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DOWNREGULATION OF ARABINOGALACTAN PROTEINS DURING SPERMATOGENESIS IN THE MOSS *PHYSCOMITRIUM PATENS*

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Abstract: Seedless plants utilize flagellated sperm cells for reproduction that develop using a series of cell walls resulting in naked motile cells. Arabinogalactan Proteins (AGPs) have been shown to play an important role in the maturation of sperm in ferns by an unknown mechanism. We sought to identify AGPs expressed in the spermatids of the moss *Physcomitrium patens* to identify this mechanism because it is amenable to genetic manipulation. We tracked the expression of 121 putative AGP-encoding genes across three time points of development with RNAseq and quantified total AGPs to compare to the fern *Ceratopteris richardii*. Unexpectedly, AGP genes and proteins were significantly downregulated in *P. patens*. BURP domain-containing genes, which are expressed in pollen of angiosperms, were highly upregulated and may serve similar roles to the AGPs of ferns. This study shows that the fern cell walls do not share as significant of a need for AGPs of developing sperm in bryophytes and this may be related to the number of flagella found in the respective lineages.

Keywords: bryophyte, BURP-domain proteins, cell wall, flagella, gametogenesis, spermatid development.

Introduction

Throughout plant evolution, key adaptations arose in major lineages allowing them to thrive in drier conditions. One feature that has undergone dramatic changes among lineages is the morphology of the sperm cell [SOUTHWORTH & CRESTI, 1997]. The male gamete of the oldest land plants form a naked coiled cell containing a pair of flagella reminiscent of sperm from related algae. The pollen grains in most seed plants represent the entire microgametophyte and only produce sperm nuclei housed within. As new lineages emerged from ancestral bryophytes, sperm cells increase their number of flagella whereas ferns have dozens, but a few pollen-bearing seed plants, Cycads and Ginkgo, can have upwards of 50,000 flagella. In these, sperm travel through a pollen tube that bursts open just shy of the egg and the flagella drive the sperm across the short distance within the ovule to the egg [RENZAGLIA & GARBARY, 2001; SOUTHWORTH & CRESTI, 1997].

Hydration of mature antheridia in seedless plants cue sperm release, initiating their journey to the egg-bearing archegonium to form the zygote. In the later stages of development, sperm transform from the characteristic boxy plant cell to take on their coiled shape while the locomotory apparatus forms at the cell anterior concurrent with the deletion of cytoplasm, facilitated by the chemically unique extraprotoplasmic matrix (EPM) [BERNHARD & RENZAGLIA, 1995; GENAU & al. 2021; LOPEZ & al. 2017]. The locomotory apparatus contains a number of microtubule based structures including the lamellar strip, the overlying

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flagella, and their anchoring basal bodies all attached to a row of connected microtubules, known as a spline [RENZAGLIA & al. 2000]. Sperm of most gymnosperms and all flowering plants, lack any need for flagella as pollinators and pollen tubes cooperate to deliver sperm nuclei directly to the egg.

Arabinogalactan Proteins (AGPs) are excreted glycoproteins containing large branching sugar residues consisting primarily of arabinose and galactose. They are encompassed within the hydroxyproline-rich glycoprotein family, along with extensins and proline-rich proteins [SEIFERT & ROBERTS, 2007]. Historically, they are defined by their small proteinaceous backbones with high proportions of hydroxyproline that act as *O*-glycosylation points [TAN & al. 2003, 2010]. However, a survey of plant genomes found much higher AGP diversity, including the formation of chimeras with kinases, formins, or cell wall modifying domains among other subfamilies [MA & al. 2017]. The authors of this survey ultimately define AGPs by the proportion of "glycomodules" to gene length. Glycomodules are the dipeptides enriched in AGP sequences consisting of Pro + [Ala, Ser, Thr]. While most have a signal peptide and many are predicted to be GPI-anchored, these do not strictly define the proteins. Those that are GPI-anchored have the ability to remain linked to the outer surface of the plasma membrane, but may also render them susceptible to cleavage by phospholipases that would release them into the wall where they may play myriad roles [BORNER & al. 2003; ELORTZA & al. 2003, 2006]. The nature of this is, however, consistently vague with little direct evidence of a molecular mechanism [LAMPORT & al. 2014].

AGPs are known to play roles in many aspects of plant biology such as cell fate determination, plant microbe-interactions, gravitropism, and more [SEIFERT & ROBERTS, 2007], but they commonly facilitate aspects of reproduction across most land plants. They are produced at various developmental events and in key tissues involved in pollen and sperm production in a diverse sampling of flowering plants [CHUDZIK & al. 2014; COSTA & al. 2015; LI & al. 1992; MA & al. 2019; QIN & al. 2007; SOUTHWORTH & KWIATKOWSKI, 1996] and gymnosperms [MOGAMI & al. 1999; YATOMI & al. 2002]. Both the generative cell, giving rise to two sperm cells, and vegetative cell, controlling the growth of the pollen tube, strongly label for AGPs in most angiosperms and they are believed to function in both pollen tube elongation and pollen-pistil interactions affecting compatibility and efficient delivery of the sperm cells in non-flagellated plant lineages [LESZCZUK & al. 2019; SEIFERT & ROBERTS, 2007]. In the fern *Ceratopteris*, varying AGP populations are differentially expressed through spermatogenesis and are intimately associated with flagella throughout their elongation in the extracellular matrix [LOPEZ & RENZAGLIA, 2014]. When the AGPs are chemically removed late in development, flagella become haphazardly arranged and the cell fails to eliminate cytoplasm or develop the microtubular backbone. The purpose of this study was to directly identify AGP encoding genes associated with spermatogenesis in flagellated plants using the model moss *Physcomitrium patens* by tracking AGP expression, family-wide, throughout antheridium development in order to test for a more specific mechanism of AGP activity.

Materials and methods

Tissue growing conditions. *Ceratopteris richardii* spores were obtained and grown according to the manufacturer (Carolina Biological, Burlington, NC, USA). *Physcomitrium patens* culture was maintained under 24 °C 24hr light at 400-500 lux every 7-12 days growing on PpNH4 media. In preparation for inducing gametangia, 2 mm balls of protonemal tissue were arranged in a 9x9 grid on BCD+Ammonium Tartarate (5 mM) media [RESKI & COVE, 2004]

and grown for 5 weeks to allow gametophore development. Gametangia were induced as described [HOHE & al. 2002]. Antheridia development was confirmed by microscopy prior to harvest. For RNA-seq samples, tissue was frozen in liquid nitrogen, ground to a fine powder and stored at -80 °C before RNA extraction. For qPCR samples, gametophore tips only were dissected, frozen, and stored at -80 °C. These samples were homogenized in RNAzol (Molecular Research Center, Cincinnati, OH, USA) using microfuge pestles followed by an application of the homogenate to a shredder spin column (Lamda Biotech, St. Louis, MO, USA).

RNA prep, RNA-seq, and analysis. RNA was extracted from quadruplicate samples of each timepoint using RNAzol, according to the manufacturer's protocol. Subsequently, samples were DNAse I treated and concentrated using a silica column (Zymo research, Irvine, CA, USA). Samples were assessed using a Qubit and 2100 Bioanalyser, ribo-depleted with a RiboZero Plant kit. Libraries were created with NEXTflex Rapid Directional qRNA-seq kit and sequenced with a HiSeq4000 (75pb paired-end, 30M reads per sample) performed by ACGT, Inc (Wheeling, IL, USA). Bcl2fastq was used to de-multiplex the raw reads. These data were further analyzed by the University of Virginia Bioinformatics Core, including quantitation and expression analyses.

For qPCR analysis, RNA quality was assessed visually on a gel and equivalent quantities were converted to cDNA using Protoscript II Reverse Transcriptase (NEB, Ipswich, MA, USA). qPCR was conducted using the Pfaffl method, after primer pair efficiencies were calculated in triplicate [PFAFFL, 2001], using PowerUp SYBR green master mix (Life Technologies, Carlsbad, CA, USA) on a QuantStudio3 thermocycler. Primer pairs are listed in Table 1.

Table 1. Primers used in qPCR analyses

Primers	Gene Locus		Efficiency	Sequence
E-256	Pp3c7_430	Fasciclin-like AGP	2.14	TCCTTCTCTCTCTACTCTTCCCACTTC
E-257				CCTGATACCTCCAATCGCCAAC
E-258	Pp3c21_10620	Periostin-Related	1.94	AGCCTTCACCATCACCAG
E-259				TAGAGCGACAACAGCGGAC
E-252	Pp3c1_4130	Fasciclin-like AGP	1.94	AGCAGCGAAGGTCTACAG
E-253				GTCGATACCATGAACAGCAAC
E-254	Pp3c13_8280	Fasciclin-like AGP	2.06	TTGCGCCCTTCTCGTTTC
E-255				GCAACAACTTTCTCGTGCAG
E-476	Pp3c10_22850	Periostin-Related	1.97	GCTTCCTAACACTACCTTGAG
E-477				CTCCAGTGAGAGCAAATACC
E-478	Pp3c26_8120	IRAK1	*ΔΔCt	GCCTACTGCTTCTTGTATTGC
E-479				TTGCATGGAGTTGTGTCTG
E-480	Pp3c5_14830	Probable LTP_2	*ΔΔCt	TCGCACCGCTTGTATCTG
E-481				CGAGCGTGAAACAAGGAAC
E-484	Pp3c16_19400	BURP domain	1.94	GATCAGAGCAAGGAAAGTCG
E-485				TATCCTTTGAGTGGGACCTG
E-470	Pp3c5_9210	AGP-31	1.96	TGAAAGATGCCGAGGTGG
E-471				TTTCGCCTTACATCCATTGC
E-272	Pp3c13_2360	Reference Gene; RP-L21e	1.94	TTTCTCTTCTTCCTCTCGCTC
E-273				TTGTGCCTGAAGGGTCTG
E-533	Pp3c8_1210	LTP_2	*ΔΔCt	AATCGGACATCATGCCTTC
E-534				CTGGAATAACTCTGCCATTGC

E-535	Pp3c12_11710	PLAT/LH2 domain	*ΔΔCt	GAGTGCCATGCAGAGCAAC
E-536				TGCGTCTATCCCACGATGA
E-537	Pp3c2_4510	BURP domain	*ΔΔCt	ACGCATCACCACTCTACAGC
E-538				CGTCCATCACCTTCCATCTC

AGP extraction. Soluble AGPs were extracted according to [POPPER, 2011]. Total protein was quantified using the BCA method and total AGPs were quantified using a radial gel diffusion assay [VAN HOLST & CLARKE, 1985]. The gel diffusion assay showed an R^2>0.99 using Gum Arabic as a standard with a dilution series between 0.031-2 µg.

Results and discussions

MA & al. (2017) identified AGPs from across the plant kingdom, and found 104 putative AGPs in the *P. patens* genome. Dynamic expression of this suite of genes was assessed using RNA-seq in plants at two points during spermatogenesis compared against non-induced samples. *P. patens* produces antheridia when exposed to low light, low temperature and long day conditions (16 dark/8 light) [HOHE & al. 2002]. Upon induction, new antheridia initials form and mature over 10 defined stages [LANDBERG & al. 2013]. However, additional antheridia initials form in succession after the previous. While the oldest antheridia may mature by 16 days after induction (stage 9), there will be a greater percentage of stage 9 antheridia later as additional sets of antheridia form and then mature. With this in mind, we sampled plants at 0 days past induction (dpi), showing only vegetative growth, at 15 dpi, when the first antheridia are nearly mature, and 28 dpi, when 2-4 antheridia in each cluster are mature and relatively few young antheridia apparent. When expression of all 104 putative AGPs were assessed, it was found that the majority were either downregulated or saw no significate change in expression (Figure 1A and archived data [JOHNSON, 2023]). Only two putative AGPs saw moderate upregulation during spermatogenesis in the initial screen. These include Pp3c26_8120 which showed a mere 1.28 Log_2 fold change (Log_2FC) at 15dpi that was sustained in the 28 dpi samples; and Pp3c10_22850 showed a 1.55 Log_2FC increase in 28 dpi vs. 0 dpi samples. A third putative AGP, Pp3c5_9210, not identified as an AGP by MA & al. (2017) was expressed strongly only in the 28dpi sample with a 2.96 log_2FC (Figure 1A). It is not surprising that some AGPs were missing from the target list as the authors acknowledged using a high threshold that likely excluded some genes. In validating these results with qPCR, we found that Pp3c26_8210 was too lowly expressed to accurately quantify in most replicates. Pp3c10_22850 saw a gradual increase in expression at 15dpi, but then a gradual downregulation at 28dpi. The third putative AGP, Pp3c5_9210 did see increased expression at both 15dpi at 1.82 Log_2(FC) and a much stronger increase of 5.77 Log_2(FC) at 28dpi (Figure 1B). The RNA-seq samples contained whole gametophores in an attempt to harvest tissue quickly, though this may have resulted in a dilution of spermatid-derived RNA with vegetative tissue. Subsequent qPCR analysis contained only gametophore tips, where antheridia are bore, giving a higher concentration of the tissues/cells of interest so a larger Log_2(FC) in our qPCR analysis is not unexpected.

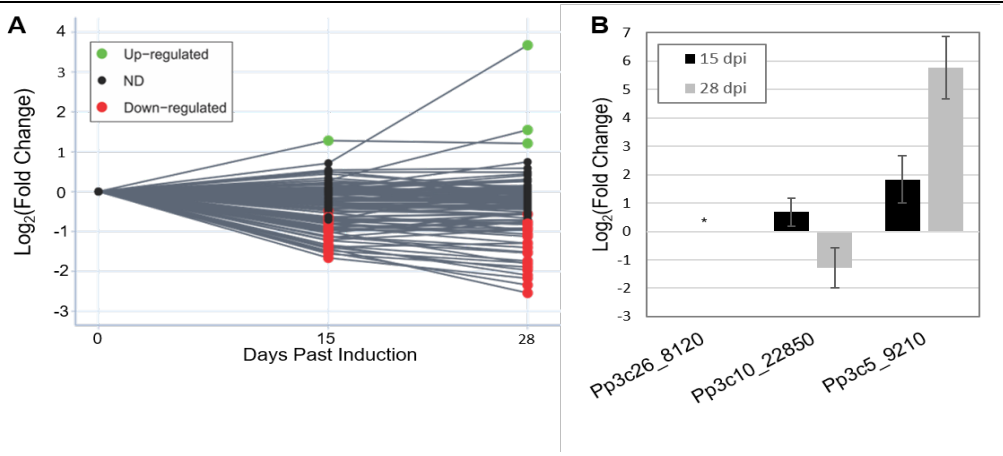

Figure 1. AGP-transcriptome dynamics during spermatogenesis in *P. patens*. **A**. normalized relative transcript counts of 104 putative AGP-encoding genes 0, 15 or 28 days past induction. Only three AGPs show a significant up-regulation, while the majority show no change (ND) and many show down-regulation. $p > 0.05$. **B**. qPCR analysis of AGPs in antheridia-induced tissue at 15 and 28 dpi.
* Pp3c26_8120 was not expressed at sufficient enough levels to measure in 3 out of 4 replicates. Bars are +/- SE. N=3-4.

Since the drop in AGP expression was initially unexpected, we wanted to confirm whether this change was reflected in total protein changes over the same time period. Total soluble AGPs were extracted and quantified (Figure 2A). While there was no significant change between 0- and 15-dpi samples, there was a significant decrease in AGPs during the maturation of sperm cells at 28 dpi, whereas we found 0.065, 0.076, and 0.036 µg AGP/µg extracted protein, respectively. We then quantified AGPs in the fern *C. richardi* to ensure that the total protein extract was reflective of the ultrastructural analysis that initially identified the increase in spermatogenesis-related AGPs [LOPEZ & RENZAGLIA, 2014]. As expected, a significant increase in AGPs was noted when sperm cells matured and at the same timepoint point that swimming sperm were observed, 11 days after sowing (das), compared to 8 das; $p<0.05$ (Figure 2B). While it should be noted that *C. richardii* sperm mature more rapidly and the gametophytes are structurally simpler and short-lived, there was more than 10 times the mass of AGPs in *C. richardii* compared to *P. patens*. AGP levels increased from 0.310 to 0.569 µg AGP/µg extracted protein as sperm matured. In conjunction with our gene expression analysis, this suggests that AGPs play a far reduced role in spermatogenesis in moss compared to *C. richardii*. However, a pair of studies investigating the cell wall makeup of maturing moss sperm cells in *P. patens* and *Aulacomnium palustre* note the presence of dynamic populations of AGP-epitopes in the spermatid walls that wanes some as maturation commences [HENRY, 2021; LOPEZ-SWALLS, 2016]. From our data, it is unclear how or when these AGPs were expressed, though there is an emerging theme that the presence of particular AGPs may arise from the presence of the particular glycotransferases that build them under given conditions rather than the AGP-encoding genes themselves [SILVA & al. 2020]. It is possible that one/some of the non-differentially expressed genes in our study codes for the AGPs found in mature sperm and the changing epitopes labelled by [HENRY, 2021] result from the differential expression of AGP-modifying glycotransferases. Our data, however, clearly show a reduction in AGP expression during spermatogenesis.

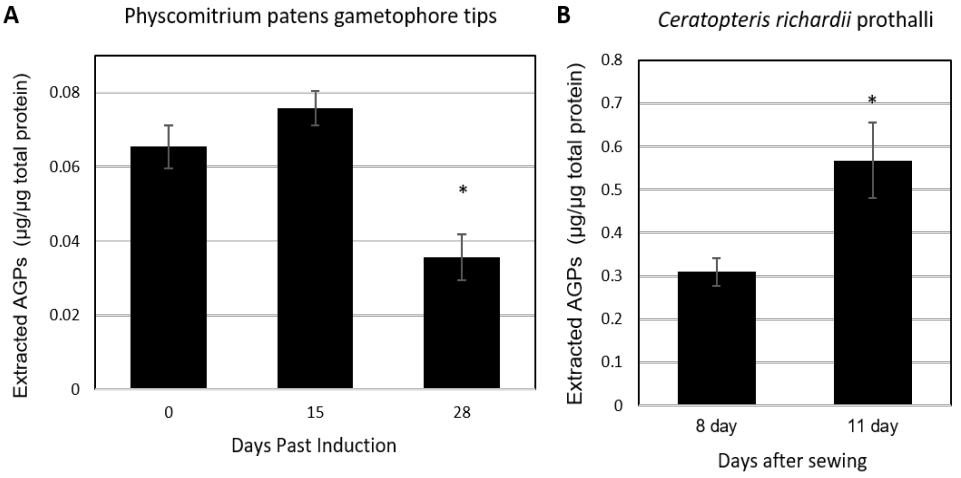

Figure 2. Arabinogalactan protein production during spermatogenesis. **A**. Extracted AGPs from developing *P. patens* gametophore tip quantified by radial diffusion assay. Note decrease in AGP levels during the maturation of sperm cells. At 28 dpi significantly fewer AGPs ($p<0.05$) are produced compared to non-induced tissue **B**. Extracted AGPs from developing *Ceratopteris richardii* prothalli, a flagellated plant known to require AGPs in sperm development. AGP levels are significantly increased ($p<0.05$) at 11 days after sewing compared to 8 days. Bars = SE; n = 3-4.

What was upregulated? Of the transcripts that were up regulated, no GO terms were significantly enriched. However, when the most up-regulated genes were assessed individually, some anticipated and unanticipated genes were identified (See Table 2 and archived data [JOHNSON, 2023]). For example, a β-tubulin component and two histone associated transcripts (Pp3c13_14080 and Pp3c26_7260) were among the top upregulated genes comparing 15 dpi to 28 dpi samples, as would be expected with the production of key microtubule-based structures and the spiral shaping of the nucleus. A key component to their maturation is the deletion of cytoplasm and organization of flagella into the extraprotoplasmic matrix. Thus, we would expect to find an upregulation of genes encoding secreted proteins, cell wall modifying enzymes and transport proteins, or possibly catabolic enzymes for cytoplasmic deletion and lysosome-related enzymes. A catabolic PLAT/LH2 protein (Pp3c12_11710), and four putative secreted proteins: a Lipid Transfer Protein (Pp3c8_1210), a pollen Ole e I Allergen (Pp3c5_9210) and two BURP domain containing proteins PpBURP4 (Pp3c16_19400) and PpBURP5 (Pp3c2_4510) were among the most highly upregulated.

What was downregulated? The genes most downregulated throughout sperm cell development are mostly associated with photosynthesis (Table 2 and archived data [JOHNSON, 2023]). This is unsurprising as induction of antheridia involves a low light, short day, low temperature treatment. However, there may be a more direct link to spermatogenesis or antheridia development as male fern gametophytes also show lower levels of photosynthesis compared to their hermaphroditic counterparts [CHEN & al. 2019]. This is also in agreement with the observation that plastids are reduced early during spermatogenesis in the liverwort *Blasia pusila* [RENZAGLIA & DUCKETT, 1987].

Table 2. Most up-regulated genes during late antheridium development. Log2 (fold-increase) in samples, ranked compared 15dpi vs. 28 dpi to emphasize late-stage spermatid development.

Gene code	Putative function	Sig rank[1]	15 dpi vs. 28 dpi	0 dpi vs. 15 dpi	0 dpi vs. 28 dpi	Basemean
Pp3c16_19400	BURP domain-containing protein	1	5.06	0.62	5.68	59.03
Pp3c12_11710	Lipase/lipooxygenase, PLAT/LH2 family protein	2	5.06	0.00	5.21	41.05
Pp3c13_14080	Linker histone H1 and H5 family	3	4.85	0.15	5.00	35.19
Pp3c8_1210	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	4	4.30	0.51	4.81	39.20
Pp3c2_4510	BURP domain-containing protein	6	3.98	0.15	4.13	19.05
Pp3c5_9210	Pollen Ole e 1 allergen and extensin family protein (A subclass of AGPs)	9	2.96	0.71	3.67	43.46
Pp3c26_470	Dehydrin family	12	2.62	0.22	2.84	251.42
Pp3c26_7260	histone H1-3	13	2.52	0.00	2.66	7.61
Pp3c8_2420	Tubulin beta chain 2	16	2.41	0.25	2.66	6.66
Pp3c10_19280	SF5-Stromal Interaction Molecule Homolog	17	2.30	0.49	2.79	63.49

[1]: Sig rank = rank of most significant (Padj) comparing 15dpi vs. 28dpi samples

Table 3. Most down-regulated genes during late antheridium development. Log2 (fold-increase) in samples, ranked compared 15dpi vs. 28dpi to emphasize late-stage spermatid development.

Gene code	Putative function	Sig rank[1]	15 dpi vs. 28 dpi	0 dpi vs. 15 dpi	0 dpi vs. 28 dpi	Basemean
Pp3c13_15990	Global Transcription Factor	5	-1.54	NA	-2.28	1106.97
Pp3c13_15980	ribulose bisphosphate carboxylase small chain 1A	7	-1.96	-2.55	-4.51	39257.61
Pp3c13_16130	ribulose bisphosphate carboxylase small chain 1A	8	-1.94	-2.03	-3.97	4418.33
Pp3c13_15790	NA	10	-1.99	-1.19	-3.18	199.05
Pp3c13_15786	ribulose bisphosphate carboxylase small chain 1A	11	-1.90	-3.28	-5.17	304.44
Pp3c13_15800	ribulose bisphosphate carboxylase small chain 1A	55	-1.24	-3.73	-4.97	7098.13
Pp3c12_22350	carotenoid cleavage dioxygenase 1	312	NS	-4.69	-3.68	101.34
Pp3c13_2310	photosystem I subunit D-2	925	NS	-1.71	-2.12	291.15
Pp3c27_2340	photosystem II subunit R	1757	NS	-1.73	-1.46	2064.85
Pp3c13_16000	ribulose bisphosphate carboxylase small chain 1A	1799	NS	-2.53	-3.01	1919.28
Pp3c18_7850	NA	3120	NS	-5.19	-5.72	259.74
Pp3c14_380	Disease resistance-responsive (dirigent-like protein) family protein	7060	NS	-2.53	-2.73	2644.46
Pp3c8_7680	NA	8096	NS	-3.67	-3.47	33208.92
Pp3c19_21160	plastocyanin 1	10180	NS	-1.44	-1.53	5099.99
Pp3c3_18750	ribulose bisphosphate carboxylase small chain 1A	18734	NS	-2.25	-2.31	3203.72

[1]: Sig rank = rank of most significant (Padj) comparing 15dpi vs. 28dpi samples

It has become clear that AGPs can play a major role serving as a Ca^{2+} store in the apoplast in a pH dependent manner through the negatively charged GlcA residues common to AGPs [LAMPORT & VÁRNAI, 2013; LOPEZ-HERNANDEZ & al. 2020; ZHANG & al. 2021]. The high proportion of AGPs found in *Ceratopteris* spermatids were suggested to support flagella positioning and elongation through this calcium signaling mechanism [LOPEZ & RENZAGLIA, 2018]. Cortical microtubule dynamics are influenced by Ca^{2+} [NICK, 2013] as are some interactions between AGPs and microtubules [NGUEMA-ONA & al. 2007; SARDAR & al. 2006], though a direct role in the polymerization of the stable microtubules found in flagella is not known. However, this mechanism could help explain why moss spermatocytes, with only 2 flagella show reduced AGP gene expression and protein as compared to the sperm of *Ceratopteris* with approximately 80 flagella [RENZAGLIA & al. 2017]. The diversity in AGP-glycosylation patterns found in tracheophytes [MUELLER & al. 2023] gives the possibility that AGPs evolved to provide a chemical environment facilitating development of these more complex sperm cells after the split from bryophytes. Furthermore, AGPs are also well known to be involved in tip growth, for which *P. patens* would have displayed significant protonema growth under vegetative conditions [LEE & al. 2005; TEH & al. 2022]. As the plants shift away from vegetative growth, the need for broad production of AGPs would also wane. [MIGNONE & BASILE, 2000] found that a strong increase in AGP production occurs as *P. patens* produces buds and transitions into gametophore growth, but in agreement with our data, the plants shift away from their production as gametophores transition into gametangia production.

Physcomitrium patens ecotypes. It was recently discovered that the most commonly studied ecotype of *P. patens*, Gransden (Gd), has accumulated (epi-)genetic changes resulting in spermatozoids with poorly formed flagella [HISS & al. 2017; MEYBERG & al. 2020]. This was attributed to consistent subculturing and encouraged the characterization of the Reute (Re) ecotype which shows healthy sperm production. To determine the nature of this in Gd, differentially expressed genes were determined and the key flaw in Gd seems to involve both the polymerization of its axoneme microtubules within the flagella as well as the adhearence of the plasma membrane to the flagella, roles AGPs could perceivably be involved in. However, off the 15 putative AGPs we identified as down-regulated in Gd, they were found to be similarly down-regulated or show no change in Re between juvenile and adult gametophore tissue, according to PEATmoss [FERNANDEZ-POZO & al. 2020]. This confirms our primary finding that AGP downregulation during gametangia development occurs in both Re and Gd. The only strongly up-regulated AGP we identified, Pp3c5_9210V3.1, was similarly upregulated in Ru and it was subsequently shown to contain a premature stop codon in Re [MEYBERG & al. 2020]. Clearly it is not necessary for normal sperm cell development. Furthermore, we confirmed that the two BURP domain containing genes were also strongly upregulated in the Reute ecotype of *P. patens* during sperm cell maturation (Figure 3). Although our developmental approach to characterizing changes in gene expression was conducted in Gd, the genes identified show similar expression patterns in both ecotypes.

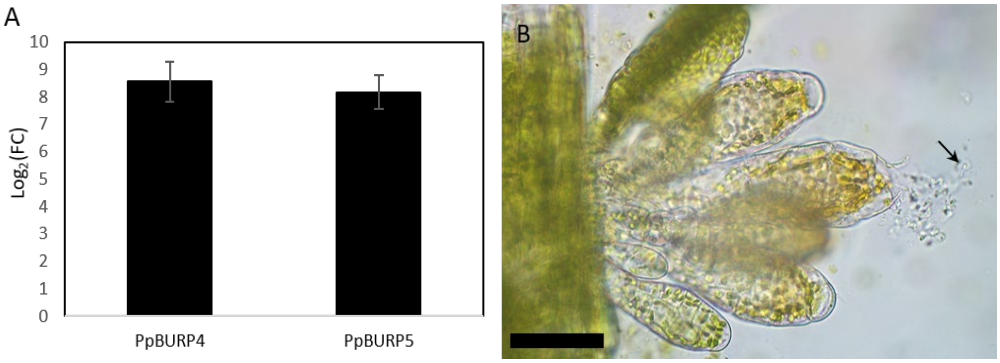

Figure 3. PpBURP4 and PpBURP5 are highly expressed during antheridia maturation in Reute ecotype. **A**. qPCR analysis of PpBURP4 and PpBURP5 in tissue 28 days past induction of antheridia formation. **B**. Image is representative of 28dpi tissue with mature swimming sperm (arrow) and a series of younger antheridia. Bar = 50 µm.

BURP-domain containing proteins. Two of the most highly upregulated genes in this study belonged to a family of genes that shows some similarities to AGPs in that they are extracellular proteins [WANG & al. 2012; XU & al. 2013] that play roles in many stress and developmental pathways. There are also a number of reports of involvement of these genes with male gametes of seed plants [KHLAIMONGKHON & al. 2021; SUN & al. 2019; TREACY & al. 1997; WANG & al. 2003]. It could be that these proteins are produced and secreted to help modify the cell walls or extra protoplasmic matrices that serve a homologous function to the AGP-rich layer in *C. richardii* spermatogenesis [LOPEZ & RENZAGLIA, 2014].

The BURP domain itself was initially characterized by its presence in BMN2, a microspore protein from *Brassica napus* [BOUTILIER & al. 1994]; USP, a nonstorage seed protein from *Vicia faba* [BASSÜNER & al. 1988]; RD22, a dehydration responsive protein from Arabidopsis [YAMAGUCHI-SHINOZAKI & SHINOZAKI, 1993]; and *PG1*β, a noncatalytic subunit of polygalacturonase isozyme I from *Solanum lycopersicum* [ZHENG & al. 1992]. The domain is located at the C-terminus and built from a pair of spaced cystine residues followed by four cysteine-histidine repeats ($CHX_{10}CHX_{23\text{-}27}CHX_{23\text{-}26}CHX_8W$) [DING & al. 2009; WANG & al. 2015]. This protein family emerged with the migration onto land but broad functional diversification only occurred after the split from lycophytes and likely showed strong diversification more recently among angiosperms [WANG & al. 2015; YU & al. 2022]. Phylogenetic analyses show most moss and lycophyte BURPs segregating into their own subfamily, BURP-IV, giving some question as to broad functional comparisons of these proteins between major plant lineages. To compound on that, each of the namesake genes for the family cluster into the three other BURP-domain containing gene subfamilies; including BMN1-like, PG1β-like, and BURPIII that includes RD22- and USP- homologues, which are all absent from lower land plants and limiting our ability to extrapolate functions of the identified genes. Regardless, the functional characterization of any BURP domain containing protein has only recently occurred whereas some members show autocatalytic peptide cyclase activity to form bioactive cyclopeptides [CHIGUMBA & al. 2022]. These are largely considered to be specialized metabolites that play defensive roles across a number of vascular plant lineages and it is unclear how these might contribute to the development of sperm cells in bryophytes. These

data highlight the need to further characterize BURP domain containing genes from seedless plants to determine their importance in spermatogenesis.

Conclusions

Sperm cells represent brief time in an organism's life cycle in which the fate of a lineage falls onto a single cell. With this, it is not surprising that plant evolution shows only incremental changes in overall structure when comparing the major lineages. It was surprising to find major differences in the importance of AGPs during spermatogenesis comparing the model moss and fern. AGPs were hypothesized to facilitate the formation of flagella in ferns and our data may actually agree as the pair of flagella in bryophytes wouldn't require as great a need for these glycoproteins as the 80+ flagella found in *Ceratopteris* sperm. This begs to determine the quantity of AGPs found in the development of Cycads and Ginkgo and their 1000s of flagella. As an alternative explanation, findings that seedless plants show the most structural diversity of AGPs among plant lineages could have provided ferns a mechanism to evolve and change the makeup of the spermatid extraprotoplasmic matrix during maturation to provide roles that other cell wall components may be serving in bryophytes, such as BURP-domain containing proteins.

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