After analyzing its sequence by BLAST search they found that the coding region of *BAN102* gene had great similarity with AGP23 gene from *Arabidopsis*. The similarities of nucleotide and amino acid sequences were 91% (170/186) and 90% (55/61), respectively.

The *BcMF8* (*B. campestris* male fertility 8) gene, possessing the features of classical AGP was later isolated from *B. campestris*. This gene was highly abundant in the fertile flower buds but silenced in the sterile ones of genic male sterile A/B line ('ZUBajh97-01A/B'). Expression patterns analysis suggested *BcMF8* to be a pollen-specific gene, whose transcript started to be expressed at the uninucleate stage and maintained throughout up to the pollination stage. Isolation and multiple alignments of the homologs of *BcMF8* gene in the family *Cruciferae* indicated that *BcMF8* was highly conserved in this family sharing high sequence identity with those of the putative pollen-expressed AGPs genes *Sta 39-4* and *Sta 39-3*, and a lower similarity with that of AGP genes *AtAGP11* and *AtAGP6*.

Besides *Sta 39-4*, *Sta 39-3*, *AtAGP11*, *AtAGP6*, and *BAN102*, a pollen-specific AGP gene *PO2* from alfalfa has been characterized by Qiu et al. (1997) using a similar differential screening technology; after performing an alignment of the deduced *BcMF8* protein sequence and its homolog from family *Cruciferae* with all these pollen-specific AGPs mentioned above and the other putative AGPs from the *Arabidopsis* database, the authors found that *BcMF8* clustered with those known pollen-specific AGPs, *Sta 39-4*, *Sta 39-3*, *AtAGP11* and *AtAGP6*. This result provided further evidence to the hypothesis that *BcMF8* was pollen-specific. Interestingly *BAN102* and *AtAGP23* are closely related to one another. Indeed, *AtAGP23* belongs to AG-peptides subclass which differs in sequence composition from classical AGPs. These results may suggest that pollen development requires different members of the AGP family (Huang et al., 2008).

5.3 Nicotiana

Mollet et al. (2002) reported that *Lycopersicon pimpinellifolium*, *Aquilegia eximia*, and *Nicotiana tabacum* were not labeled with MAb JIM13 at their tube tips nor did the Yariv reagent bind there and arrest pollen tube growth, as opposed to *Lilium longiflorum* and *Annona cherimola*. The authors stated that the presence or absence of AGPs at the tube tip appeared to be species dependent. However, they do not exclude the possibility that other AGPs which do not possess the epitopes recognized by these antibodies and/or are not bound with β -GlcY may be present at the pollen tube tips of these species.

Qin and coauthors (Qin et al., 2007), also working with *Nicotiana*, showed that abundant AGPs were present in all areas of the pollen tubes after labeling with JIM13, including the tip region. In pollen tubes, immunogold particles were mainly distributed in the cell wall and cytoplasm, especially around the peripheral region of the generative cell wall. β -D-Glucosyl Yariv reagent, which specifically binds to AGPs, caused slow growth of pollen tubes and reduced immunogold labeling of AGPs with JIM13 *in vitro*. These data suggest that AGPs participate in male gametogenesis and pollen tube growth and may be important surface molecules in generative and sperm cells.

5.4 Malus

By searching for anther-specific genes involved in male gametophyte development in apple (*Malus x domestica* Borkh. cv. Fuji) by differential display-PCR, three full-length cDNAs were isolated, and the corresponding genomic sequences were determined by genome walking. The identified genes were intron-less with 228- to 264-bp open reading frames and shared 82–90% nucleotide sequence. Sequence analysis identified that they encoded a putative AGP and were designated *MdAGP1*, *MdAGP2*, and *MdAGP3*, respectively. By RT (reverse transcriptase)-PCR the authors showed that the *MdAGP* genes were selectively expressed in stamens. Promoter analysis confirmed that the *MdAGP3* promoter was capable of directing anther or pollen specific expression of the GUS reporter in tobacco and apple. Furthermore, expression of ribosome-inactivating protein under the control of the *MdAGP3* promoter induced complete sporophytic male sterility as expected (Choi et al., 2010).

6. Mode of action of AGPs; What are the possibilities?

As we have shown, most of the evidence involving AGPs in pollen development is based on immunolocalization of sugar epitopes, in the genetic manipulation of individual AGP backbone peptides or in the binding of AGPs to β -Yariv reagent, a synthetic reagent which binds AGPs in general, perturbing its biological function. Although significant to find modes of action for the AGPs, these observations are currently difficult to interpret. Also important is to compare the biology of AGPs with that of analogous molecules in animals, trying to match up to their modes of action.

6.1 Arabinogalactan proteins as cell wall components

Although precise functions of AGPs remain elusive, they are widely implicated in plant growth and development. When the Yariv reagent is added to seedlings or to cells in culture, a strong inhibition or even a growth arrest is observed, one possibility for this is the involvement of AGPs in cell wall polymer biosynthesis (Seifert & Roberts, 2007).

In plants most cells expand by diffuse growth; whereas root hairs and pollen tubes expand by tip growth. It is now well documented the high expressed number of AGP genes in the root hair transcriptome (Johnson et al., 2003) and on the pollen tube expressed genes profile (Wang et al., 2008). AGPs are deposited into the tip of growing pollen tubes (Dardelle et al., 2010; Pereira et al., 2006) and tip-growing pollen tubes are inhibited by genetic interference with GPI-anchor biosynthesis (Lalanne et al., 2004) and also by β -Yariv reagent treatment, leading to ectopic callose deposition and pectin alteration (Mollet et al., 2002). These results together entail the involvement of AGPs in growth and development.

The presence of AGP carbohydrate epitopes and AGP backbone peptides in secondary wall thickenings suggests that AGPs might be secreted to the cell surface in parallel with cellulose synthase, and they might be released from their GPI anchor and incorporated into cell wall thickening (Seifert & Roberts, 2007).

A well-characterized classical tomato AGP containing a glycosylphosphatidylinositol plasma membrane anchor sequence was used to elucidate functional roles of AGPs.

Transgenic tobacco BrightYellow-2 (BY-2) cells stably expressing GFP-LeAGP-1 were plasmolysed and used to localize LeAGP-1 on the plasma membrane and in Hechtian strands. Cytoskeleton disruptors and β -Yariv reagent were used to examine the role of LeAGP-1 as a candidate linker protein between the plasma membrane and the cytoskeleton. This study used two approaches. First, BY-2 cells, either wild-type or expressing GFP-microtubule (MT)-binding domain, were treated with β -Yariv reagent, and effects on MTs and F-actin were observed. Second, BY-2 cells expressing GFP-LeAGP-1 were treated with amiprophosmethyl and cytochalasin-D to disrupt MTs and F-actin, and effects on LeAGP-1 localization were observed. Collectively, these studies indicated that GPI-anchored AGPs function to link the plasma membrane to the cytoskeleton (Sardar et al., 2006).

6.2 Arabinogalactan proteins as gradients

Plant reproduction involves a series of interactions between the male gametophyte (the pollen grain or pollen tube) and the extracellular matrix molecules secreted by different cell types along the pollen tube growth pathway in the pistil. These interactions are believed to signal and regulate the pollen tube growth process to effect successful delivery of the sperm cells to the ovules where fertilization takes place. AGPs are believed to play a broad range of functions, ranging from providing structural integrity to mediating cell-cell interactions and communication. Upon germination on the stigma, pollen tubes elongate in the stylar transmitting tract, aided by female factors, with speed and directionality not mimicked in *in vitro* pollen tube growth cultures. It was shown that a stylar transmitting tissue arabinogalactan protein from *N. tabacum*, a TTS (Transmitting Tissue Specific) protein, stimulates pollen tube growth *in vivo* and *in vitro* and attracts pollen tubes grown in a semi-*in vivo* culture system. Within the transmitting tissue, TTS proteins display a gradient of increasing glycosylation from the stigmatic end to the ovarian end of the style, coincident with the direction of pollen tube growth (Cheung et al., 1995; Wu et al., 1995).

Gradients of morphogens are a hallmark in animal development, chemoattractants are important to microbial as well as to animal cell motility systems and developmental pathways; they are also believed to function in the directional growth of pollen tubes (Reger et al., 1992). The gradient of increasing TTS protein glycosylation coincident with the direction of pollen tube elongation is a unique protein-based sugar gradient observed in the female reproductive tissue.

6.3 Arabinogalactan proteins as signaling molecules

AGPs have frequently been hypothesized to be sources of soluble signal molecules, in the form of sugar chain fragments, it is now well established that sugars act as signaling molecules (Hanson & Smeekens 2009) and/or as sources of lipid signal molecules, in the form of the lipid chains that are liberated upon fracture of the GPI anchor, and which may diffuse in the plane of the plasma membrane. Despite the scarcity of experimental evidence to support such roles, it is indeed the combination of AGP main features, namely cell surface localization, likely presence in membrane lipid rafts through their GPI anchor, possible controlled release from the plasma membrane upon stimuli, and the complexity of their

sugar content, that makes AGPs, or specific fragments of AGPs, as likely candidates to perform signaling functions in plants, and in plant reproductive processes, in particular. In mammals, species specificity in fertilization occurs through the interactions between sperm cell and egg cell, with cell surface proteins acting as key determinants (Vieira & Miller, 2006). Although flowering plants and mammals have evolved very divergent mechanisms for fertilization and reproductive species recognition, it is highly likely that in both cases GAPs may be used to regulate key steps in fertilization phenomena.

PTs with their unique type of growth restricted to the tip and with an intense secretion and endocytic activity needed for this rapid cell expansion at the apex are always depending on the communications with the pistil molecules. Most probably, the AGPs released to the membrane as part of this exocytic activity are fundamental pieces in the signaling network that directs with precision, pollen tubes to their target, the embryo sac cells.

7. Conclusions and future perspectives

Experiments performed integrating reverse genetics and other experimental approaches, led us to believe that the observed fertility reduction in *agp6 agp11* double null mutant was due to abortion of pollen grains during development (Coimbra et al., 2009). We have further characterized the anthers and pollen of *agp6 agp11* and concluded that both AGPs, AGP6 and AGP11, are necessary for the proper pollen tube growth as well as for preventing untimely pollen grain germination (Coimbra et al., 2010). Further details on the biology of pollen-specific AGPs are expected to emerge from a microarray experiment performed on RNA isolated from pollen tubes of *agp6 agp11* mutant, and which is currently under analysis.

Whether AGPs are predominantly structural, or nutrient-providers, or signaling molecules, is yet to be determined. We are committed to search for AGP-specific ligands recently identified for tobacco pistil AGPs (Lee et al., 2008), reason why we performed microarrays in the double null mutant, *agp6 agp1* pollen tubes, to try to bring some clarification for the biological way of action of this ubiquitous class of plant proteoglycans.

Key future challenges are also the elucidation of the enzymatic machinery that synthesizes the AG carbohydrate structure and the molecular nature and biological role of endogenous AG-specific carbohydrate hydrolases. AGP galactosyltransferase (GalT) activities in tobacco and *Arabidopsis* microsomal membranes were studied with an *in vitro* GalT reaction system. This *in vitro* assay reported to detect GalT activities using AGP peptide and glycopeptide acceptor substrates provides a useful tool for the identification and verification of AGP-specific GalT proteins/genes and an entry point for elucidation of arabinogalactan biosynthesis for AGPs (Liang et al., 2010).

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