Characterization of an *Arabidopsis thaliana* Anther-Specific Caleosin

By

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ABSTRACT

Anther and pollen development are important to plant reproduction processes leading to seed production. The floral bud of the model plant organism, *Arabidopsis thaliana*, consists of 6 anthers which encase copious amounts of pollen. Within the anther locules, a variety of nutrients are provided to pollen in order to support its development. This study aims to offer a deeper understanding of the role of anther caleosins in the Brassicaceae by investigating the expression, localization, and putative function of the anther-specific caleosin (AT1G23240). Through the use of various molecular and microscopy techniques (e.g., quantitative reverse transcriptase polymerase chain reaction, Western blot analysis, laser confocal microscopy, and transmission electron microscopy), it was determined that AT1G23240 was expressed at late stages of anther development - mainly within the endoplasmic reticulum and tapetosomes of tapetal cells, and the protein was also detected on the pollen coat of mature pollen. This elucidation of anther caleosin expression will help in better understanding its role and significance in the overall plant reproduction process, possibly in pollen-stigma interactions, as well as its potential in biotechnological applications in regards to fertility within key crop kinds of the Brassicaceae family.

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PREFACE

Within this thesis, techniques such as the qualitative polymerase chain reaction, Western blot analysis, confocal laser scanning microscopy, and transmission electron microscopy were used to elucidate the developmental expression, localization, and potential function of the *Arabidopsis thaliana* anther-specific caleosin AT1G23240. Figures and text used within this thesis are intended for later submission and publication of my findings.

While I carried out the majority of the work within this thesis, I would like to thank the following individuals for their important contributions to the completion of my research:

Dr. John Chan, a postdoctoral fellow before my time in Dr. Laurian Robert's lab, initiated the investigation of anther-specific caleosin, AT1G2340. He developed the transgenic construct lines for AT1G2340 tagged with a citrine yellow fluorescent protein reporter (AT1G23240:YFP) and RNAi constructs, which were used as a starting point for my research project; and

Ms. Denise Chabot and Mr. Keith Hubbard assisted me in developing and completing the imaging of *Arabidopsis thaliana* anther and pollen samples using confocal laser scanning microscopy and transmission electron microscopy.

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DESCRIPTION OF *ARABIDOPSIS THALIANA* GENES

AT1G23240: The *Arabidopsis thaliana* locus encoding the anther-specific caleosin characterized in this thesis.

Short form for AT1G23240: caleosin

Translational fusion (AT1G23240:YFP): Caleosin-YFP

Arabidopsis thaliana glyceraldehyde-3-phosphate dehydrogenase C subunit (*GAPC1*). NCBI GenBank AK226804. <https://www.ncbi.nlm.nih.gov/nuccore/AK226804>.

Arabidopsis thaliana Ubiquitin 11 (*UBQ11*) gene - NCBI GenBank U84968.
<https://www.ncbi.nlm.nih.gov/nuccore/U84968>.

Arabidopsis thaliana caleosin-related family protein mRNA -NCBI GenBank NM102173.
[https://www.ncbi.nlm.nih.gov/nucleotide/NM_102173?report=genbank&log\\$=nuclalign&blast_rank=1&RID=BRTX195V014](https://www.ncbi.nlm.nih.gov/nucleotide/NM_102173?report=genbank&log$=nuclalign&blast_rank=1&RID=BRTX195V014)

ABBREVIATIONS

BCP – Bicellular Pollen
CL – Coatless Pollen
CLSM – Confocal Laser Scanning Microscopy
GAPC – Glyceraldehyde-3-phosphate dehydrogenase C subunit
EIF41 – Eukaryotic translation initiation factor 4A1
ER – Endoplasmic reticulum
RNAi – RNA interference
PC – Pollen Coat
RT-qPCR – Reverse transcription quantitative polymerase chain reaction
TAG – Triacylglycerides
TCP – Tricellular Pollen
TEM – Transmission Electron Microscopy
TET- Tetrad
TUB2 – Tubulin β -2
UBQ11 – Ubiquitin 11
UNM – Uninucleate Microspores
YFP – Yellow Fluorescent Protein

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1. INTRODUCTION

1.1. *Arabidopsis thaliana*

The model plant *Arabidopsis thaliana* (Arabidopsis), also referred to as thale cress or mouse ear cress, is an herbaceous dicot plant belonging to the Brassicaceae family. There are several characteristics that make Arabidopsis a useful plant model system. It is a self-compatible, self-fertilizing, small diploid plant that produces a copious amount of flowers and seeds at maturity. Abundant within the northern hemisphere, Arabidopsis possesses a rapid life cycle, has a short generative time, and is relatively easy to grow (Krämer, 2015). It has been found to be closely related to dicot plants used in crop development and commercial purposes such as Brassica crops (e.g. canola and broccoli). The complete sequence of the relatively small genome (114.5 Mb/125 Mb total) of Arabidopsis comprised of 5 chromosomes has been reported (Arabidopsis Genome Initiative, 2000). Arabidopsis can be easily transformed using *Agrobacterium tumefaciens* which enables the use of various genetic manipulation techniques such as RNA interference (RNAi), T-DNA tagging, site-directed mutagenesis, etc. Due to these attributes, Arabidopsis has been the subject of a vast amount of research that also resulted in extensive molecular and genetic resources that can be found for example at the Arabidopsis Research Center (ABRC, <http://abrc.osu.edu>), the Arabidopsis Information Resource (TAIR, <http://www.arabidopsis.org>) and Araport (<https://www.araport.org/>). As such, Arabidopsis is a very useful model organism to perform plant reproduction research.

1.2. *Arabidopsis thaliana* reproduction

Plant reproduction is crucial for the production of seeds and offspring. The *Arabidopsis thaliana* pistil and stamens are central floral organs involved in the overall process of plant reproduction. The pistil is the female organ of a flower and comprises the ovary, style, and stigma; while the stamen is the male organ and comprises the anther and filament, these organs play an important role in the flower development and plant reproduction of angiosperms. The anther is the site where pollen grains are produced and mature (Alvarez-Buylla et al., 2010). The pollen grains develop within 4 anther locules lined by a cell layer called the tapetum which is comprised of individual cells holding subcellular organelles such as endoplasmic reticulum (ER), Golgi bodies, tapetosomes ((proteinaceous organelle mainly comprised of triacylglycerides (TAG)), and elaioplasts (lipid droplets) (Figure 1). The tapetum provides nutrients and structural components in the form of lipids, flavonoids, alkanes, and proteins essential for pollen development, maturation, and function (Hsieh and Huang, 2007). Mature pollen grains are surrounded by a cell wall consisting of the intine and exine (Figure 2). The intine is an inner layer comprised of pectin and cellulose, while the exine is composed of a biopolymer complex, called sporopollenin, consisting of fatty acids, carotenoids, and phenylpropanoids provided by the tapetum (Heslop-Harrison, 1971; Quilichini et al., 2014). Sporopollenin provides the pollen grain with a tough outer layer and assists in adhesion to stigmatic surfaces to initiate pollen-stigma interaction and plant reproduction (Rejón et al., 2016; Zinkl et al., 1999). Later in anther development, the tapetum degenerates via programmed cell death, tapetal debris fills the anther locule and some material (e.g. protein, lipids, and pigments) is delivered to the pollen grain and fills cavities within the exine (Figure 2). This extra layer is called the tryphine, also commonly referred to as the pollen coat (PC), and provides protection against pollen dehydration and UV

Figure 1. Whole anther *Arabidopsis thaliana* using differential interference contrast microscopy

Differential interference contrast microscopy overlapped with confocal laser scanning microscopy of a whole *Arabidopsis thaliana* anther showing the locule spaces containing the pollen grains and surrounded by an intact tapetum. The epidermis is distinguished with a laser exciting and emitting auto-fluorescent chlorophyll in red.

Figure 2. Transmission electron microscopy of *Arabidopsis thaliana* pollen grain

Transmission electron microscopy thin-section of an *Arabidopsis thaliana* mature pollen grain showing the pollen coat (tryphine), exine, and intine layers. Scale bar represents 0.5 μm . Picture was taken by Ms. Denise Chabot.

light, and helps during pollination and pollen-stigma interactions (Rejón et al., 2016). At anther maturity, it dehisces and releases the pollen. Once the pollen grain lands on the stigma, the pollen coat flows to create a contact zone with the papillar cell where exchange of information occurs. In a compatible reaction, water flows from the stigma to hydrate the pollen grain which then germinates and forms a pollen tube that will grow through the stigma papillar cell wall, down the style towards the ovary. Once the pollen tube reaches the embryo sac it delivers two sperm cells for double fertilization to generate a new embryo (seed) (Lord and Russell, 2002; Wilhemi and Preuss, 1999).

1.3. Seed and anther caleosins and oleosins

Caleosin is a member of a family of lipid-associated proteins which are found within the seeds and anthers of several higher order plant systems including *Arabidopsis thaliana*. Caleosins resemble lipid-binding oleosins which are seed and anther oil body surface proteins.

Oleosins were first characterized in seeds within the oil body monolayer (TAG matrix surrounded by phospholipid monolayer) suggesting the organization of this organelle possesses more complexity (Chen et al., 1998). In *Arabidopsis thaliana*, seed oleosins were shown to be the most abundant protein in oil bodies (Wu et al., 1999). They were found on the ER near oil bodies and later in development specifically associated with seed storage oil reserves suggesting a key functional purpose in dessication-tolerant seeds (Murphy et al., 2001). Seed oleosins mainly contain three structural areas: an amphipathic N-terminus, a central hydrophobic region, and an amphipathic C-terminus (Abell et al., 1997). Within the hydrophobic domain, there is a proline knot motif which is a highly conserved region in related gene families such as caleosins.

The role of the proline knot is thought to target oleosins to the bilayer of oil bodies (Abell et al., 1997; Murphy et al., 2004). Oleosins play a crucial role in the stability of oil bodies during seed dehydration/rehydration, as the formation and integrity of oil bodies were lost when oleosin levels were reduced by trypsin treatment impacting TAG accumulation as well as desiccation and seed dormancy (Tzen and Huang, 1992 and Leprince et al., 1998).

Caleosin shares a similar role to that of oleosins in stabilizing oil bodies and later facilitating the degradation of stored TAG upon seed germination (Chen et al., 2004; Murphy et al., 2004). It has also been found to be present in the membrane layer of oil bodies and associated with calcium pools in the lumen of the ER (Frandsen et al., 1996; Murphy, 2001). However, caleosin differs from oleosin in that it has been shown to also exhibit peroxygenase activity and metal ion-binding activities (Frandsen et al., 2001; Partridge and Murphy, 2009). Seed caleosins further play an important role in plant defense since the peroxygenase activity is involved in the production of oxylipins which initiate plant stress responses (e.g., jasmonic acid production) in the presences of abiotic and biotic stressors (Eckardt, 2008). Furthermore, it was postulated that seed caleosins contribute in oil body transport from the ER to storage and transport vesicles (Frandsen et al., 2001). Caleosin also varies from oleosin in that it contains a calcium-binding EF-hand domain located at the N-terminus. The EF-hand promotes calcium ion-binding and is thought to be associated with the abscisic acid signalling pathway and osmotic stress responses (Sanders et al., 1999; Knight, 2000).

Caleosin and oleosin-related proteins have also been found in the anthers, pollen, and the tapetum (Murphy and Ross, 1998; Kim et al., 2002; Zienkiewicz et al., 2010). In the pollen, caleosin and oleosin are found associated with oil bodies in the interior of the pollen grain and function in stabilizing lipid droplets (TAG droplets) (Kim et al., 2002; Zienkiewicz et al., 2010).

The tapetum contains oil droplets made up of an oil matrix surrounded by layers of phospholipids and oleosins, which are first generated in the rough endoplasmic reticulum and mature into tapetosomes (Hsieh and Huang, 2007). These oil droplets are degraded (still containing full length oleosins proteins) once the tapetum undergoes programmed cell death and tapetosome debris travels to the pollen coat. Tapetal oleosins are thought to act as a lipid-emulsifying agent coating the pollen and protecting it from desiccation and/or aiding in the uptake of water (due to oleosin's amphipathic properties) during pollen germination once on the stigma (Hsieh and Huang, 2007). In *Arabidopsis thaliana*, there are nine known anther-specific oleosins (Huang et al., 2013).

With regards to caleosin, in *Arabidopsis thaliana*, there are two caleosin genes: AT1G23240 and AT1G23250 shown to be expressed in the anther (University of Toronto's Arabidopsis eFP Browser <http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>). However, there is no data to support pollen expression for these genes (based on the microarray analysis of Honys and Twell, 2004 and eFP Browser). Caleosin AT1G23240 was chosen for further examination due to its much higher level of expression in the anther. The eFP Browser data indicates that caleosin AT1G23240 shows a 15.9-fold higher absolute transcript expression in stamens in comparison to AT1G23250. As shown in Figure 3, AT1G23240 is a 210 amino acid (aa) sequence with no predicted signal peptide, which comprises two key domains: a calcium-binding EF-hand domain and a hydrophobic domain (Purkrtova et al., 2015; Chen and Tzen, 2001). The calcium-binding EF-hand is a 35 aa domain positioned near the N-terminal of the caleosin protein. It functions as a calcium-binding motif which is thought to aid in signal transduction (Bhattacharya et al., 2004).

Figure 3. Caleosin protein sequence schematic

Schematic representation of the caleosin (AT1G23240) protein sequence showing key domains – calcium binding EF-hand domain, hydrophobic domain with the proline knot motif.

The hydrophobic domain is 38 aa residues in length and is adjacent to the EF-hand domain, it is the conserved domain found in all caleosins. Though the caleosin hydrophobic domain is comparably smaller than the 70 aa hydrophobic region found in tapetal oleosins, it is important to the overall protein structure and to lipid interactions (Chapman et al., 2012; Murphy, 2001). Lastly, a relatively small 9 aa proline knot motif is embedded within the hydrophobic domain and it is important to lipid droplet and membrane targeting as in oleosins (Murphy, 2001). Anther and seed caleosin variants differ in domain positioning and size, as well as protein length.

1.4. Thesis rationale

One of the key goals of this research is to investigate pollen genes and proteins in order to gain a better understanding of pollen-stigma interactions. Pollen coat proteins are known to be important to these interactions and previous work in the lab has focused primarily on tapetum-produced pollen coat-located T-oleosins (Foster et al., 2002; Lévesque-Lemay et al., 2016). To investigate whether anther caleosins might be similarly targeted to the pollen, a preliminary analysis of microarray data was performed on caleosin-related genes. It revealed that although neither the AT1G23240 nor AT1G23250 transcripts were detected in the pollen (Honys and Twell, 2004), the caleosin encoded by AT1G23240 was highly expressed in the anther (http://bar.utoronto.ca/efp2/Arabidopsis/Arabidopsis_eFPBrowser2.html). It was therefore chosen for further analysis.

The central goal of this thesis/research study is to investigate the anther-expressed caleosin AT1G23240 in order to better understand its expression, localization, and role using Western blot analyses, quantitative reverse transcriptase polymerase chain reaction, confocal laser

scanning microscopy, and transmission electron microscopy/immunocytochemistry. To gain insight into its function, RNAi lines developed to knockdown expression of caleosin were characterized.

Thus, this thesis work was conducted to meet the following objectives:

1. Determine the caleosin gene and protein expression and localization throughout anther development and during the mature pollen stage; and
2. Initiate the elucidation of caleosin function.

2. MATERIALS AND METHODS

2.1. Plant material and transformation

Arabidopsis thaliana (L.) Heynh Columbia was grown in a growth cabinet with 16 h light/8 h dark at 20°C and 70% humidity. Plants were watered daily and fertilized bi-weekly with 20-20-20 (Nitrogen-Phosphorus-Potassium).

Agrobacterium-mediated transformation as described by Clough and Bent (1998) was used to introduce two different constructs into *Arabidopsis thaliana*. The first construct, Caleosin-YFP, represents a translational fusion between the coding regions of AT1G23240 and the yellow fluorescent protein (YFP) constructed as described by Tian et al. (2004). It comprised of a 619 bp region upstream of the caleosin start ATG, the caleosin coding sequence fused in-frame to the citrine YFP, and a 996 bp fragment downstream of the stop codon. The caleosin promoter/caleosin-YFP fragment was inserted into the pRD420 binary vector (Datla et al., 1992) replacing the GUS gene and used for plant transformation. After initial screening of 12

transformants for anther YFP activity, two lines (CCAL 2B4 and CCAL 2B6) were chosen for further analysis. All experimental results were validated using both these lines.

The second construct CCAL-RNAi was a caleosin RNAi construct generated and initially screened by Dr. John Chan, a former postdoc in the lab. The RNAi fragment targets the following sequence in the first exon of the caleosin gene:

5'- CGGCTTCTTCGATCATTATA

2.2. DAPI staining

Arabidopsis thaliana flower buds of sizes ranging from 0.2 mm to 2.5 mm were collected with the help of a Leica EZ4 dissecting microscope (Concord, ON, Canada). This was done to determine the staging of pollen development at tetrad (TET), uninucleate microspore (UNM), bicellular pollen (BCP), and tricellular pollen (TCP), in order to harvest anthers at appropriate stages. Fine-tipped forceps were used to isolate anthers from these flower buds and the anthers were placed on a glass slide in 100 µl of DAPI-Pollen Isolation Buffer (2 µg/ml 4',6-diamidino-2-phenylindole, 100 mM NaPO₄ pH 7.5, 1 mM EDTA, 0.1% (v/v) Triton X-100®) for 1 min at room temperature (RT). After placing a coverslip, slight pressure was applied to free the pollen encased in the locules of the anthers. Processed slides were imaged using the fluorescence setting equipped with an ultraviolet filter set of a Zeiss LSM 510 DUO, using a FLUAR x 20/0.75 objective and the Zen 2009 software package (Zeiss, Toronto, ON, Canada). In order to visualize the DAPI staining, samples were excited at 405 nm and emitted at a band pass filter of 450 nm.

2.3. RT-qPCR

Total RNA was extracted from wild type *Arabidopsis thaliana* anthers of each developmental stage from at least three different plants (corresponding to the biological replicates) using the RNeasy Plant RNA mini-kit (Qiagen, Toronto, ON, Canada). The RNA extracts were verified to have a concentration >200 ng/μl and high purity (RNA integrity number > 8.0) with the use of an Agilent 2100 Bioanalyzer (Agilent, Mississauga, ON, Canada). Subsequently, RNA was converted to cDNA using the Advance cDNA synthesis kit (Wisent BioProducts, Saint-Jean-Baptiste, QC, Canada). For the *Arabidopsis thaliana* anther reverse transcription quantitative polymerase chain reaction (RT-qPCR), constitutive housekeeping genes glyceraldehyde-3-phosphate dehydrogenase 1 (*GAPC1* - AT3G04120), ubiquitin 11 (*UBQ11* - AT4G05050), eukaryotic translation initiation factor 4A1 (*EIF4A1* - AT3G13920), and tubulin β-2 (*TUB2* - AT5G62690) were tested to evaluate whether their expression remained stable throughout anther development. *GAPC1* (GenBank: AK226804) and *UBQ11* (GenBank: U84968) were found to meet this criterion and were chosen for comparative analysis to the target caleosin gene (GenBank: NM102173; AT1G23240). Primers were devised with the use of the PrimerQuest Tool (Integrated DNA technologies <https://www.idtdna.com/PrimerQuest/Home/Index>) and obtained from Millipore-Sigma (Oakville, ON, Canada). The primer sets for the constitutive controls and target consisted of the following:

GAPC1-Fwd: 5'-TCAGACTCGAGAAAGCTGCTA

GAPC1-Rev: 5'-GATCAAGTCGACCACACGG

Ubiquitin11-Fwd: 5'-AATGTGAAGGCGAAGATCCAA

Ubiquitin11-Rev: 5'-ACGGAGACGGAGGACGACGAGAT

Caleosin-Fwd: 5'-TGGCGGCTTTTGTTGCTATA

Caleosin-Rev: 5'-CGGCGAGAATCCCTTTCC

Amplification reactions for each developmental stage were run in triplicates. Reactions were carried out in MicroAmp 96 well plates (Thermo Fisher Scientific) and contained 500 µg cDNA, 1 µl of 100 nM forward and reverse primers, and 10 µl of Wisent Biosystem Hi-Rox Advanced MasterMix (SYBR green) in a final volume of 20 µl. The qPCR amplifications were performed using a StepOne Plus qPCR (ThermoFisher Scientific) system and the PCR conditions were: 95°C for 2 min, 40 cycles of 95°C for 5 s and 62.5°C for 25 s. A dissociation phase was performed to observe melt curves of amplicons of the reactions. Three separate independent biological replicates of each *Arabidopsis thaliana* anther developmental stage were analyzed.

Delta-delta CT comparative analysis was performed to quantify the data output obtained from the Step One Plus qPCR system. The averages were calculated for each technical replicate of each biological replicate at the individual developmental stages for the *caleosin* gene and the two constitutive controls, *GAPC1* and *UBQ11*. Once the averages were determined, a ratio was obtained by adjusting each of the developmental stages to the TET average. Thus, the TET developmental stage was set to a value of '1' and used as the reference stage in adjusting the expression levels of the other stages (UNM, BCP, and TCP) of anther development (Pfaff, 2001; Livak and Schmittgen, 2001). The relative quantity of each housekeeping gene was separately compared to caleosin and then averaged. Thereafter, the standard deviation was calculated by capturing the relative quantity ratios of each housekeeping gene and caleosin for each

developmental stage. The standard deviation of the relative quantity ratios between controls and the target gene were used to identify statistical significance.

2.4. Calceosin Western blot analysis

2.4.1. *Arabidopsis thaliana* anthers

Wild type *Arabidopsis thaliana* anther protein extractions were performed using at least three different plants for each developmental stage via maceration in 1x SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer (60 mM Tris Stacking gel buffer pH 6.8, 10% (v/v) glycerol, 2% SDS, 1% Bromophenol Blue + 3.3% (v/v) β -mercaptoethanol) (Lévesque-Lemay et al., 2016). Protein extracts were ran at 190 V in a 12% (w/v) acrylamide SDS-PAGE gel and then transferred to a PVDF membrane (BioRad, Mississauga, ON, Canada) using 1x transfer buffer comprised of 25 mM Tris, 192 mM glycine, 20% (v/v) methanol for 60 min at 100 V at a temperature of $\sim 4^{\circ}\text{C}$. Two identical gels were run simultaneously, one gel was stained with Coomassie Brilliant Blue G (BioRad, Mississauga, ON, Canada) to ascertain equal protein sample loading and the second gel was transferred onto the PVDF membrane. Subsequent to transfer, the PVDF membrane was submerged in Roche (Hoffman-La Roche Ltd, Mississauga, ON, Canada) blocking reagent (1% w/v) + TBST (20 mM Tris, 150 mM NaCl pH 7.6 with 0.05% (v/v) Tween® 20) for 1 h with constant rotation. Fresh 1% Roche blocking reagent + TBST containing the primary antibody at a dilution of 1:1000 was then added and incubated overnight in a cold room ($\sim 4^{\circ}\text{C}$) with constant rotation. The membrane was washed twice with TBST solution for 10 min and then incubated in 1% Roche blocking reagent for 10 min before applying the secondary antibody ((HRP-conjugated goat anti-rabbit secondary antibody

(1:20,000) (BioRad, Mississauga, ON)). The PVDF membrane was incubated with the secondary antibody at RT for 1 h with constant rotation. The blot was then washed four times for 10 min, and the chemiluminescence reaction was performed with the use of the ECL Plus Western Blotting Detection System (GE Healthcare, Mississauga, ON, Canada). Precision Plus Protein™ All blue pre-stained protein standard (BioRad, Mississauga, ON) was used as the molecular weight ladder. The rabbit primary antibody was raised against the specific caleosin peptide sequence QDKNGLLSEKSVRAIC (Millipore Sigma) found within the C-terminal region of At1G23240 and was produced by the National Research Council of Canada (Montréal, QC, Canada).

2.4.2. *Arabidopsis thaliana* pollen

Arabidopsis mature pollen grains were collected onto a filter using a vacuum suction device described by Johnson-Brousseau and McCormick, 2004. The pollen grains were contained in 1.5 ml micro centrifuge tubes and washed/re-suspended with cyclohexane, and then transferred into a Corning® Costar® Spin-X column (MilliporeSigma) to separate the flow through (pollen coat (PC) proteins) from the residual tissue (coatless pollen (CL)) (Lévesque-Lemay et al., 2016). After centrifugation at 5000 RPM, the PC-cyclohexane extract was evaporated using a SVC100H SpeedVac (ThermoFisher Scientific). The pelleted dry PC protein, as well as the coatless pollen remaining on the membrane of the column, was suspended in 1x SDS loading buffer. Western blot analysis was performed as described above.

2.5. Confocal laser scanning microscopy

2.5.1. *Arabidopsis thaliana* anthers

Anthers at the TET, UNM, BCP and TCP stages were placed in Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA, USA), and confocal laser scanning microscopy (CLSM) was carried out using a Zeiss LSM 510 DUO with a Plan APOCHROMAT x40/1.4 objective and the Zen 2009 software package (Zeiss, Toronto, ON, Canada). Two laser settings were used to distinguish chlorophyll and tapetal cell content. To visualize YFP and chlorophyll of fresh anthers, samples were excited at 488 nm and emitted at a band pass filter of 505-530 nm or a long pass filter of 650 nm, respectively. Differential interference contrast images were taken in order to overlap with the confocal images.

2.5.2. *Arabidopsis thaliana* pollen

CLSM of mature pollen was carried out using a Zeiss LSM 800 AiryScan using a Plan APOCHROMAT x63/1.46 objective and the Zen 2012 software package (Zeiss, Toronto, ON, Canada). To distinguish the YFP signal of the Caleosin-YFP translation fusion from the auto-fluorescent exine of fresh mature pollen, samples were excited at 488 nm and detected in emission wavelength bands of 511-583 and 588-700 nm respectively.

2.6. Immunogold labeling and transmission electron microscopy

Transmission electron microscopy (TEM) was performed with the use of a TEM Hitachi H-7000 equipped with an ORIUS SC200 digital camera using Digital Micrograph software version 1.8.3 (Gatan Inc., Pleasanton, CA, USA). Anthers of wild type or transgenic *Arabidopsis thaliana* plants expressing the Caleosin-YFP translational fusion were collected in a 0.5 ml

microcentrifuge tube and fixed in 4.0% (w/v) paraformaldehyde, 0.8% (v/v) glutaraldehyde for 1 h at RT. Subsequently, the anthers were washed with 100 mM NaPO₄ pH 7.2, under vacuum for 1 h at RT and microwaved using a Pelco Biowave ® 34700 microwave (Ted Pella, Redding, CA, USA) at 250 W at 37°C for 1 min at 0% power, 40 s at 100%, and 3 min at 0%. Samples were subjected twice to serial dehydration through an ethanol series of 30%, 50%, 70%, 80%, 90%, 95%, and 100%. Each dehydration step was microwaved at 250 W at 37°C for 45 s at 100% power. Infiltration was performed three times using LR White resin (Electron Microscopy Sciences, Hatfield, PA, USA) at 450 W at 43°C, 100% power for 2.5 min. Samples were then placed in a conventional oven for 24 h to polymerize the resin. These steps were performed under vacuum apart from the serial dehydration stage and polymerization.

Embedded anther samples were prepared into 1 µm thick sections with the use of a Diatome 45 MA1895 diamond knife (Hatfield, PA, USA) and Reichert-Jung Ultracut E ultra-microtome (Leica, Concord, ON, Canada). Sliced sections were placed onto carbon coated grids and incubated in 1% (w/v) glycine-Phosphate Buffered Saline (PBS) (10 mM NaPO₄ pH 7.4, 149 mM NaCl) for 1 h at RT. The sections were then washed three times for five min with PBS and blocked with 2.5% (w/v) bovine serum albumin (BSA), 1.0% (v/v) Tween® 20, and 0.1% (v/v) fish gelatin in PBS for 15 min at RT. Sections were then incubated overnight at 4°C in a PBS mix comprised of the caleosin primary antibody at a dilution of 1:400 (v/v), 2.5% (w/v) BSA, and 1.0% (v/v) Tween® 20 in PBS. The sections were washed three times for five min with PBS at RT prior to introduction of the 15 nm colloidal gold labeled goat anti-rabbit secondary antibody (Abcam, Toronto, ON, Canada) at 1:20 (v/v) in 2.5% (w/v) BSA, 1.0% (v/v) Tween® 20 in PBS. The grids were then incubated for 1 h and washed with PBS twice for 5 min and

once with distilled water at RT. Lastly, the grids were treated for 12 min with 5% (w/v) uranyl acetate and given a final water wash.

3. RESULTS and DISCUSSION

3.1. *Arabidopsis thaliana* anther development

DAPI staining was performed on *Arabidopsis thaliana* anthers isolated from floral buds of increasing maturity to identify the bud length corresponding to the different pollen developmental stages (Figure 4). The examined range consisted of bud lengths of 0.2 mm to 2.5 mm (largest bud size just before petals appear). Early in development, the process of meiosis results in a tetrad of four separate microspores bound in a callose wall (Fernández Gómez et al., 2015). TET microspores were observed in floral buds with lengths ranging from 0.5-0.9 mm. This corresponds to stage 7 of *Arabidopsis thaliana* flower development indicated in research performed by Sander et al., 1999. As development progresses, the callose wall is degraded releasing single UNMs consisting of a large centralized single nucleus. UNMs were observed in floral buds with lengths ranging from 1.1-1.4 mm relating to stage 9 of flower development (Sander et al., 1999). Following pollen mitosis I, two nuclei (generative and vegetative) are formed within BCP (Fernández Gómez et al., 2015). The generative nucleus stains strongly, while the vegetative nucleus stains more diffusely. BCP pollen was seen in floral buds with lengths ranging from 1.5-2.0 mm relating to stage 10-11 of flower development (Sander et al., 1999). Lastly, within the latest stage of development, pollen mitosis II splits the generative nucleus into two bright sperm nuclei and a condensed vegetative nucleus forming the male gametophytic unit of the TCP (Fernández Gómez et al., 2015). Floral buds ranging from

Figure 4. DAPI staining of *Arabidopsis thaliana* pollen

DAPI staining of *Arabidopsis thaliana* pollen throughout development. TET corresponds with stage 7 flower development, UNM corresponds with stage 9 flower development, BCP corresponds with stage 10-11 flower development, and TCP corresponds with stage 12 flower development. TET – tetrad, UNM – uninucleate microspore, BCP – bicellular pollen, TCP – tricellular pollen.

2.0-2.5 mm were shown to contain TCP pollen relating to stage 12 of flower development (Sander et al., 1999).

3.2. Anther caleosin gene expression during anther development

RT-qPCR was performed to examine the RNA transcript levels of *caleosin* (AT1G23240) in wild type *Arabidopsis thaliana* anther tissues at the different developmental stages corresponding to the pollen staging described in Figure 4. In order to compare *caleosin* RNA expression throughout anther development, two constitutively expressed housekeeping genes were used as controls (Plaff, 2001; Livak and Schmittgen, 2001). Based on an analysis performed by Czechowski et al. 2005, *GAPC1* and *UBQ11* were found to possess consistent constitutive expression throughout plant development. *GAPC1* is an essential enzyme in the process of glycolysis and converts D-glyceraldehyde 3-phosphate (G3P) into 3-phospho-D-glyceroyl phosphate (Rius et al. 2008). Ubiquitin is involved in a variety of cellular processes such as protein modification, proteasomal degradation, and cell signalling/regulation (Sharma et al., 2016). These housekeeping genes were also found to show consistent expression throughout anther development in *Arabidopsis* and were thus chosen to serve in comparisons with the anther caleosin gene. The results of a typical RT-qPCR reaction are shown in Figure 5. Unlike both the *GAPC1* and *UBQ11* control genes, *caleosin* exhibited variations in expression profile throughout anther development. In comparison to the low level of *caleosin* expression observed within the TET and UNM stages (there was no statistically significant difference in expression between TET and UNM), BCP and TCP anthers showed an 18.5 +/- 1.99 and 10.35 +/-1.35 fold changes respectively (Figure 6).

Figure 5. Quantitative polymerase chain reaction amplification plot

Quantitative polymerase chain reaction amplification plot of constitutive internal reference controls (*GAPC1* and *UBQ11*) and target AT1G23240 (caleosin) throughout *Arabidopsis thaliana* anther development.

Figure 6. Relative quantitative polymerase chain reaction amplification histogram

Relative quantitative polymerase chain reaction amplification of constitutive controls (*GAPC1* and *UBQ11*) and target gene AT1G23240 (caleosin). Error bars represent standard deviation of relative quantities between normalized controls and target. The y-axis represents the fold change in expression, while the x-axis represents the different stages of anther development. TET – tetrad, UNM – uninucleate microspore, BCP – bicellular pollen, TCP – tricellular pollen.

3.3. Anther caleosin protein expression during anther development

To further characterize caleosin protein expression in wild type *Arabidopsis thaliana* anthers, a Western blot analysis was performed on protein extracts from each anther developmental stage, using a primary antibody raised specifically against the caleosin AT1G23240 protein. As shown in Figure 7, the caleosin protein (predicted size of 23851 Da) was detected in BCP and TCP anthers, but not at the TET and UNM stages. The immune-reactive caleosin band was found to be at a slightly higher molecular mass than predicted and this phenomenon has been observed previously with oleosins, and postulated to be the result of their high basic isoelectric point (pI) (caleosin's pI: 10.27) (Wang et al., 1997; Murphy and Ross 1998; Lévesque-Lemay et al., 2016). The caleosin protein levels were higher in BCP than the TCP stage consistent with the gene expression levels revealed in Figure 7. These results are similar to those obtained with an olive anther caleosin that was found to be expressed in the microspore and bicellular pollen stages, as well as in the mature pollen grain (Zienkiewicz et al., 2011). However, the results presented in Figure 7 do not identify where the *Arabidopsis* caleosin is found within the anther and additional experiments were performed to investigate its subcellular localization.

3.4. Subcellular localization of the caleosin protein

3.4.1. Confocal laser scanning microscopy

The Caleosin-YFP translational fusion is comprised of the caleosin native promoter, the in-frame coding regions of AT1G23240 and the YFP citrine reporter, as well as the nopaline synthase polyadenylation signal (Figure 8). YFP is attached as a C-terminal translational fusion to the full length caleosin protein. Citrine is a derivative of enhanced YFP which is the result of an

Figure 7. Developmental Western Blot of wild type *Arabidopsis thaliana* anthers

Developmental Western blot analysis of wild type *Arabidopsis thaliana* anthers using the caleosin-specific antibody. 'M' indicates the Precision Plus Protein™ All blue pre-stained protein standard. TET – tetrad, UNM – uninucleate microspore, BCP – bicellular pollen, TCP – tricellular pollen.

Figure 8. Caleosin-YFP translational fusion sequence schematic

Schematic representation of the Caleosin-YFP translational fusion showing the caleosin native promotor region, caleosin coding region (expanded to depict full length protein with key domains), YFP citrine reporter region, and nopaline synthase termination region (Nos T).

Diagram is not to scale.

additional mutation at location Q69M (Shaner et al., 2005). This reporter was selected due to the fact that it has been reported to have a greater insensitivity and stability in environments with high salinity and acidity in comparison to the conventional fluorescent proteins such as GFP and YFP (Heikal et al., 2000; Griesbeck et al., 2001; Shaner et al., 2005). Furthermore, citrine has been found to be an appropriate marker to show expression in crucial subcellular compartments such as the ER (Griesbeck et al., 2001).

CLSM was performed on transgenic *Arabidopsis thaliana* lines expressing the Caleosin-YFP translational fusion to determine its subcellular location within the anther throughout development (Figure 9). As shown in Figure 9, the top row of panels depicts the staging of anther development via DAPI staining of pollen for each stage, while the two subsequent rows show a close-up view of a section of the anther highlighting the tapetum surrounding the anther locule space and the anther wall for both a wild type plant and a Caleosin-YFP transgenic line. A whole anther with dramatized colouring of the anther wall/filament (red) and Caleosin-YFP expression (yellow) is shown at the top of Figure 9. During early anther development, the Caleosin-YFP fusion was not detected in either the TET or UNM stages. However, YFP activity was present within the tapetal layer starting at the BCP stage. As the tapetum degenerates during the late bicellular and tricellular stages, Caleosin-YFP activity was observed moving into the locule space among the pollen grains.

To investigate whether caleosin becomes part of the mature PC, CLSM was also performed on mature pollen grains from transgenic Caleosin-YFP *Arabidopsis thaliana* plants. As shown in Figure 10, mature pollen grains from these lines consistently showed fluorescence associated with the PC and possibly the exine layer. This is in contrast to the mature wild type *Arabidopsis* pollen which shows only the autofluorescence of the exine layer represented as a

Figure 9. Confocal laser scanning and differential interference contrast microscopy of wild type and *Caleosin-YFP Arabidopsis thaliana* anthers with correlative DAPI stained pollen

Confocal laser scanning and differential interference contrast microscopy of anthers showing pollen and tapetal cells from wild type and Calcosin-YFP transgenic *Arabidopsis thaliana* plants during development. The top row shows DAPI staining of wild type pollen at different developmental stages, while the middle and bottom row depicts the corresponding wild type and transgenic Calcosin-YFP anther stages, respectively. TET – tetrad, UNM – uninucleate microspore, BCP – bicellular pollen, TCP – tricellular pollen. Scale bars represent 20 μm .

Figure 10. Confocal laser scanning microscopy of wild type and Caleosin-YFP *Arabidopsis thaliana* pollen

Confocal laser scanning microscopy of mature pollen grains from wild type (top row) and Caleosin-YFP transgenic (bottom row) *Arabidopsis thaliana* plants. Auto-fluorescent exine is shown in deep orange and the Caleosin-YFP is shown in yellow. Scale bars represent 10 μ m.

deep orange colour. It also suggests that once the tapetum undergoes programmed cell death, the caleosin protein is shuttled to the outer layer of pollen. These results are very similar to

observations reported by Lévesque-Lemay et al., 2016 where full-length T-oleosins (AT5G07530 and AT5G07550) fused to GFP were also shown to first be expressed in the tapetum, then to be released in the anther locule to finally become part of the pollen coat. This transfer of proteins to the pollen coat following tapetum degeneration was also demonstrated using GFP fusions for AT5G62080 and AT5G07230, two type III lipid transfer secretory proteins (Huang et al., 2013).

3.4.2. Western blot analysis

Western blot analyses were performed to confirm the results obtained by CLSM. Firstly, a developmental analysis was performed on transgenic plants expressing the Caleosin-YFP translational fusion using anther tissue from the different developmental stages. Consistent with the CLSM analysis (Figure 10), the caleosin-specific antibody detected both the endogenous caleosin protein (24 kDa) and the YFP translational fusion (predicted molecular weight of 51 kDa) in BCP and TCP anthers of the transgenic lines (Figure 11). Occasionally, the antibody recognized additional proteins showing up as a band above and below caleosin, and these are unlikely to be related to caleosin since they also occur in TET and UNM where no caleosin mRNA was detected. The fusion protein was not observed in untransformed Arabidopsis BCP anthers. Secondly, to determine whether the Caleosin-YFP fusion was primarily associated with the pollen coat (PC), a Western blot analysis was performed using mature pollen PC extracts and the resulting coatless pollen (CL) in both untransformed and Caleosin-YFP transgenic Arabidopsis. The caleosin-specific antibody did indeed detect the endogenous caleosin protein

Figure 11. Developmental Western Blot of Caleosin-YFP *Arabidopsis thaliana* anthers

Developmental Western blot analysis using the caleosin-specific antibody of *Arabidopsis thaliana* anthers expressing the Caleosin-YFP translational fusion. The red star indicates the endogenous caleosin protein and the blue star designates the translational fusion protein. ‘M’ indicates Precision Plus Protein™ All blue pre-stained protein standard. TET – tetrad, UNM – uninucleate microspore, BCP – bicellular pollen, TCP – tricellular pollen. WT BCP – BCP anthers from untransformed plants.

Figure 12. Western Blot of wild type and Caleosin-YFP *Arabidopsis thaliana* pollen coat and coatless pollen

Western blot analysis using the caleosin-specific primary antibody of mature pollen coat (PC) and coatless pollen (CL) of *Arabidopsis thaliana* anthers from wild type (WT) and Caleosin-YFP plants. 'M' represents Precision Plus Protein™ All blue pre-stained protein standard. The red star indicates the endogenous caleosin protein and the blue star designates the translational fusion protein.

(24 kDa) in the untransformed *Arabidopsis* PC extract (Figure 12). As expected, the antibody detected the endogenous caleosin protein in the transgenic line, but it also revealed the presence of the translational Caleosin-YFP fusion (50 kDa) in the PC extract. The endogenous caleosin protein could also be observed in the untransformed plant CL sample and this is likely due to an incomplete extraction of the pollen coat leaving some pollen grains in the CL fraction with remaining pollen coat, combined to the fact that there was more proteins in that lane. Unlike caleosin, the extra protein bands observed in Figure 11 were not observed with pollen extracts and may correspond to cross-reacting protein found elsewhere in the anther.

In summary, the Western blot analyses of the Caleosin-YFP plants confirmed the results obtained by CLSM (Figure 10) and indicate that the caleosin protein is not likely found within the mature pollen grain, but is primarily located in the pollen coat. This also supports the data presented by University of Toronto's *Arabidopsis* eFP Browser where no transcript expression is found in the mature pollen grain and only in developing anthers.

3.4.3. Immunocytochemistry

Immunocytochemistry using the anti-caleosin antibody was performed with wild type *Arabidopsis thaliana* BCP and TCP stage anthers, as well as late stage pollen, to further confirm the subcellular localization of caleosin. As shown in Figure 13, secondary immunogold colloidal particles were observed within the tapetum and on mature pollen grains. In the tapetal cells of the BCP anther stage, it appears that caleosin is associated with structures resembling the ER and possibly regions of the tapetosomes. ER accumulation in *Brassica* and *Arabidopsis* tapetal cells

Figure 13. Transmission electron microscopy immunogold labeling of wild type *Arabidopsis thaliana* late stage tapetum and pollen tissue

Transmission electron microscopy immunogold labeling, using the anti-caleosin antibody, of tapetal cells and pollen grains from untransformed *Arabidopsis* plants. The control is untransformed *Arabidopsis* tissue incubated with only secondary immunogold labeling antibody. OB - oil body, IN - intine, EX - exine, PC - pollen coat, MT - mitochondria, TA - tapetosome, EL - elaioplast, ER - endoplasmic reticulum. Scale bar represents 1 μ m.

was found to often occur in close association with tapetosomes which are tapetal-specific organelles that accumulate alkanes, flavonoids, and lipid-specific proteins (i.e., triglycerides, caleosins, oleosins) destined for delivery onto the pollen surface (Hsieh and Huang 2007; Murphy 2001). The gold particles associated with the tapetosomes (Figure 13) may be labeling regions shown to contain ER marker proteins (Hsieh and Huang, 2005). The ER has previously been shown to be in close association to tapetosomes in Brassica and Arabidopsis (Hsieh and Huang, 2007; Murphy 2001). Seed caleosin was also found to be localized on lipid bodies and on ER that are associated with vesicle trafficking (Chen et al., 1999).

With respect to the bicellular and tricellular pollen, although caleosin detection was found primarily associated with the PC (which is difficult to fix while maintaining immunogenicity), gold particles were also found attached to the intine and exine layers, as well as inside the pollen grain. Very few gold particles could be detected in the control experiments lacking the primary antibody (Figure 13).

An immunocytochemical analysis using the anti-caleosin antibody was also performed on anthers of the same stages obtained from transgenic plants expressing the Caleosin-YFP fusion (Figure 14). In this analysis, the tapetal cells are further along in the degeneration process, but gold particles still were found to be associated to ER-like structures or remnants of tapetosomes. With respect to the pollen, at both the BCP and TCP stages, gold particles were mostly associated with the PC, although some could be detected more or less randomly within the pollen cytoplasm.

The immunogold labeling results presented in Figures 13 and 14 are similar to those obtained with a caleosin observed in olive anthers, where an immune-reactive protein(s) was found to be associated with the tapetal ER, released into the locule of the maturing anther and then re-located

Figure 14. Transmission electron microscopy immunogold labeling of Caleosin-YFP

***Arabidopsis thaliana* late stage tapetum and pollen tissue**

Transmission electron microscopy immunogold labeling, using the anti-caleosin antibody, of tapetal cells and mature pollen grains from *Arabidopsis* plants expressing the Caleosin-YFP fusion. The control is Caleosin-YFP tissue incubated with only secondary immunogold labeling antibody. OB - oil body, IN - intine, EX - exine, PC - pollen coat, MT - mitochondria, TA - tapetosome, EL - elaioplast, ER - endoplasmic reticulum. Scale bar represents 1 μ m.

to the pollen coat (Zienkiewicz et al., 2010 and 2011). However, in these analyses, the antibody also detected proteins within the pollen grain as well as in the pollen tube. It is possible that this difference is due to the fact that the anti-caleosin antibody that was used in these experiments had been raised against the N-terminal sequence of a constitutively expressed *Arabidopsis thaliana* caleosin-related protein (AT2G33380, CLO-3, PRXG3, RD20) involved in stress responses (Partridge and Murphy, 2009), and that this antibody cross-reacts with different caleosin-like proteins. There are also *Arabidopsis* caleosins that are expressed in the pollen such as AT1G70670 (Honys and Twell, 2004).

In summary, the results observed in the immunocytochemical analyses (Figures 13 and 14) essentially reflect those of both the CLSM (Figures 9 and 10) and the Western blot analyses (Figures 11 and 12) showing that caleosin protein is first detected within the tapetal cells and subsequently on the pollen coat.

3.4.4. Characterizing caleosin function

To investigate the function of caleosin in plant reproduction, the first step was to search for T-DNA insertional mutants Salk lines (ABRC, <http://abrc.osu.edu>) which might disrupt the expression of the caleosin gene. After evaluating the available lines, it was determined that there were no T-DNA insertion mutants likely to completely disable the expression of AT1G23240. RNAi represents an alternative tool that can be used to down-regulate gene expression and it has previously been used in the study of anther genes. For example, in the study performed by Huang et al., 2013, RNAi was utilized to knockdown individual *Arabidopsis* anther LTPs and although the transgenic plants appeared normal, at a microscopic level, it was found that the

developed pollen had an abnormal/impaired intine structure and that it impacted pollen viability due to an increased susceptibility to dehydration damage. Similarly, RNAi was successfully applied to reduce the expression of an *Arabidopsis* tapetum-specific caffeoyl CoA O-methyltransferase-like gene named AtTSM1 leading to sterility (Fellenberg et al., 2008). Given that in both these instances RNAi of an anther-expressed gene led to a visible phenotype, it is possible that a similar outcome could be obtained by reducing the expression of the tapetally-expressed caleosin. Thus, *Arabidopsis thaliana* was transformed with an RNAi construct targeted to the first exon of the caleosin gene (developed by postdoctoral fellow John Chan). Western blot analysis was performed using the anti-caleosin antibody to identify *Arabidopsis thaliana* transformants with reduced or complete loss of caleosin protein expression. Out of 12 RNAi transgenic lines, four (Plt 4, 5, 8 and 9) were shown to produce significantly less caleosin protein than wild type in BCP and TCP anthers (Figure 15). Plants 3, 6, 7, 10, 11, and 12 succumbed to infection and were not assayed. The results obtained are preliminary, further testing and controls (e.g. loading control) is required to validate knockdown expression found in Plt 4, 5, 8, and 9. From initial observations of phenotypic expression, the top candidate lines appear to be fertile although they were shown to have fewer siliques and there were a few incidences of abnormal floral bud development (results are not shown).

Figure 15. Western blot analysis on Caleosin RNAi lines late stage anthers

Western blot analysis using the anti-caleosin antibody of BCP and TCP anthers from different *Arabidopsis thaliana* RNAi transgenic plants (Plt1 – Plt9). BCP – bicellular pollen, TCP – tricellular pollen, WT – untransformed plants. ‘M’ represents Precision Plus Protein™ All blue pre-stained protein standard.

4. GENERAL CONCLUSION and FUTURE DIRECTIONS

4.1. General conclusion

Caleosin is a lipid-associated protein involved in peroxidase, lipid-, and metal ion-binding activities. It has similar properties and homology to the well-characterized oleosin protein family. In this thesis, Western blot, qPCR, TEM-immunogold labeling, and CLSM analyses were performed successfully to demonstrate that *Arabidopsis thaliana* caleosin (AT1G23240) is expressed primarily during the BCP and TCP stages of anther development. Caleosin is first expressed in the tapetal cells at the BCP stage where it appears to be associated to the ER and the tapetosomes. Upon tapetum degeneration, the protein then moves into the anther locule space holding the pollen grains. Lastly, as the pollen matures, the caleosin protein re-localizes to the pollen coat. In addition, to decipher the role of caleosin in *Arabidopsis thaliana* reproduction, RNAi lines were screened to identify plants with reduced caleosin protein expression and three lines were found to show significant reduction in the expression of caleosin. These lines combined with the elucidation of caleosin expression described above will serve to facilitate further investigations of the role of this protein in tapetal and pollen development, or for example, pollen-stigma interactions.

4.2. Future directions

4.2.1 Analysis of RNAi lines

Now that RNAi lines showing a reduction of caleosin protein expression have been identified, these transgenic lines could be further characterized to identify any possible consequences such as defects in the tapetum or tapetosomes, sterility of pollen, etc. At present, these lines appear to

be fertile although they seemed to have fewer siliques and there were a few incidences of abnormal floral bud development. Phenotypic variance between the top candidate RNAi lines and wild type plant inflorescence should be analyzed and observed throughout development as described by Fellenberg et al., 2008. If RNAi of caleosin affects fertility, it may cause reduced seed set. To test fertility, a further step should include collecting and weighing the seed from RNAi and wild type lines to assess any changes to overall seed production.

In order to validate RNAi expression results obtained by Western blot analysis, qRT-PCR could be performed to ascertain whether caleosin levels were also reduced in these knockdown lines. This can be done by comparing the expression profile of the top RNAi candidate lines to that of the untransformed *Arabidopsis thaliana* at both the BCP and TCP anther stages. To ensure the validity of these results, technical and biological replicates would be used similarly to the experimental design highlighted in the qRT-PCR analysis mentioned above.

Lastly, the caleosin RNAi lines could be analyzed using transmission electron microscopy to search for anther phenotypes during the BCP and TCP stages, as well as in the mature pollen. This would allow for the comparative analysis of the subcellular structure of the tapetal layer and pollen (including the pollen coat) in RNAi lines compared to wild type *Arabidopsis thaliana*. Any abnormal developmental event would in turn aid in the elucidation of overall caleosin function.

4.2.2 Subcellular co-localization of the ER

During this research, there was an attempt to use a subcellular chemical marker for the ER ((ER-Tracker™ Red (BODIPY™ TR Glibenclamide)) to establish if it would co-localize with caleosin-YFP translational fusion protein. Although different approaches were tested, the

experiment did not yield acceptable results. Further attempts could be made using different ER-specific markers and/or the technique could be further optimized. Alternatively, the plants expressing the Caleosin-YFP fusion could be crossed with transgenic Arabidopsis plants containing a tapetum-expressed fluorescent marker (compatible with YFP) linked to organelle (e.g. ER, Golgi) marker proteins. Co-localization of the different fluorescent marker proteins could be examined by CLSM.

4.2.3 Biotechnological applications

Since caleosin is transferred to the pollen coat, it may play a role in pollen-stigma interactions. Accordingly, reducing caleosin expression could potentially lead to male sterility by affecting tapetosome and/or pollen coat formation, or by interfering with pollen-stigma interactions. The consequences could include non-viable pollen, decreased seed set, and/or delayed pollen-stigma interactions. Down-regulation of tapetal and pollen oleosins has been found to have such effects (Huang et al., 2013 and Rejón et al., 2016).

Alternatively, since caleosin appears to move to the pollen coat during the late stage of pollen development, it could be used to shuttle specific proteins to the pollen coat that could possibly impact pollen-stigma interactions and for example, cause pollen rejection. In modern day agriculture, hybrid seed technology is a regularly utilized practice to increase the performance, yield, and tolerance to biotic and abiotic stresses of staple crops such as maize and canola. For example, this can be done by developing an elite line with a proven high crop yield and then cross-pollinating with a plant possessing desired genomic or phenotypic traits such as weed/insect resistance or greater uniformity which then can be passed onto the next generation of seeds during reproduction. In relation to the Brassicaceae, hybrid canola lines approved for

environmental release in Canada that possess fertility restoration and herbicide tolerance traits can be produced using molecular approaches. Given the potential function of caleosin, down-regulation of this gene could lead to male sterility and could maybe be used in a hybrid system or in cases where pollen production needs to be hindered.

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