TRANSCRIPTOMIC ANALYSIS IDENTIFIES POTENTIAL REGULATORS INVOLVED IN PROGRAMMED CELL DEATH AND REMODELLING OF LACE PLANT LEAVES (APONOGETON MADAGASCARIENSIS)

by

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Dedicated to my grandmother, Mary Rowarth, whose memory keeps me moving forward.

Thank you for all of your love and support in helping make me the man I am today.

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ABSTRACT

Programmed cell death (PCD) is a highly controlled, regulated process of deleting cells. Plant PCD is either developmentally regulated or environmentally induced due to biotic or abiotic stress. Leaves of the lace plant *Aponogeton madagascariensis* undergo developmentally regulated PCD to form perforations in areoles framed between longitudinal and transverse veins throughout the leaf lamina. The lace plant is an emerging model system to study PCD due to its spatiotemporal predictability of PCD on thin, almost translucent leaves, making them ideal for live cell imaging and the existence of protocols for sterile propagation and experimentation. The molecular mechanisms that control animal PCD are well defined but less understood in plant PCD. The objectives of this thesis were to identify potential regulators of lace plant PCD using RNA sequencing analysis and test the role of selected genes using pharmacological experiments, western blotting, qPCR, anthocyanin quantification, reactive oxygen species (ROS) detection and observing changes in leaf perforations. By taking advantage of the visible cell death gradient, cells destined to die (PCD) and cells destined to survive (NPCD) were separated using laser capture microdissection. Transcriptomes of different stages of lace plant leaf development, NPCD and PCD cells were assembled to profile differentially expressed genes during PCD and leaf remodelling. Genes encoding heat shock protein 70 (Hsp70) and autophagy-related protein 16 (Atg16) were highly expressed in early leaf development when PCD is active. Treatment with PCD regulator ROS increased Hsp70 levels 2-fold and caspase-like activity but no effect on perforations formed. Antioxidants yielded opposite effects, inhibiting the number of perforations. Treatment with an Hsp70 inhibitor increased Hsp70 levels 4-fold and yielded similar results to antioxidants, while inhibiting anthocyanin accumulation. Autophagy promotion by rapamycin increased Atg16 levels and inhibited anthocyanin and perforation formation, while autophagy inhibitors had the opposite effect. Results from this work generated a workflow for future lace plant omics studies and improved the understanding of Hsp's and autophagy's (through Atg16) role in PCD during lace plant leaf remodelling. The ultimate goal of lace plant research is to improve our understanding of PCD to manipulate the process for future applications in medicine and agriculture.

LIST OF ABBREVIATIONS USED

10KP	Ten Thousand Plant Transcriptomes Initiative
3MA	3-methyladenine
AA	Ascorbic acid
ACC	Aminocyclopropoane-1-carboxylic acid
ANOVA	Analysis of variance
AS	Alternative splicing
Atg	Autophagy related
AVG	2-aminoethoxyvinyl glycine
AVI	Anthocyanin vacuolar inclusion
Bag	Bcl-2 athanogene
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
BGP	BioGenome Project
bHLH	Basic-helix-loop-helix
BI-1	Bax inhibitor 1
bp	Base pair
C3RE	Cyanidin-3-rutinoside equivalents
CaM	Calmodulin
Caspase	Cysteine aspartate-specific protease
cDNA	Complementary DNA
CLP	Caspase-like protease
ConA	Concanamycin A
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Cyt c	Cytochrome c
DEG	Differentially expressed gene
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	double-stranded RNA (ribonucleic acid)
EBP	Earth Bio Genome Project
ECL	Enhanced chemiluminescence
EdgeR	Empirical Analysis of Digital Gene Expression
EPCD	Early-programmed cell death
ER	Endoplasmic reticulum
FPKM	fragments per kilobase of exon model per million mapped reads
g	acceleration due to gravity, 9.81 m/s2
GFP	Green fluorescent protein
GO	Gene Ontology

HR	Hypersensitive response
HRP	Horseradish peroxidase
HS	heat shock
HSP	Heat shock protein
kDa	kiloDaltons
LPCD	Late-programmed cell death
Mb	Mega base pair
MC	Metacaspase
mRNA	messenger RNA (ribonucleic acid)
MS	Murashige and Skoog
mTOR	Mammalian target of rapamycin
N50	median length of a set of sequences
NAA	1-napthaleneacetic acid
NAC	No Apical Meristem
NBT	Nitro blue tetrazolium
NCBI	National Centre for Biotechnology Information
NGS	Next-generation sequencing
NPCD	Non-programmed cell death
PCD	Programmed cell death
PCR	Polymerase chain reaction
PE	phosphatidylethanolamine
PES-Cl	chlorophenylethynylsulfonamide
PI3K	Phosphoinositide 3-kinase
qRT-PCR	reverse transcription quantitative PCR
RIN	RNA integrity number
RNA	ribonucleic acid
RNA-Seq	RNA sequencing
RNAi	RNA interference
ROS	Reactive oxygen species
RT-PCR	reverse transcription PCR
scRNA-Seq	single cell RNA-Seq
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sHsp	small heat shock protein
TE	tracheary element
TF	transcription factor
TMM	Trimmed Mean of M-values normalization
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling
UV	ultraviolet
VPE	vacuolar processing enzyme

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CHAPTER 1: INTRODUCTION

This introduction is submitted as a review paper and is currently under review (with a condensed Abstract) titled as:

Rowarth N. M., Dauphinee A. N., Tattrie S. B., Lacroix, C. R., Gunawardena A. H. L. A. N. (2022) Filling in the gaps: A road map to establish a model system to study developmental programmed cell death. *Botany;* submission ID: cjb-2022-0110.

1.1. ABSTRACT

Out of ~450,000 species of green plants (Viridiplantae), only a handful are model systems for studying biological processes like programmed cell death (PCD). However, it is still debatable if this is the optimal approach moving forward. Therefore, it is essential to introduce new model systems to broaden and illuminate our understanding of the universality of plant PCD. This review focuses on the potential of one aquatic monocot – *Aponogeton madagascariensis* – or the lace plant, to be a model of choice for plant PCD. PCD plays a significant role in plant development and defence. Thus, identifying key regulators or a conserved pathway across plant systems and PCD processes is a priority in the field. Furthermore, plant PCD has been studied mainly using *in vitro* systems. Therefore, developing a novel model to study PCD in an *in vivo* system would be more optimal and reliable. A fascinating example of developmental PCD is perforation formation in the lace plant leaves, which form in specific sites called areoles framed by

the vasculature. Cells actively undergoing PCD (PCD cells) within areoles lose anthocyanins which are potent antioxidants. In contrast, cells adjacent to veins 'Non-PCD' (NPCD cells) retain anthocyanin and persist as the leaves mature. PCD modulates lace plant leaf development by producing perforations between longitudinal and transverse veins, offering a unique model system for studying the timing and regulation of the cell death process next to control NPCD cells simultaneously in one field of view. The spatial-temporal pattern of PCD in these thin and semi-translucent leaves during perforation formation, along with the available axenic culture system, would provide a highly suitable *in vivo* system to identify critical regulators and mechanisms of developmental PCD in planta. The subcellular events of lace plant PCD are well characterized. However, less is known about the genetics involved in lace plant PCD. Developing future technologies such as single-cell omics coupled with the natural accessibility of PCD cells will help identify regulators of plant PCD. Gaining a detailed knowledge of these regulators has many potential applications, from reducing post-harvest losses to producing stress-resistant crops. Additionally, studying this system will aid in unveiling the evolution of genetic mechanisms involved in perforation formation, determining the universality of PCD mechanisms across plants and testing how anthocyanin composition can manipulate plant PCD. The priorities for further development of this model involve sequencing the lace plant genome, establishing efficient transformation protocols and identifying endogenous anthocyanin species, including determining their medicinal properties. Here, we discuss the practical methodologies and challenges associated with developing the lace plant as an established

system to study developmentally regulated PCD and provide a road map for model system development.

1.2. MODEL PLANT SPECIES

1.2.1 What makes a model plant species?

Model plants have research value based on the expected knowledge of biological processes gained in that species and how they may apply to other species (Borrill, 2020). Small size and genome size, short growth period, fast generation time (intrinsic properties) and genetic amenability are attributes that characterize a model plant. In addition, model plants require ancillary practicalities such as validated protocols for DNA, RNA and protein extractions, purifications and transformation (derived properties). Another tier of model status can be reached by having research groups contributing to an ever-growing communal annotated genomic database or genetic strain repositories (communal properties; Borrill, 2020; Chang et al., 2016; Provart et al., 2016). As described in Kress et al. (2022) and Sun et al. (2021), there are approximately ~450,000 species of Viridiplantae (green plants and green algae) and fewer than 300 'chromosome scale' genome assemblies representing ~812 species found in International Sequencing Database Consortium (INDSC; Arita et al., 2021). Of the Viridiplantae, there are ~350,000 angiosperm species (543 sequenced genomes identified by the INDSC), 1,000 gymnosperm species (11 sequenced), 13,000 seedless vascular plant species (5 sequenced) and 20,000 bryophyte species (8 sequenced) and 22,000 (249 sequenced) of

green algae (Kress et al., 2022). As of 2022, there are less than 300 'chromosome scale' genome assemblies. The number of non-model plants that have been sequenced by next-generation sequencing (NGS) is a promising avenue for increasing the number of model plant species in the future.

Much progress in plant biology research is carried out using the model *Arabidopsis thaliana* (Holland and Jez, 2018). *Arabidopsis* has exponentially advanced our understanding of plant processes such as stress, defence, development, signalling and evolution. At the same time, the rapid use of NGS for studying gene function has widened the scope for new emerging plant models and their respective research communities (Unamba et al., 2015). The *Arabidopsis* system features optimal intrinsic properties for a model species such as a small, easy-to-grow organism with a short life cycle, a small diploid genome and few chromosomes. However, it is naive to assume that *Arabidopsis* alone is suitable to represent biological processes across multiple groups of plants (Chang et al., 2016).

Arabidopsis is only one out of ~450,000 identified species of green plants (Chang et al., 2016). Therefore, in order to gain a greater understanding of the development and stress tolerance of economically important crop plants, there needs to be an emphasis on broadening the horizon of non-model species. Traditionally, monocot model plants are studied to make biologically parallel discoveries compared to eudicots for important grasses like *Oryza sativa* (rice), *Triticum aestivum* (wheat) and *Zea mays* (maize). However, there are few aquatic monocots with sequenced genomes like the duckweeds

Lemna minor and *Spirodela polyrhiza* (Laird and Barks, 2018) to help diversify accessible angiosperm models (Borrill, 2020; Chang et al., 2016; Haberer et al., 2016).

Due to the anchoring effect of NGS projects to model plants like *Arabidopsis,* the non-model plants or plants with one non-optimal characteristic such as long-life cycle, long growth period, or complex genome have gained less attention. As a result, the genomic information for biological processes of non-model plants is not as comprehensive (Unamba et al., 2015). In the era of NGS, unveiling specific gene characteristics is a unique advantage for revealing new gene interactions and enzyme pathways of non-model plants. Additionally, NGS has contributed to further elucidate the processes of gene expression in non-model plants without access to a fully sequenced genome (Unamba et al., 2015). Few plants possess intrinsic, derived and communal model organism properties like *Arabidopsis*, *Solanum lycopersicum*, maize and rice. However, non-model plants have unique physiologies, development, phylogenetic relationships, secondary metabolite composition, or socioeconomic values that drive research interest.

1.2.2. Model monocots

Arabidopsis (an eudicot) is used as a functional genomics system to study an array of biological plant processes that have generated relevant crop improvements but is limited in its ability to investigate monocot-specific processes (Brkljacic et al., 2011). Compared to eudicots, monocots are different in terms of cell wall hemicellulose, including a lower pectin composition, and have a seed aleurone layer, as well as a different meristem and

fibrous root architecture (Brkljacic et al., 2011). The genomes of three model cereals, maize, rice and wheat have been mostly sequenced and resolved (Alexandratos et al., 2012). These model organisms have large research communities and specific genetic library databases (e.g., http://www.gramene.org/, http://www.maizegdb.org/, https://wheat.pw.usda.gov/GG3/). These economically important grasses are well studied, but their large size, their longer life cycle and extensive space requirements for research are limiting factors.

Purple false brome, *Brachypodium distachyon*, and green foxtail, *Setaria viridis,* have core biological model attributes that researchers may desire in a model system for studying economically essential crops like wheat and maize (Garvin, 2007; Pant et al., 2016). Their small size, smaller genomes and recognition as grass satellite model systems make them resemble the *Arabidopsis* model. *Brachypodium distachyon* utilizes C3 photosynthesis (like rice, wheat and *Arabidopsis*), is transformable by *Agrobacterium* (available protocols: http://brachypodium.pw.usda.gov/; http://www.brachytag.org/) and easy to propagate. It has an 8–12-week generation time, small physical size and small genome (272 Mb, Table 1.1.; Brkljacic et al., 2011; Larré et al., 2010; The International Brachypodium Initiative, 2010; Watt et al., 2009). *Setaria viridis*, a small C4 plant, is an alternative grass monocot model to maize. Its advantages include its small size, short lifecycle and high fecundity, self-compatibility, small diploid genome (395 Mb; 2N=18) and transformation protocols (Bennetzen et al., 2012; Saha and Blumwald, 2016; Xianmin et al., 2014). These small monocots *B. distachyon* and *S. viridis* are proposed as

models for being genetically applicable to agronomically important grass species and can be used directly to extract information about agriculture and biofuel production and tease

Table 1.1. Individual genomic information of selected model and non-model plant groups. Most data are adapted from Chen et al. (2016). *; https://phytozome.jgi.doe.gov/pz/portal.html.

Species **(common name)**	**Ploidy**	**Genome size (Mb/2C)**	**Chromosomes**	**References**
Angiosperm Eudicot				
Arabidopsis thaliana	2X	135	10	*
Vitis vinifera (grape)	2X	487	38	*
Brassica napus L. (Rapeseed)	4X	1130	38	(Chalhoub et al., 2014)
Angiosperm, Monocot, aquatic				
Spirodela polyrhiza (greater duckweed)	2X	158	40	(Wang et al., 2014)
Lemna minor (common duckweed)	2X	472	20	(Van Hoeck et al., 2015)
Zostera marina (L.) (eelgrass)	2X	202	12	(Olsen et al., 2016)
Angiosperm, Monocot, terrestrial				
Brachypodium distachyon (purple false brome)	2X	272	10	(Garvin, 2007; Scholthof et al., 2018)
Hordeum vulgare (barley)	2X	5,100	14	*
Miscanthus sinensis (Chinese silver grass)	2X	5,500	38	(Rayburn et al., 2009)
Oropetium thomaeum (resurrection plant)	2X	245	18	(VanBuren et al., 2015)
Oryza sativa (rice)	2X	372	24	*
Paris japonica (Andromeda japonica)	8X	148,000	40	(Pellicer et al., 2010)
Setaria viridis (green fox tail)	2X	395	18	(Bennetzen et al., 2012)
Triticum aestivum (wheat)	6X	17,000	14	*
Zea mays (corn)	2X	2,300	20	(Schnable et al., 2009)
Gymnosperms				
Picea abies (Norway spruce)	2X	20,000	12	(Nystedt et al., 2013)
Seedless vascular plants				
Azolla filiculoides (water fern)	2X	740	44	https://www.azollagenome.net
Ceratopteris richardii (C-fern)	2X	22,000	78	(Sessa et al., 2014)
Selaginella moellendorffii (spike moss)	2X	106	20	(Banks et al., 2011)
Bryophytes				
Antheroceros agresis (field hornwort)	2X	83	12	(Szövényi et al., 2015)
Ceratodon purpureus (fire moss)	2X	340		(Thornton et al., 2005)
Marchantia polymorpha (liverwort)		226		*
Physcomitrella patens (earth moss)	2X	473	54	*

apart monocots' repetitive genomes. The intrinsic and communal model properties of these non-crop grasses are similar to *Arabidopsis* (Cheng et al., 2016), resulting in potential less future reliance on *Arabidopsis* models for agricultural or monocot-specific studies. It is also theorized that *B. distachyon* and *S. virdis* can be used as model grass systems to identify key genes to manipulate C4 photosynthesis. The use of genetic manipulation to introduce C4 photosynthesis into C3 plants like rice is currently under investigation (Brkljacic et al., 2011; Ermakova et al., 2020).

1.2.3. What do aquatic monocots have to offer?

Aquatic plants which include bryophytes, seedless vascular plants, angiosperms and one known gymnosperm, are a heterogeneous group of plants with the common feature of having adaptations to living either submerged in, emerged from, or floating on aquatic habitats (Acosta et al., 2021; Kozlowski et al., 2015). Aquatic monocots comprise ~11% of the monocots (~6,600 of ~60,000 species) and represent ~2% of all angiosperms (Zhao et al., 2020). The monocot group Alismatales makes up the majority of the aquatic angiosperms and provide an opportunity to study plants surviving in environments of high pressure, low light, low temperature, reduced gas diffusion and low oxygen availability (Maberly and Gontero, 2018). In addition, aquatic angiosperms are helpful for model studies due to the flexible nature of their thin submerged leaves to reduce mechanical stress, the dual use of CO_2 and HCO_3^- for carbon sources, gas-filled stomata, thin cuticles and the presence of epidermally bound plastids to help compete for limited light (Veen and Sasidharan, 2021).

Aquatic plants offer a growing body of information on how monocots and their leaves cope with their environment compared to terrestrial plants. Sequenced genomes of aquatic monocots include seagrass *Zostera marina* (*L.*) and duckweed *L. minor* (Lam et al., 2014; Olsen et al., 2016; Van Hoeck et al., 2015). The seagrass genome is the first marine angiosperm genome sequenced, which revealed insights into genomic losses and gains involved in physiological adaptations required for the plant's shift to a marine aquatic lifestyle (Olsen et al., 2016). Duckweed is a fast-growing, efficiently genetically transformed small diploid plant (2 cm; 472 Mb) used as a model aquatic plant for biofuels and environmental stress assays (VanBuren et al., 2015).

This review provides further evidence to support the lace plant as a suitable model species for studying programmed cell death (PCD). Here, we discuss key outcomes of bringing the lace plant closer to an established model system and build a potential road map for further development.

1.3. CLASSIFICATIONS AND THE UNIVERSALITY OF PCD

1.3.1. Plant PCD phases

It is prudent to identify commonalities of a biological process among species to establish how a potential model or non-model system can have connectivity with other systems. Across plant processes, PCD plays an essential role in developing reproductive and vegetative organs and is categorized into three phases: initiation, effector (synonymous

with execution) and degradation (Daneva et al., 2016; Van Durme and Nowack, 2016). The induction phase of PCD starts with designated cells receiving intracellular or extracellular signals from pathogens, neighbouring cells, or as a result of stress. Differentiation of plant cells can involve PCD and can initiate from a spatial or temporal context and can be triggered by age, hormone concentration, or transcriptional activity (Daneva et al., 2016). Common transcriptional activities that control developmental PCD have been documented (Daneva et al., 2016; Kabbage et al., 2017; Rantong and Gunawardena, 2015) and they tend to be cell-type specific.

In contrast, downstream effector molecules tend to be more specific to a PCD process (Olvera-Carrillo et al., 2015). Olvera-Carrillo et al. (2015) utilized bioinformatics to profile reporter genes for *Arabidopsis* PCD processes and showed a shared set of several cell death-associated genes (*metacaspase-9, aspartyl protease-3, bifunctional nuclease-1 and RNase-3*, amongst others) that are up-regulated across a majority of developmental PCD processes exclusively different from stress-induced PCD.

The effector phase consists of intracellular communication activating the necessary molecular machinery for cell death. Common intracellular signalling cascades can include changes in intracellular concentrations of reactive oxygen species (ROS), ions, protons and cytochrome c (cyt c; Daneva et al., 2016; Van Durme and Nowack, 2016). In addition, the reorganization of the actin cytoskeleton is an effector for PCD signalling in animals and plants and can be mediated by intracellular Ca^{2+} changes (Gourlay and

Ayscough, 2005; Staiger and Franklin-Tong, 2003). Organelle signalling and hormones are less well understood in how they act as effectors of PCD.

The degradation phase that follows is irreversible. A signalling cascade of molecules leads to the timely and precise destruction of cellular functions. Cellular bodies disintegrate and membranes permeabilize. Proteins such as nucleases and vacuolar processing enzymes (VPEs) are released alongside tonoplast permeabilization leading to an organized cell deletion. Caspases are absent in plant genomes, but many proteases play a role in plant PCD processes and show caspase-like protease activity is present in plants and can induce cell death (Van Doorn et al., 2011; Salvesen et al., 2016). A review by Balakireva and Zamyatnin (2019) highlighted where and how plant proteases (metacaspases, VPEs and papain-like cysteine proteases (PLCPs)) help regulate PCD, but the specific proteases involved differ or are less understood across different plant species and PCD processes (Balakireva and Zamyatnin, 2019).

1.3.2. Plant PCD classifications

Plant PCD has long been compared to animal apoptosis as 'apoptosis-like' because they share similar features such as cytoplasmic reduction, nuclear condensation, DNA fragmentation, mitochondrial cyt c release and the involvement of caspase-like proteases for degradation (Balakireva and Zamyatnin, 2019; Huysmans et al., 2017; Kacprzyk et al., 2011; Van Doorn et al., 2011; Lord and Gunawardena, 2012; Wertman et al., 2012). However, these apoptotic hallmarks are not consistent in plant PCD and are not always unique to apoptosis (Minina et al., 2021). Plant PCD is starkly different from animal

apoptosis because it involves plant hormones like ethylene (Lombardi et al., 2012; Yakimova et al., 2006) in plant signalling PCD pathways and plant-specific organelles such as chloroplasts, cell walls, and larger vacuoles. Chloroplasts are involved in energy production and, coupled with the cell wall, are capable of producing ROS, which triggers PCD (Petrov et al., 2015; Rodríguez-Serrano et al., 2012). On the other hand, vacuoles clear cellular contents through autophagy and release hydrolytic contents (Hatsugai et al., 2006; Rantong and Gunawardena, 2018; Yamada et al., 2005). Plant cells also possess a rigid cell wall that prevents cell fragmentation and lack key animal apoptotic activities like Bcl-2 family proteins, true caspases, the formation of apoptotic bodies and their subsequent engulfment of phagocytes (Minina et al., 2021). Animal apoptosis and plant PCD may have evolved from a common ancestor to cater to their exclusive cell modalities, morphologies and contents (Minina et al., 2021) as there is evidence of pro and anti-apoptotic animal proteins exhibiting functionality in plant systems (Lord and Gunawardena, 2012).

Animal PCD categories are relatively well defined, while new categories of plant PCD continue to emerge across plant processes. Animal cell death is classified into three morphological cell death types: apoptosis (Type I), autophagic cell death (Type II) and necrosis (Type III; Kroemer et al., 2005; Galluzzi et al., 2018). Apoptotic cell death is characterized by cellular volume reduction, condensation of chromatin and nuclear fragmentation, conservation of organelle ultrastructure and plasma membrane retraction. Cell death advances when apoptotic bodies form (Kerr et al., 1972; Kroemer et al., 2005; Elmore, 2007). Autophagic cell death is characterized by an increase of double

membraned vesicles called autophagosomes that shuttle cellular cargo to fuse with lytic bodies like lysosomes for degradation (Kroemer et al., 2005). Necrotic cell death is a passive process usually triggered by extreme or irreversible stress where cellular volume increases and organelles swell, the plasma membrane ruptures and cellular bodies spill outside the cell (Dauphinee et al., 2014; Kroemer et al., 2005).

Plant PCD classifications are ongoing and under debate (Bozhkov and Lam, 2011; Minina et al., 2021). Plant PCD was initially classified with animal PCD as a reference point for apoptotic-like cell death, senescence and vacuole-centric PCD (Fukuda, 2000). Apoptotic-like plant cell death was characterized by the retraction of the plasma membrane relative to the cell wall and cytoplasmic condensation. Later, Van Doorn and Woltering (2005) pointed out how plant PCD examples lack true apoptosis characteristics: (i) plants do maintain plasma membrane integrity through stress and do not form apoptotic-like bodies, apart from necrosis, (ii) DNA fragmentation is not always universal across plant PCD processes, (iii) plant cells do not shrink with stress like animal apoptosis and (iv) true Bcl-2 and true caspase-like proteases are not present in plant systems and (v) there exists caspase-independent apoptotic pathways in animal apoptosis (Van Doorn, 2011).

Van Doorn et al. (2011) proposed two plant cell death classifications: ‘vacuolar cell death’, where the cell is degraded by autophagy and hydrolases post-tonoplast rupture and ‘necrotic cell death’, where the plasma membrane ruptures early, the protoplast shrinks, organelles swell and an unprocessed corpse is left, lacking an autophagic

process. Van Doorn (2011) later proposed two new categories of "autolytic PCD" where cytoplasmic clearing occurs after tonoplast collapse and "non-autolytic PCD" where there is no cytoplasmic clearing after tonoplast rupture. The idea of apoptosis-like PCD was once widespread to describe plant PCD but it is now considered a misnomer as plant cells lack the formation of apoptotic bodies and their clearing of cells after plasma membrane degradation (Minina et al., 2021). Additionally, the use of apoptotic-like PCD can imply a conserved evolutionary link between prokaryotes and eukaryotes where although morphologically similar, not all of these PCD processes utilize caspases (Dauphinee and Gunawardena, 2015; Hakansson et al., 2011). The number of, and means to, define different plant PCD classifications is still under contention. What is agreed upon is that more morphological, biochemical and molecular investigations into model and non-model plant PCD processes are needed to sort these 'atypical' types of plant cell death (Van Doorn, 2011; Van Doorn et al., 2011; Bozhkov and Lam, 2011).

1.3.3. Commonalities in Plant PCD regulators

The exclusivity and mutual overlap of morphological, biochemical, transcriptional and proteomic changes during different processes of plant PCD lead us to ask: at what point can cell death processes be considered universal or alike? Jiang et al. (2021) reviewed recent progress in uncovering molecular regulation of plant developmental PCD events. Plant PCD processes involving male and female reproductive tissues such as tapetum cell deletion and nucellus degradation is regulated by the crucial timing of transcription factors. For example, tapetum cell deletion is promoted by a few bHLH transcription

factors. In rice, a specific tapetum degeneration retardation (OsTDR) factor activates a needed cysteine protease gene that induces tapetal PCD (Jiang et al., 2021; Xie et al., 2020). In *Arabidopsis*, the precise timing of tapetal cell PCD is controlled by MYB80 to repress cysteine protease-1 expression (Phan et al., 2011). In addition, the transcription factor MADS29 can regulate the degradation of the nucellus in rice, which regulates the expression of a cysteine protease gene (Jiang et al., 2021; Yin and Xue, 2012).

In plant vegetative tissues, PCD plays a vital role in cell differentiation. Xylogenesis results in tracheal elements that distribute water and solutes across plant tissues. Vascular NAC domain factors control the secondary cell wall regulators MYB83 and MYB46, and the downstream proteases xylem cysteine proteinase 1, 2 and metacaspase9 that execute autolysis in developmental PCD (Jiang et al., 2021; Zhong et al., 2010). Another example of developmental PCD involvement is the root cap. The root cap protects stem cells but must produce new cells to maintain shape and slough old cells through developmental PCD. The transcription factor ANAC033 has been documented to control lateral root cap differentiation and cell death (Fendrych et al., 2014). In *Arabidopsis*, NAC087 and NAC046 induce PCD in the root cap by activating the expression of BFN-1 (Huysmans et al., 2018). Senescence is the final stage of development for many plant tissues and is classified under PCD, controlled by many signalling networks. For example, senescence in *Arabidopsis* leaves is influenced by ethylene and the ethylene insensitive factor-2 (EIN2) activates EIN3, which indirectly regulates BFN-1 to promote the PCD cascade (Daneva et al., 2016; Jiang et al., 2021; Matallana-Ramirez et al., 2013).

Since plant cells are fixed and encased by the cell wall, they are not removable by phagocytosis as in animal cells (Daneva et al., 2016). Instead, autolysis occurs during and after cell death allowing cell clearance like in tracheal element differentiation, although the exact mechanism is less well understood (Daneva et al., 2016; López-Fernández and Maldonado, 2015). Across plant taxa and tissues, there are multiple paths for external or intrinsic signals to trigger plant cells to prepare, initiate and execute PCD depending on the differentiating cell type (Daneva et al., 2016). For example, in rice endosperm development, PCD progresses in a highly organized fashion from the central to the peripheral region in a temporal pattern preceded by mitochondrial membrane permeabilization and VEIDase activity (Kobayashi et al., 2013).

From embryogenesis to leaf senescence, PCD can take place throughout the lifecycle of a plant (Kacprzyk et al., 2011). Different plant PCD processes can show several molecular and morphological characteristics that may indicate that multiple overlapping PCD pathways evolved and exist in one plant species (Kacprzyk et al., 2011). It is predicted that novel *in vivo* plant systems for studying PCD will further advance the field's understanding of plant PCD regulation (Kacprzyk et al., 2011).

1.4. STUDYING PCD IN PLANT MODELS

As sessile organisms, plants rely on PCD to defend and recover from environmental biotic and abiotic stressors such as heat, salinity and pathogens, which ultimately dictate stress tolerance, lifespan and distribution. Building on our ability to manipulate plant

PCD is of growing interest to tackle declining plant health due to climate change and post-harvest losses (Kacprzyk et al., 2021). There is consequently growing momentum in further expanding our understanding of PCD and autophagy in plant stress responses and development (Thanthrige et al., 2021).

Plant papain-like C1A cysteine protease activity has been documented to promote stress induced autophagic-cell death during embryogenesis in microspores of *Hordeum vulgare* (barley). Treatment with cysteine protease inhibitor E-64 delayed PCD and, in turn, enhanced barley embryogenesis after exposure to cold stress (Bárány et al., 2018). *Brassica napus* (Rapeseed) embryogenesis is also associated with metacaspase activity promoting autophagic cell death and decreasing microspore embryogenesis. These findings show that PCD-process-specific proteins are suitable biotechnological targets to improve crop breeding and growth (Berenguer et al., 2021).

Other plant models emerged for studying PCD in economically important species such as Norway spruce (*Picea abies*). This plant is known for its large embryonic mass-suspensor system where the PCD mechanism is easily characterized under the microscope and its genome is sequenced (Bozhkov et al., 2004; Filonova et al., 2000; Nystedt et al., 2013; Reza et al., 2018). Reza et al. (2018) observed the upregulation of *Bax inhibitor-1* (BI-1) in dying suspensor cells of Norway spruce. Inhibiting BI-1 expression by RNAi resulted in induced necrotic cell death indicating a role for BI-1 in maintaining autophagic cell death and preventing runaway necrosis. BI-1 has also been found to interact with Atg6 and influence autophagic flux and hypersensitive response cell death in *Nicotiana*

benthamiana (Reza et al., 2018; Xu et al., 2017). Lesion simulating diseases-1 (LSD-1) protein regulates PCD in *Arabidopsis* and has been shown to regulate stress acclimation in many economically important plants (Bernacki et al., 2019).

Cereals and other monocot seeds like *B. distachyon*, maize, wheat and rice make unique models for studying the consumption of endosperm during embryogenesis (López-Fernández and Maldonado, 2015). In addition, maize and rice serve as models for studying PCD during aerenchyma formation. *Zinnia elegans* and *Arabidopsis* mesophyll cells can also be studied for their ability to differentiate into tracheary elements during xylogenesis (Iakimova and Woltering, 2017).

A difficult hurdle is that plant PCD can occur in a small population of cells surrounded by healthy cells that are difficult to access or probe within a complex developing tissue (Kacprzyk et al., 2011; Reape et al., 2008). Due to the experimental difficulty of probing for inaccessible plant cells initiating developmental PCD, the use of cell suspension cultures has attempted to overcome the complexity of developing plant tissues (Kacprzyk et al., 2011; Malerba and Cerana, 2021). Plant cell cultures possess desirable qualities like uniformity, reproducibility and accessibility of dividing cells (Malerba and Cerana, 2021). Cell cultures also have advantages with tools to study PCD, such as live microscopy and ease of visibility for observations of morphological changes and staining techniques on isolated cells. Successful plant cell cultures of *Arabidopsis*, *N. tabacum* (tobacco) and *Z. elegans* have been used to study PCD processes (Babula et al., 2012, Iakimova and Woltering, 2017; Van Doorn et al., 2011). The trade-off in using cell

culture for studying PCD is the lack of natural cell differentiation. *Zinnia elegans* leaf mesophyll cell cultures can be induced to differentiate into tracheary elements with hormones such as auxin and cytokinin (Iakimova and Woltering, 2017). However, suspension cell experiments is considered *in vitro* study of PCD (Dauphinee and Gunawardena, 2015; Iakimova and Woltering, 2017; Wertman et al., 2012). Cell cultures represent a simplified and controllable system to analyze intracellular features to study the progression of plant PCD, yet different types of PCD can be observed in cultures using similar stress inducers (Malerba and Cerana, 2021; Sychta et al., 2021). Whether a conserved core mechanism exists in whole plants or cell cultures, there has been little research done *in vivo* to understand the changes that take place in organelles during developmental plant PCD (Lord et al., 2011; Lord and Gunawardena, 2011; Wertman et al., 2012).

Single-cell RNA sequencing (scRNA-Seq) has successfully profiled individual single plant cell types in *Arabidopsis* roots and the stems of the non-model aquatic monocot duckweed (Abramson et al., 2022; Denyer et al., 2019). scRNA-Seq can quickly profile many cell types in developing plant tissues. However, some cell types, like quiescent centres in roots, have proven challenging to parse out (Denyer et al., 2019). Although profiling transcriptomes is only one omics approach to help finely unravel core PCD regulators, it shows promise to distinguish PCD genes uniquely critical to plant PCD mediation, even in non-model organisms (Alfieri et al., 2022). Finding a suitable plant model that provides easily accessible differentiating cells undergoing PCD would give the field a valuable system to better resolve truly conserved pathways in plant PCD.

The emerging model system of the lace plant (*Aponogeton madagascariensis*) has the potential to fill this demand for a simplified, accessible *in vivo* plant model to help study PCD (Dauphinee and Gunawardena, 2015; Gunawardena, 2008; Gunawardena and Dengler, 2006). The lace plant generates leaves that form natural holes or perforations through the lamina in a grid-like fashion between the leaf veins through developmental PCD. In addition, these perforating leaves are naturally thin and nearly translucent during early development. They are ideal for live-cell imaging to simultaneously observe adjacent cells destined to survive or die. In the following section of this review, we summarize the appealing features of the lace plant as an emerging plant PCD system and how it can contribute to the current body of knowledge.

1.5. THE LACE PLANT: WHAT WE KNOW ABOUT THIS EMERGING MODEL

Aponogeton madagascariensis is one of 57 species of *Aponogeton* (Aponogetonaceae) but the only one that forms perforations during leaf development (Van Bruggen 1985, 1998). Although a few species with perforated leaves in Araceae also belong to the Alismatales, it is unknown if perforation formation has a common evolutionary origin (Gunawardena, 2008). Therefore, the evolutionary advantage of leaf perforations in aquatic plants is unclear. However, several hypotheses have been proposed, such as promoting thermoregulation, camouflage, defence from herbivores and mechanical protection from water drag (Gunawardena and Dengler, 2006).

The lace plant model system is ideal for studying PCD due to the accessibility and predictability of PCD in developing adult leaves. Lace plant leaves are also thin and translucent, making them ideal for live-cell imaging. Finally, the sterile propagation of whole plants in axenic environments creates an opportunity for pharmacological studies (Gunawardena, 2008).

1.5.1. Predictability of perforation formation

The predictable spatiotemporal nature of PCD within lattice-like veins of mature lace plant leaves presents a unique opportunity to develop a model system to study PCD (Gunawardena et al., 2004). The first 3-4 leaves that emerge from the corm do not produce perforations and are observed to senesce relatively quickly after they reach maturity. The leaves that follow (known as adult leaves) are enriched with a red pigment from the antioxidant anthocyanin and form perforations. The developmental stages of leaves are subdivided into pre-perforation, window, perforation formation, perforation expansion and mature (Figure 1.1.). Newly emerged leaves are in the pre-perforation stage; they are furled and the mesophyll cells of the lamina are full of anthocyanin. The initiation of PCD in window stage leaves is visible as a loss of anthocyanin pigmentation. In the perforation formation stage, the deletion of dying cells begins to progress outwards from the center of the areole, a region framed by longitudinal and transverse veins. During perforation expansion, the hole formed by PCD continues to expand until it reaches 4-5 cells from the veins. At maturity, the perforation is complete and PCD has ceased (Figure 1.1.). Mesophyll cells at the perforation border transdifferentiate into

epidermal cells protected from water loss or infection by a suberin layer (Gunawardena et al., 2007).

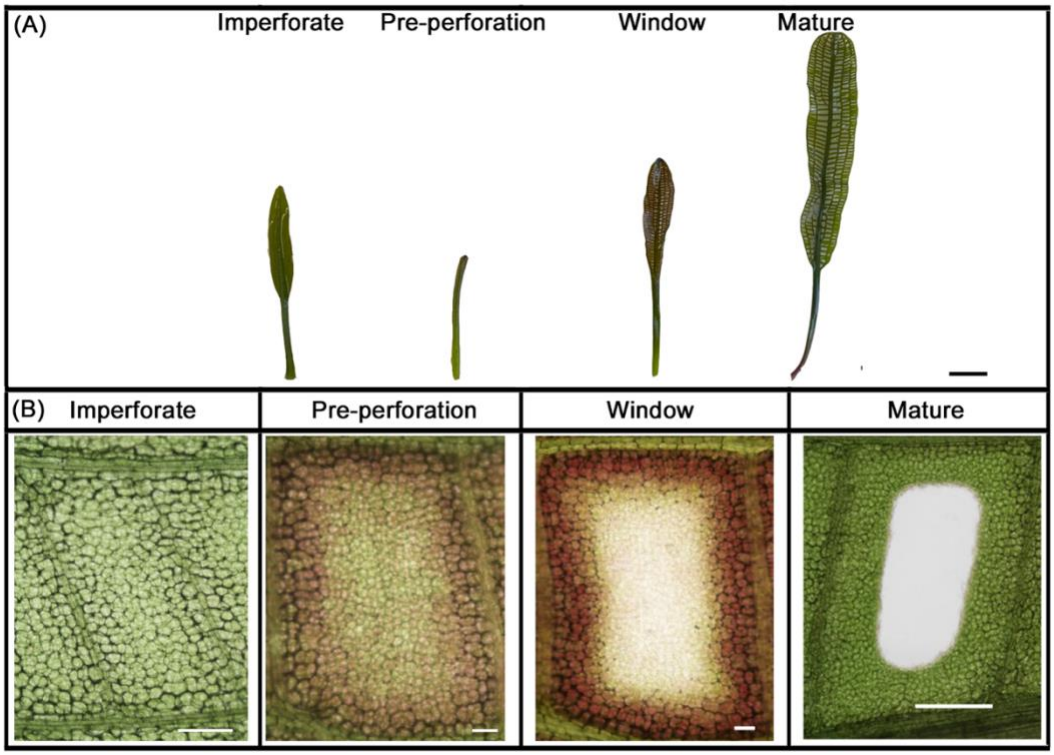

Figure 1.1. Lace plant leaf developmental stages. Individual leaf stages harvested from whole lace plants (A) and micrographs of areoles (B) show the progress of PCD. Imperforate leaves are the first 3-4 leaves to emerge from the corm and they do not accumulate anthocyanin or form perforations. Successive leaves, called adult leaves, emerge as pre-perforation stage furled leaves. As leaves unfurl, they enter the window stage, where anthocyanin is visible only in areas near veins. Next, the centrally located cells (PCD cells) enter PCD to initiate the formation of a hole. In the mature stage, PCD has ceased and a hole is visible within the areoles. The 4-5 cell layers axial to the veins (NPCD cells) survive. *Scale bars*: A, 2 cm; B, 70 µm.

1.5.2. Live cell imaging of lace plant PCD

Aquatic lace plant leaves are ideal for live cell imaging due to their near translucent nature, which has been useful for characterizing the chronological subcellular events that take place during PCD (Figure 1.2.; Dauphinee et al., 2017, 2019; Lord et al., 2013; Lord

and Gunawardena, 2011). Wertman et al. (2012) previously detailed the chronological order of lace plant developmental PCD using a combination of conventional light microscopy, transmission electron microscopy and laser scanning confocal microscopy. Cells central to the areole undergo the first sign of PCD differentiation with the loss of anthocyanin pigment, but the cellular signalling that controls this change is unknown. This change in pigment is also observed in the senescence of petals in *Arabidopsis,* theorized to be a result of changes in selective permeability or pH of the vacuole (van Doorn, 2004; Wertman et al., 2012). Next, in early-PCD cells (EPCD cells, Figure 1.2.C, Online Resource A.1.) comes the loss of chlorophyll along with a decrease in chloroplast size and number (Wertman et al., 2012; Wright et al., 2009), also observed in *Arabidopsis* leaf senescence (Lim et al., 2007). Actin microfilaments reorganize from thin and organized to thicker and disorganized in arrangement before degradation, a standard feature found in early plant PCD cells, including *P. abies* suspensor deletion (Filonova et al., 2000; Smertenko and Franklin-Tong, 2011). This feature is believed to occur to prime microfilaments for being targeted by upstream caspase-like proteases (Wertman et al., 2012). TUNEL positive PCD cell nuclei indicate DNA fragmentation is detectable in lace plant PCD cells during actin degradation, followed by changes in the tonoplast (Wertman et al., 2012). Tonoplast rupture is common in plant PCD processes like tracheary element differentiation (Iakimova and Woltering, 2017), suspensor deletion (Reza et al., 2018) and aerenchyma formation (Ni et al., 2014). Once the tonoplast ruptures, vacuolar aggregates cease their Brownian movement (Wertman et al., 2012), the nucleus condenses, the mitochondrial membrane potential is lost and the plasma

membrane retracts (~20 minutes after tonoplast rupture). Early and late-PCD cells (LPCD, Figure 1.2.C, Online Resource A.2.) have aggregates in their vacuole and there

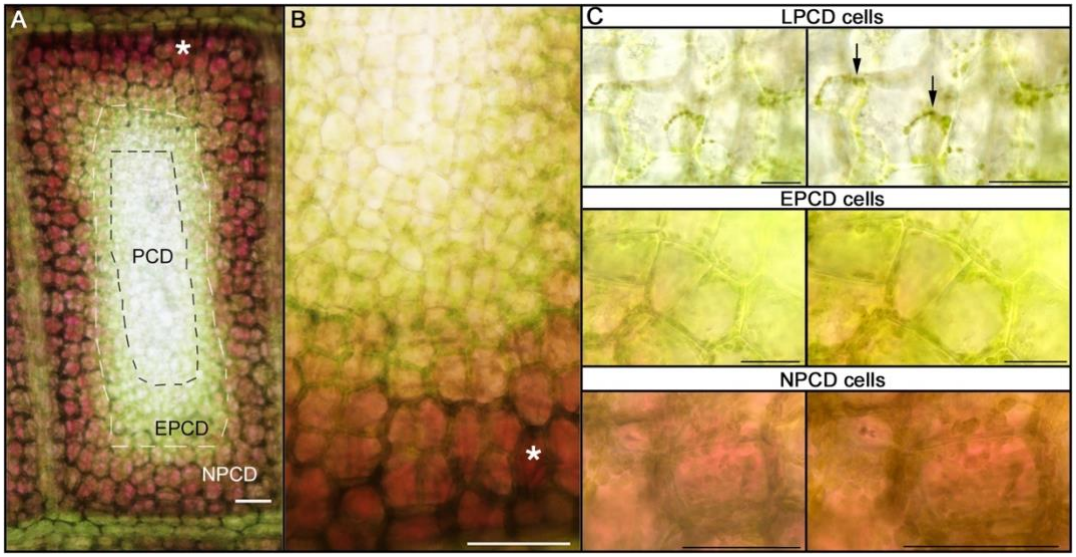

Figure 1.2. NPCD and PCD cell morphologies within window stage leaf areoles. The gradient of anthocyanin and progression of PCD within the window stage leaf (A-B)**.** Non-PCD (NPCD) cells are bound by leaf veins and maintain chlorophyll pigmentation and mesophyll accumulation of anthocyanins (*) leading to survival. Early-PCD cells (EPCD; bound by *white dashed lines*) are devoid of anthocyanin but still contain chlorophyll. Late-PCD cells (PCD; bound by *black dashed lines*) are devoid of any pigmentation and committed to PCD, commonly observed with chloroplast ring formation around the perinuclear space (*black arrows*; C). Supplementary videos of C in Appendix A, Online Resources A.1.-A.3. *Scale bars*: A, 2 cm; B, 70 µm; C, 30 µm.

is evidence that chloroplasts are brought to the vacuole by autophagy (Dauphinee et al., 2019; Wright et al., 2009). In addition, an interesting observation in early and LPCD cells is the formation of perinuclear chloroplast aggregations that are common during developmental PCD (Dauphinee et al., 2014; Lord et al., 2013; Wright et al., 2009). The same process is also observed in tobacco protoplasts and leaf aerenchyma formation in *Typha angustifolia* (Lord et al., 2011; Ni et al., 2014; Wertman et al., 2012; Wright et al., 2009). However, it is unknown if this morphological manifestation is required for

coordinated genetic expression for timely PCD execution or a consequence of vacuolar swelling.

Cells proximal to the vasculature retain their anthocyanin (in the mesophyll layer) and chlorophyll pigmentation, maintaining homeostasis throughout the formation of perforations (non-PCD cells, Figure 1.2.C, Online Resource A.3.). Non-PCD (NPCD) cells maintain function during perforation formation providing a suitable control group to test and observe intracellular changes in EPCD and LPCD cells. This natural gradient of PCD is accessible in a single field of view, a distinct advantage over other plant PCD systems.

1.5.3. Sterile culture system

Although lace plants can be maintained in aquariums, they are propagated in sterile controlled environments to avoid environmental disturbances and to maintain a consistent leaf morphology and PCD pattern (Dauphinee and Gunawardena, 2015). This transfer to a controlled sterile environment represents a trade-off where lace plant leaves are generally smaller than aquarium plants. Lace plants are propagated as cleaned corms under established protocols in G47 magenta box containers (Figure 1.3.) or 1-litre glass jar vessels (Figure 1.4.). Cultures are supplemented with Murashige and Skoog (MS) media containing 1% agar and 3% sucrose (Figure 1.3.) and grown at 24°C under 12h light:12h dark cycle without additional hormones to promote growth. Pharmacological whole-plant treatments in axenic cultures make for consistent conditions for optimal (i)

protein and RNA extractions and (ii) monitoring leaf growth and morphological changes (Figure 1.3.). Whole plants can therefore be propagated and subjected to different types of treatment for weeks without infection, signs of necrosis, or accumulation of

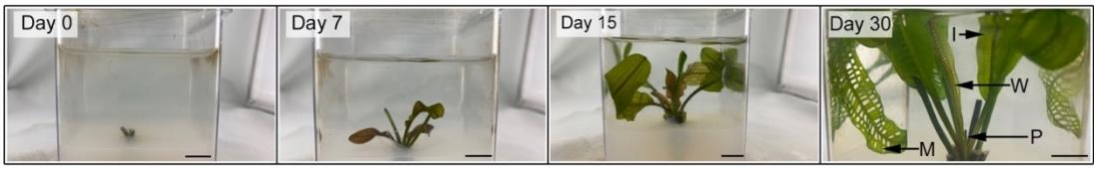

Figure 1.3. Whole plant growth under axenic conditions in 150 ml magenta boxes. New corm embedded in solid 1% agar MS media in magenta boxes (Day 0). Leaves emerge in a heteroblastic series beginning with imperforate leaves (I), followed by adult leaves emerging as furled pre-perforation leaves (P), which will develop into window leaves (W) where PCD is active and anthocyanin gradients are visible. By day 30, at least one of each developmental leaf stage is present in the culture. At least three perforated mature leaves (M) are visible, indicating that the whole plant is ready for pharmacological experimentation. *Scale bars*; 2 cm.

Figure 1.4. Tracking newly furled leaf growth in 1-litre glass culture jars. Furled adult or pre-perforation leaves (*black arrows*) emerge from the corm successively after 3-4 imperforate leaves have already emerged (Day 1). The laminar tissue of pre-perforation leaves has a red pigmentation from anthocyanin. Pre-perforation leaves grow and develop into window stage leaves (Day 4; *black arrow*) when leaves have unfurled; anthocyanins in the middle portion of the areoles start to fade and a visible gradient of red pigment remains. *Scale bars*; 2 cm.

biproducts. In comparison, embryos of Norway spruce used to study suspensor deletion PCD need to be stimulated for development with growth regulators, a limitation the lace plant system does not experience (Högberg et al., 1998). The constant recycling of removing overgrown shoots and cleaning lace plant mother corms before transplanting to

a new culture magenta box provides highly repeatable experiments in terms of reducing genetic variation.

1.5.4. Summary of molecular lace plant PCD findings to date

Twenty years of lace plant research has provided the plant PCD community with a series of observations of events that characterize lace plant leaf remodelling on a morphological, biochemical and molecular level. The inhibition of ethylene biosynthesis produces leaves with fewer perforations (Dauphinee et al., 2012; Rantong et al., 2015). Downstream of ethylene, caspase-1-like activities triggered by the release of a mitochondrial signal have been postulated but not identified (Lord et al., 2013). The inhibition and promotion of lace plant Hsp70 in early developing leaves also affect anthocyanin levels, caspase-like protease activity and the formation of perforations. However, the exact mechanism of its connection to PCD remains to be elucidated (Rowarth et al., 2020). The morphological and cellular changes during lace plant leaf development are well categorized. However, the genetic control underpinning lace plant PCD remains elusive (Rantong et al., 2016; Rantong and Gunawardena, 2015), partly due to a lack of genetic information for the Aponogetonaceae family. However, advancements in comparative RNA sequencing (RNA-Seq) analysis between PCD and NPCD-like cells in other plants has helped profile key DEGs that resemble PCD regulators (Rowarth et al., 2021).

To date, lace plant experiments have taken advantage of sterile culture systems, long-term live cell imaging, protoplast extractions, successful western blotting protocols and RNA extractions from whole leaves or cell populations using laser capture microdissection (Figure 1.5., Online Resource A.4.; Rowarth et al., 2020, 2021). Currently, the lace plant model system is limited from reaching its full potential due to the lack of genomic data, mutants and a robust protocol for genetic transformation. For

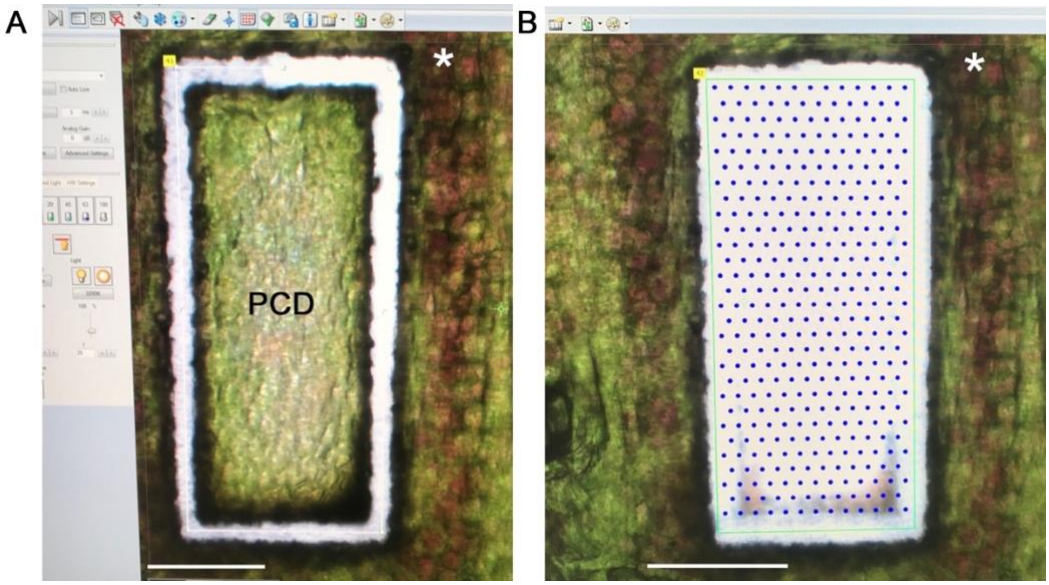

Figure 1.5. Laser capture-microdissection and catapult of leaf tissue. The user interface of the Zeiss Laser capture micro dissector shows fresh window stage leaf tissue under 4X magnification. Laser cuts around PCD cells (PCD), separating them from healthy NPCD tissue (*) (A). The dotted region selects for desired tissue to be catapulted by air pulse into a microcentrifuge tube (B). *Scale bars:* 150 µm. Example laser protocol shown in Appendix A, Online Resource A.4.

example, *Agrobacterium tumefaciens* strain GV2260 has been used to transform eudicots and monocots and, in optimal environments, was ~25% successful in transforming lace plant shoot apical meristem (SAM) explants (unpublished data, Gunawardena lab 2017). On the other hand, callus tissue transformation produced a limited number of regenerated leaves, making it a less viable process than SAM transformation.

1.6. MODEL STATUS FOR THE LACE PLANT

1.6.1. New avenues to explore in lace plant PCD knowledge

Even with the established protocols for propagation and live cell imaging, the lace plant system still needs improvement in terms of protocols to expand genomic and molecular information. For example, elucidating essential genes that regulate lace plant PCD was challenging due to little prior molecular work, sequencing data, or valid transformational protocols. Recently, progress was made to tackle this issue through a *de novo* RNA sequencing project of the lace plant leaf transcriptome. In addition, optimizing RNA and protein extraction and detection workflows have enabled lace plant research to expand into the characterization of proteins.

1.6.2. Building the lace plant to model status

Dauphinee and Gunawardena (2015) highlighted the new avenues that can now be undertaken with the lace plant system. These future avenues were highlighted in the context of comments made by Mandoli and Olmstead (2000), who stated the importance for researchers to be transparent and communicate the limitations of developing their respective model systems. In the next sections, we discuss the latest findings of our lace plant PCD research and how we have addressed a few of the roadblocks to date.

1.6.3. An optimized protocol for RNA extraction

Complex secondary biproducts like phenolics and polysaccharides can potentially lead to suboptimal RNA extractions in plant tissues for downstream molecular applications like RNA-Seq (Moser et al., 2004). The removal of the midrib, which contains laticifers and secondary compounds, has dramatically increased the quality of extracted RNA for applications like DNA-amplified fragment length polymorphisms (cDNA-AFLP) (Rantong et al., 2016). For our global transcriptome comparison of lace plant leaf stages (Rowarth et al., 2021), we utilized a ReliaPrep RNA Kit from Promega for cells and tissues to produce mRNA samples with consistent RIN numbers (RIN ≥ 6.5) and sequencing quality scores (Q30) for extractions from leaves at different stages of development as well as NPCD and PCD cells before cDNA conversion.

Single-cell sequencing (scRNA-Seq) will further advance our work to characterize cell types within the NPCD and PCD cell populations. Many scRNA-Seq studies have resolved root, meristem, or inflorescences to characterize specific cell type trajectories (Abramson et al., 2022; Denyer et al., 2019). The use of scRNA-Seq in the lace plant system may provide an opportunity to obtain spatial gene expression change information during cell differentiation from mesophyll cells to PCD cells on a developmental time scale similar to what was achieved in duckweeds. Our well-established RNA extraction protocol using axenically-grown whole lace plants sets the foundation for future RNA-Seq experiments using pharmacological treatment. Performing differential gene expression analysis focusing on genes involved in autophagy, heat shock proteins, or

caspase-like protease activity between NPCD and PCD cells will help identify critical genes involved in lace plant PCD regulation.

1.6.4. Transcriptome analyses of lace plant leaves, NPCD and PCD cells by RNA-Seq

Based on our comparative analysis, NPCD and PCD cells differentiate by balancing plant hormone and transcription factor activities that both promote or inhibit the PCD pathway in lace plants. Alternative splicing (AS) of mRNA variants occurs with 20-30% of genes in *Arabidopsis* and *O. sativa,* demonstrating a critical role of AS in gene expression (Baralle and Giudice, 2017; Campbell et al., 2006; Gassmann, 2008; Wang and Brendel, 2006) and promotion of plant development and disease resistance (Reddy et al., 2013). Furthermore, AS events linked to Hsp81-2, 1-aminocyclopropane-1-carboxylic acid synthase and WRKY33 have been demonstrated to mediate stress-induced PCD in *Vitis amurensis* (Xu et al., 2014) and in *Gossypium davidsonii* under salt stress (Zhu et al., 2018a). These genes are also differentially expressed between NPCD and PCD cells of the lace plant. The results supports the notion that quantifying AS profiles coupled with a LC/MS study across the leaf and cell type transcriptomes would prove helpful in identifying mRNA compositions that may mediate lace plant PCD.

Our *de novo* RNA-Seq analysis has generated ~22,000 non-redundant protein-coding genes, but this transcriptome's completeness is unknown. In order to update these gene annotations, there must either be an improved assembly or an improved gene prediction protocol. One possibility includes single-molecule long-read genome sequencing using

PacBio Iso-Seq and Nanopore RNA-Seq to identify new genes and isoforms to revise previously sequenced isoforms (Van Bel et al., 2019; Wang et al., 2016). The use of complementary sequencing methods will serve to increase the completeness of the whole lace plant genome (Van Bel et al., 2019).

1.6.5. An optimized protocol for protein quantification

Protocols for protein extraction, detection via western blotting and normalization by Ponceau S stain have been optimized for the lace plant and led to the characterization of several protein levels in whole leaves by Dauphinee et al. (2017) and Rowarth et al. (2020). Individual leaves at different developmental stages from plants used in pharmacological studies provide enough biomass to yield sufficient protein for detection via western blotting. However, distinguishing protein levels between NPCD and PCD cells has been challenging. Laser microdissection of NPCD/PCD cells from one leaf does not provide enough material for further proteomic or mass spectrometry studies, but pooling biomass across several leaves may be helpful. Focusing on optimizing conditions for laser capture microdissection on larger leaf areas will help characterize which native proteins are synthesized between the NPCD/PCD cell boundary in lace plant leaves.

Protein subcellular localization using confocal microscopy has been useful within the NPCD/PCD cell gradient to characterize proteins involved in PCD. In addition, monoclonal antibodies have been used successfully to probe for several proteins (Dauphinee et al., 2019 and Online Resources A.8. and A.9.) within fixed cells. However,

confirming quantification levels between PCD and NPCD cells may prove challenging given that PCD cells are more susceptible to the permeation of stains and antibodies than NPCD cells.

1.6.6. The lace plant genome and genetics

The lace plant model has advantages over *Arabidopsis*, *L. minor* and *Z. mays* for studying plant developmental PCD. However, more information on the genome must be obtained before it can be established as a *bona fide* model. A sequenced "reference genome" of the lace plant will provide a complete reference library for future comparisons with other plant species. The *Aponogeton* group of genomes still falls under the "dark clades" of green plant life that possess little to no genomic information (Kress et al., 2022). At the same time, the gold standard approach to build an optimal reference genome is changing and future technologies will allow chromosome and structural scale sequence information (Ballouz et al., 2019; Kress et al., 2022). The goals of genome collection projects like The Earth BioGenome Project (EBP) and Ten Thousand Plant Transcriptomes Initiative (10KP) are to produce genome sequences that represent each plant taxonomic family (Cheng et al., 2018; Lewin et al., 2018). Here we will briefly give an overview of the current understanding of the lace plant and *Aponogeton* genome information.

Little is known about the genomes of the Aponogetonaceae family. However, Šmarda et al. (2014) estimated ploidy level, genome size and chromosome counts in a few *Aponogeton* species (Table 1.2.; Šmarda et al., 2014). The nature of polyploidy and

euploidy of aquatic angiosperms (Les and Philbrick, 1993) and monocots or the differences in cultivars used may explain the wide range in ploidy levels and genome size across Aponogetonaceae. As well, the inconsistency of methods used to extract genomic material and karyotype from lace plant tissue for detection may be a source of error (Šmarda et al., 2012, 2014). Additionally, the plastomes of five different *Aponogeton* species have been analyzed and found to be suitable protein-coding genes for future intrageneric barcoding (Mwanzia et al., 2020). The Gunawardena lab is currently estimating the genome size of three different lace plant isolates using flow cytometry in collaboration with Drs. Sonja Yakovlev, Université Paris-Sud, Béatrice Satiat-Jeunemaitre, Institut de Biologie Intégrative, Michaël Bourge and Nicolas Valentin, Imagerie-Gif, Plateforme de Cytométrie, France. Even though high ploidy levels and high repeat genomes harm the efficiency of NGS genome assembly, this can be overcome by using self-fertilized generations or novel bacterial artificial chromosome (BAC) libraries (Polashock et al., 2014; Unamba et al., 2015).

Table 1.2. Individual genomic information of select members of Aponogetonaceae compared to the lace plant. All data were adapted from (Šmarda et al., 2014).

Aponogetonacea species	Ploidy level	Genome size (Gb/2C)	2N chromosomes
Aponogeton madagascariensis (lace plant)	10X	5.083	78
Aponogeton undulatus			70-74
Aponogeton natans			80
Aponogeton crispus			32
Aponogeton longiplumulosus	10X	5.357	78

There is an overall gradual increase in the number of high-quality plant genomes to study (Van Bel et al., 2021). Each update of genome annotations improves the completeness of assemblies, as shown in the *Theobroma cacao* and *Cucumis sativus* genomes (Sun et al., 2021). The interest in plant genomes has been driven by the economic importance of crops like *Z. mays,* even though this plant is one of many of the more complex and challenging crop genomes to assemble and perform comparative analyses (Van Bel et al., 2019). Plant-specific characteristics such as cell walls and production of secondary metabolites can make DNA extraction more challenging than animal cells (Friar, 2005). Also, autopolyploidy, allopolyploidy and proportion of transposon elements are correlated to large genome sizes (Lee and Kim, 2014; IWGSC et al., 2018). Examples of sequencing multiple strains into a pan-genome, as in *O. sativa* and *B. distachyon,* increases the discovery of assembled genes that cannot be achieved by using a single reference genome alone (Gordon et al., 2017; Zhao et al., 2018). Generating a pan-genome for a species is one way to capture variation and complexity fully; this may be one feasible approach to achieve *de novo* genome sequencing of the lace plant for which we have multiple strains.

To fully characterize the lace plant as a new model species, its genome must be sequenced to gain a better understanding of the biological processes being studied. The past decade has seen significant advances in sequencing technologies that have expanded the plant genomics field. As a result, genomes of many model and non-model species have been sequenced (Davis, 2004). The genomes of approximately 788 plant species in reference or draft form (613) have been published (Bolger et al., 2017; Schmidt et al.,

2017; Sun et al., 2021; Van Bel et al., 2021). Most of these genomes are in favour of those species with few chromosomes and small genome sizes thereby minimizing complexity and sequencing costs but moving toward bigger genomes like Norway spruce, Rapeseed, and *Paris japonica* (Table 1).

Although we have not attempted to sequence the lace plant genome to date, getting an overview of how complex monocot genomes have been sequenced would benefit our plan of attack. The duckweed family (*Lemnaceae*) has benefited from NGS to develop duckweed transcriptomes and genomes. The small genomes of *S. polyrhiza* (158 Mb), *L. minor* (481-800 Mb) and *L. gibba* (450 Mb) have been assembled. In contrast, the bigger genomes of *Landolita wolffiella* and *Wolffia* genus are not yet available due to the higher number of repeat elements and genome size (An et al., 2018).

Short-read sequencing for draft genome assemblies is useful for genome estimates and identifying repeat content but not for chromosomal and structural details (Kress et al., 2022). The sequencing hurdles of repetitive regions, long retrotransposons and large genomes render short-read NGS less effective for genome assembly resulting in fragmented assemblies (An et al., 2018; Phillippy, 2017). Long-read technology such as PacBio single-molecular real-time (SMRT) is recommended to overcome this issue and produce full-length transcripts. Oxford nanopore sequencing can also generate up to 200 kb reads that are easier to assemble and provide coverage over transposons in complex genomes. Complex genomes of important crops like maize, wheat, *Sorghum* and barley

may be challenging to assemble (Table 1.1., Haberer et al., 2016) but are nonetheless invaluable tools for crop research and breeding.

The manipulation of genomes and advances in sequencing technologies allow the accession of complex genomes. In the coming decade, low-cost long-read genomic sequencing and improved scaffolding techniques fortify the notion that large genomes will be less of a barrier to producing a reference genome (Jiao et al., 2017). The combined use of short-read, long-read, single-cell read, chromosome conformation capture scaffolding (Hi-C) and Strand-Seq (Sun et al., 2021; Kress et al., 2022) increases the quality of polyploid assemblies. Hi-C data can scaffold long-read contigs to make pseudochromosomes. Examples of improved polyploid genomes include *Chenopodium quinoa* (Jarvis et al., 2017), *G. arboretum* (Huang et al., 2020) and *Arachis hypogaea* (Chen et al., 2019). Complementary sequencing methods can be used to tackle large complex genomes, but the assembly methods often fail to produce a complete assembly due to lack of cooperation, or the objective complexity of the system is underrepresented. In addition, standard assembly pipelines developed for reference organisms may not be scalable for other complex genomes like the lace plant (Sun et al., 2021). An integrated strategy of multiple sequencing approaches and flow-sorted individual chromosomes sometimes reduces complexity.

As reviewed by Kress et al. (2022), there is a need for a gold-standard sequencing and annotation protocol for these 'dark clade' reference genomes like *Aponogeton*. With a large and complex genome, the first plan of attack is to assess gene space using short-

read sequencing (Kress et al., 2022). The chromosomal level genome assemblies will need advanced technological resources for plant groups with large genomes. As sequencing, assembly and annotation costs fall and long-read sequencing improves, we are confident that future chromosomal level genome assembly will be feasible for plants with a large genome (Kress et al., 2022).

1.6.7. Gene editing

The establishment of a gene editing method and creating mutant lines to study gene function is needed for the lace plant to reach comprehensive model status along with *Arabidopsis* (Holland and Jez, 2018). Successful transformation protocols of monocots like duckweeds have been achieved. The introduction of exogenous genes, the regeneration of transgenic cells and the selection for and regeneration of transgenic plants have been successfully achieved in duckweeds. Such methods include *Agrobacterium*-meditated transformation, microinjection, electroporation and microprojectile bombardment (Yamamoto et al., 2001; Yang et al., 2020). Other monocot transformation examples include switchgrass (*Panicum virgatum*) calli derived from seeds with *A. tumefaciens* EHA105. Additionally, *S. viridis* can be transformed using a floral dip technique using a suspension culture of tobacco extract and AGL1 *A. tumefaciens* and acetosyringone (Martins et al., 2015). Oil palm (*Elaeis guineensis*) protoplasts can be transformed using polyethylene glycol (PEG)-mediation to generate non-chimeric callus with a high degree of success (Masani et al., 2014).

Agrobacterium-mediated transformation for monocot plants has had limited success due to their inability to counter wound damage with the release of phenolic compounds. However, exogenous supplementation of monocot systems with phenolics such as acetosyringone during transformation improves the efficiency of *vir* gene expression during co-cultivation in rice callus (Xi et al., 2018). Successful transformation of callus tissue harvested from a mother corm, confirmed by the detection of GFP fluorescence in newly-formed lace plant shoots was achieved using 5% sucrose, 100 µM acetosyringone, 10 µM aminoethoxyurinal glycine, 15 µM phloroglucinol pH 5.5 over a period of 12 weeks with a ~25% success rate (unpublished data, Gunawardena Lab 2017). Transformation with the EHA105 strain of *Agrobacterium* was also successfully achieved in seven different duckweed species with varying levels of efficiency (0.14-82.5%) (Liu et al., 2019).

One can also manipulate the accumulation of transcripts using RNA interference (RNAi) or over-expression. Delivering double-stranded RNA into plant tissue using laser-assisted RNA delivery was successfully achieved with citrus leaves (Killiny et al., 2021). However, RNAi methods can be flawed due possible off-targeting effects and may not be the optimal mode of studying gene function (Kola et al., 2015; Senthil-Kumar and Mysore, 2011). For a more stable transformation and a better understanding of genes in Aponogetonaceae, a CRISPR/Cas9 system for genome editing should be developed. CRISPR/Cas9-mediated targeted mutagenesis has been successfully optimized in the duckweed *L. aequinoctialis* via EHA105 *Agrobacterium* transformation with a rice ubiquitin promoter within the vector (Liu et al., 2019). Liu et al. (2019) discussed that the

increased transformation efficiency in *L. aequinoctialis* over *L. minor* was due to an optimized sonication and vacuum filtration protocol. This method has also been successful in cowpea (*Vigna unguiculata*), chickpea (*Cicer arientinum*) and banana (*Musa* cv. AAB) (Bakshi et al., 2011; Indurker et al., 2010; Subramanyam et al., 2011) and could also be used to improve lace plant transformation. One limitation to establishing CRISPR/Cas9-mediated transformations is the occurrence of chimeric cells within mutant plants.

Additionally, the CRISPR/Cas9 system can require labour-intensive trial and error runs to successfully generate large constructs and multiple cassettes (Wang et al., 2017). Although transformation has been successfully achieved in lace plant callus tissues, isolated SAM culture transformation was characterized by a lower induction and faster regeneration time (unpublished thesis work; Dauphinee, 2017). Consequently, direct transformation of window stage leaf areoles may be more practical than generating transformed leaves from callus tissue. Advanced gene editing like CRISPR-Cas9 or 13 has many potential benefits for studying PCD control in the PCD/NPCD gradient of lace plant leaves, including (i) recovery or silencing of functional proteins, (ii) promotion of specific AS events, or (iii) tracking the shuttling of specific RNA inside cells with fluorescent detection. However, one of the limitations of using this biotechnology in studying plant PCD is the unknown biochemical effects on the Cas9/13 catalytic domain within eukaryotes (Wang et al., 2017).

Aquatic monocots like duckweed and *Lilium tenuifolium* (water trumpet) have been transformed successfully (Dauphinee and Gunawardena, 2015; Qi et al., 2014; Yamamoto et al., 2001), but the lace plant is the only member of Aponogetonaceae where transformation has been attempted (Dauphinee and Gunawardena, 2015). The biosynthesis of ethylene and phenolics may be contributing to inhibiting the success of our transformation constructs into lace plant SAMs. Treating the inoculation medium with ethylene biosynthesis inhibitor 2-aminoethoxyvinyl glycine (AVG) and phloroglucinol for lace plant transformations has yielded minor but not significant improvements (Dauphinee et al., 2012; Lacroix et al., 2011).

Plant cell suspension cultures can be a more reliable system for more straightforward transformations using individual cells (Santos-Ballardo et al., 2013). Therefore, establishing an isolated callus or protoplast protocol for the lace plant would be more efficient for single-cell-based studies. Also, lace plant laminar protoplast protocols could provide high-throughput inspections of lace plant PCD cell signalling involving hormones, secondary metabolites and environmental stressors (Dauphinee and Gunawardena, 2015; Nanjareddy et al., 2016). Lord and Gunawardena (2011, 2010) established a protoplast isolation protocol using mature leaf stage lamina excised from the midrib while perforations are still expanding. Using lace plant protoplasts Lord and Gunawardena (2011) were able to characterize morphological differences and commonalities between developmental and heat induced PCD and necrosis.

1.6.8. Secondary metabolites in the lace plant

Secondary metabolites were once considered unfunctional end product metabolic plant waste until they were increasingly investigated for their pharmaceutical and industrial uses for humans and metabolism as well as defense for plants (Anulika et al., 2016; Li et al., 2020a). Aquatic plants can produce an array of compounds like peptides, alkaloids, flavonoids, phenolics, tannins and essential oils that are commercially important (and medicinally active) in antibiotics, antioxidants and anti-inflammatory and anti-cancerous compounds, to name a few (Saxena et al., 2021). Anthocyanins are potent antioxidants and secondary metabolites that play a central role in regulating PCD during lace plant leaf development (Dauphinee et al., 2017) and are the most abundant flavonoids in plant vacuoles (Pervaiz et al., 2017; Gunawardena et al., 2021). Lace plant leaf extracts also have been shown to have anti-cancer properties (Gunawardena et al., 2021). Due to the role of anthocyanin in lace plant PCD cell differentiation and leaf remodelling, studying lace plant anthocyanin composition is of particular interest. With established protocols for extracting anthocyanins from lace plant leaves, more resources will be directed towards characterizing secondary metabolite synthesis in lace plants in the future.

Even without complete genomic information, some non-model plants possess significant research value by characterizing the biosynthesis of their secondary metabolites (Unamba et al., 2015). For example, ginsenosides were isolated from ginseng (*Panax*) to identify the mode of action of their respective anti-cancerous, anti-diabetic and anti-inflammatory uses (Choi et al., 2005; Haralampidis et al., 2002). The abundance and composition of

secondary metabolites reflect the environmental challenges organisms are exposed to during their evolutionary history. Our knowledge of secondary metabolites was poor due to the lack of understanding of their biosynthesis and regulation. NGS, bioinformatics and metabolomic analyses have been used to identify putative genes involved in secondary metabolite biosynthesis. For example, *Podophyllum hexandrum* and *Podophyllum peltatum* tissue analysis identified enzymes underpinning the synthesis of podophyllotoxin (a chemical scaffold for anti-cancer drugs) and mass spectrometry identified the anti-cancerous aporphine alkaloid pathway in *Podophyllum* species (Marques et al., 2014). Similarly, transcriptomic and proteomic dual analysis characterized the final steps of the monoterpenoid indole alkaloid pathway in *Catharanthus roseus,* which led to the horizontal characterization of the biochemical pathway in *N. benthamiana* and isolation of similar compounds (Miettinen et al., 2014).

As outlined above, the anthocyanins in lace plants may have intrinsic medicinal, antioxidant and anticancer properties (Gunawardena et al., 2021). Crude anthocyanin extracts from window stage leaves have been shown to inhibit the growth of MDA-MB-231, OVCAR-8 and SKOV-3 cancer cells and induce apoptotic behaviour in the former (Gunawardena et al., 2021). Although this was the first evidence of anti-cancer properties in lace plant leaf extracts, it is still unclear which flavonoid compounds are critically responsible for these properties and what their role is specifically in PCD control. RNA-Seq analysis of leaf stages, PCD and NPCD cells revealed that anthocyanin biosynthesis enzymes are differentially expressed across leaf development and NPCD cell differentiation (Rowarth et al., 2021). In pre-perforation and window stage leaves, where

anthocyanin pigments are most abundant, mRNA accumulation is highest for genes coding for enzymes involved with enzymes anthocyanidin-3-O-glucosyltransferases, anthocyanidin-5,3,-O-glucosyltransferases, anthocyanin-5-aromatic acyltransferases and anthocyanidin-3-glucoside-6-hydroxycinnamoyl glucoside. Transcriptomic and proteomic approaches in non-model plants continue to generate functional genomic resources and provide an understanding of the molecular mechanisms underlying plant secondary metabolite biosynthesis (Unamba et al., 2015). A gene knockdown study of these highlighted enzymes will help elucidate which anthocyanins play a role in lace plant development and (or) suggest a mode of action with regard to medicinal uses.

1.7. PERSPECTIVES AND APPLICATIONS OF LACE PLANT PCD

The lace plant has been recognized as a unique emerging model for studying perforation formation during leaf development (Gunawardena, 2008; Kacprzyk et al., 2011, 2021). The fact that this phenomenon is such a rare occurrence in the plant kingdom begs the question of whether or not the lace plant can be used as a PCD model compared to established plant PCD models.

PCD is ubiquitous across plants, but the molecular machinery that regulates its response, timing and execution can vary across models and PCD processes. Many genes and signalling networks are increasingly being characterized across established plant models from transcriptional networks, nucleases and protease cascades. What remains in question

is finding (if any) what subcellular genetic, morphological, proteomic, or biochemical changes are common or unique across plant PCD processes and plant groups.

Using the unique cell death gradient to observe morphological variability in adjacent and accessible PCD and NPCD cells simultaneously in one field of view will contribute to the ongoing debates on cell death classifications (Lord and Gunawardena, 2011; Wertman et al., 2012). Lace plant PCD may elucidate regulatory pathways better manipulated in agriculturally important species processes like leaf senescence which share intracellular morphological changes similar to PCD cells during perforation formation (Wertman et al., 2012; Wright et al., 2009). The potential anti-cancerous properties of anthocyanins from window stage leaves may contribute to biomedical applications and our knowledge of how they can regulate PCD in other plant systems (Gunawardena et al., 2021). Previous cross-species experiments show that mammalian pro-death Bax and anti-death Bcl-XL affect PCD and tobacco stress resistance (Lacomme and Santa Cruz, 1999; Qiao et al., 2002), suggesting common conserved modulators in the PCD process exist (or once did) between animals and plants. Testing functionally similar genes of interest between animal and plant models and the lace plant system will likely lead to a broader, more universal understanding of PCD regulation.

The Bcl-2-associated athanogene (Bag) protein family is ubiquitous with homologs existing in yeast, humans, metazoans and plants, all containing an evolutionarily conserved BAG domain (Kabbage et al., 2017). Animal Bags are involved with Bcl-2, promoting antiapoptotic activity (Kabbage et al., 2017; Takayama et al., 1995) and act as

co-chaperones to influence Hsp70 protein folding (Lüders et al., 2000; Takayama et al., 1997). *Arabidopsis* Bag protein homologs can be organized into those which possess a ubiquitin-like motif (UBL; Bag1-4) and those which possess a calmodulin-binding motif (CaM; Bag5-7) (Doukhanina et al., 2006). Plant Bags have been shown to mediate plant stress responses, PCD and autophagy (Kabbage et al., 2017; Li et al., 2016a; 2016b), but their direct function in relation to plant Hsp70s is not elucidated (Kabbage et al., 2017). Given that several Bag homologs are differentially expressed during lace plant leaf development (Rowarth et al., 2021) and that Hsp70 plays a role in lace plant PCD (Rowarth et al., 2020), we are currently investigating the functionality of Bag protein activity in lace plant *in vivo*. The work will help build on our understanding of Bag proteins and their conserved nature across animal and plant PCD.

Furthermore, testing NPCD and PCD cells under different environmental stressors will improve our understanding of morphological variations in response to different thresholds of cell death signals. These comparisons will help clarify the morphological classifications of plant PCD (Minina et al., 2021; Van Doorn et al., 2011). The involvement of organelles in PCD regulation is also beginning to be more understood (Van Aken and Van Breusegem, 2015). How and when mitochondria and chloroplasts cooperate during PCD is being investigated. For example, the dynamics of perinuclear aggregation of chloroplasts in early PCD cells (which does not occur under heat, HCl or NaOH stress induced PCD) provide an opportunity to investigate if this phenomenon plays a vital role in lace plant PCD (Dauphinee et al., 2014; Wertman et al., 2012).

The first sign of PCD initiation within the areole is a reduction in the amount of anthocyanin pigmentation. However, whether this is the result of anthocyanin being shuttled out of the vacuole, degradation, or direct pH changes in the vacuole is not clear (Dauphinee et al., 2017; Wertman et al., 2012). Using adjacent NPCD and PCD cells, future experiments involving the manipulation of anthocyanins through knockdown experiments will help determine if specific anthocyanin compounds play an important role in plant PCD cell differentiation.

Another important area of plant PCD research is unravelling the role of autophagy. Autophagy has been documented to play a dual role in cell death by promoting or inhibiting PCD across different critical processes (Dauphinee et al., 2019; Minina et al., 2014a). In lace plants, autophagy is active in NPCD and PCD cells and autophagy-related genes Atg8, Atg16 and Atg18 are differentially regulated at different stages of leaf development and under influence of autophagy modulators (Dauphinee et al., 2019; Mishra et al., 2017; Rowarth et al., 2021). Furthermore, lace plant autophagy modulation using promoter rapamycin and inhibitor wortmannin has been found to significantly affect anthocyanin accumulation and perforation formation (Chapter 4, manuscript under review). Within plant tissues, the role of autophagy is even more cryptic as it can influence PCD in a cell type and in a time-dependant manner, as seen in root cap cells in *Arabidopsis* (Feng et al., 2022). The tractable model of differentiating cells within the lace plant gradient can be employed to characterize the role of autophagic flux during leaf development.

The transition of an ancestral aquatic plant to land and back to an aquatic environment in aquatic monocots may have resulted in a gain/loss of genetic components related to immunity, drought stress, or even mechanisms for PCD for underwater adaptations. Aquatic monocots such as lace plants, duckweeds and aquatic eudicots *Utricularia* species (bladderworts and *Lotus*) represent an opportunity to identify PCD pathways convergently lost or gained in aquatic species compared to their terrestrial ancestors, as demonstrated in plant immunity pathways (Baggs et al., 2020).

Arabidopsis has been used successfully for biological, computational modelling approaches for differential equation-based, kinetic and genome-scale metabolic mapping (Holzheu and Kummer, 2020). As the plant community moves toward using new modelling techniques to solve other significant biological questions, the lace plant PCD gradient would be an excellent example for developing computational models to predict which cells will undergo PCD and determine how key regulators of PCD are integrated to precisely coordinate cell death.

1.8. SUMMARY AND CONCLUSIONS

The lace plant system has contributed to the elucidation of PCD processes shared across species, but also unique features can also help further elucidate PCD (Dauphinee et al., 2014; Lord and Gunawardena, 2011; Wertman et al., 2012; Wright et al., 2009). Recently, there have been advances in sequencing draft genomes for plants with large complex genomes such as Norway spruce, *Paris japonica* and transformation protocols

for the recalcitrant species like Rapeseed (Li et al., 2021). Combining these new tools will facilitate the assembly of a draft genome for the lace plant and functional analyses of genes essential for leaf morphogenesis. The knowledge gained from this cellular gradient of PCD and anthocyanin pigmentation within developing leaves will leverage the lace plant as a more widespread model for studying plant PCD processes.

The lace plant transcriptome has provided important information on the molecular mechanisms and genetics underpinning developmental PCD. Sequencing genomes across Aponogetonaceae members and the lace plant will be critical to perform comparative genomics and tease apart what controls leaf perforation formation in lace plants but not other members of *Aponogeton*. Additionally, the future of lace plant research should utilize CRISPR/Cas9, single-cell transcriptomics and proteomics to characterize more precisely the roles of candidate genes that may control plant PCD.

The PCD process in lace plants has been studied for two decades and advances in the NGS will likely continue to further our understanding of this complex but 'natural' mechanism. For example, the sharp gradient of anthocyanin pigments within areoles of window stage leaves makes it possible to pinpoint cells for single-cell capture methods and high-throughput omics technology. There is also potential interest in harvesting lace plants as a source of anthocyanins. This review highlighted how the lace plant can be used for research applications and how it is on track to become a model organism to study PCD. The long-term goal of lace plant research is to improve our understanding of

PCD with the ultimate goal of manipulating the process for applications in medicine and agriculture.

1.9. AUTHOR CONTRIBUTIONS

NMR participated in the design of the study, collected all literature and data for the manuscript, contributed to the visualization of figures, wrote the first manuscript and contributed to manuscript revisions. AHLANG conceived the study, participated in its design, coordination and helped with manuscript revisions as well as supervising all of the figures. SBT contributed to the visualization of the figures, the review and editing of the manuscript. AND contributed to the visualization of figures, the review and editing of the final manuscript. CRL contributed to the review and editing of the final manuscript.

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1.11. THESIS OUTLINE

1.11.1. Thesis objectives

The first objective of this thesis work is to elucidate potential regulators of lace plant PCD by using RNA-Seq analysis. To do this we generated *de novo* transcriptomes of lace plant developmental leaf stages and isolated NPCD and PCD cell populations to identify differences in gene expression during leaf remodelling and differentiating cell types within leaf areoles. For the second objective, two selected differentially expressed genes (DEGs) were investigated for their role in lace plant PCD and leaf remodelling using pharmacological experimentation and molecular techniques. The following passages describe how individual projects were conducted to achieve the objectives:

1.11.2. RNA-Seq Analysis of Lace Plant Transcriptomes

The chronological order of intracellular events during lace plant PCD is well characterized. Yet, the genetic mechanisms that mediate lace plant leaf development and PCD cell differentiation is less well understood. The lace plant cell death gradient system is a powerful emerging model to study plant developmental PCD adjacent to NPCD control-like cells. Here, we conducted a transcriptome gene expression analysis of separated lace plant PCD versus NPCD cells using laser capture microdissection followed by RNA-Seq. Differential expression analysis was used with the goal to identify highly expressed candidate genes that encode for proteins involved in lace plant cell

death initiation, mediation and execution. Additionally, we analyzed transcriptomes of several lace plant developmental leaf stages to identify highly expressed genes involved in lace plant leaf remodelling between perforating and non-perforating leaves.

1.11.3. Hsp70 as a Regulator of Lace Plant PCD

Heat shock proteins (Hsps) are a ubiquitous family of molecular chaperone that are involved in mediating cell homeostasis and cell signalling by refolding nascent and stressed proteins before irreversible denaturation. Plant Hsp70s have not only been found to play a role in stress tolerance and development but also both inhibiting and promoting plant PCD. Due to the lace plant Hsp70 homologue being highly expressed in lace plant window stage leaves by RNA-Seq we sought to investigate its possible role in lace plant PCD. Whole plant experiments were carried out using known PCD regulators (ROS and antioxidants) and an Hsp70 inhibitor chlorophenylethynylsulfonamide (PES-Cl) as treatment to determine effects on lace plant perforation formation. Post-treatment, Hsp70 levels of leaf stages were probed using western blotting, qRT-PCR, while leaf anthocyanin, ROS, caspase-like peotease-1 activity and perforation count were measured to compare effects of Hsp70 modulation. If Hsp70 inhibition by PES-Cl has an effect on lace plant PCD signalling then treated plants will generate mature leaves with a significantly different number of perforations.

1.11.4. Autophagy and Atg16 Involvement in Lace Plant Leaf Development

Autophagy has been shown to play a dual role during plant PCD processes either promoting or inhibiting cell death but only characterized in a cell-type specific manner in *Arabidopsis* root cap development and Norway spruce suspensor deletion. Autophagy has also been shown to be active across the lace plant cell death gradient during leaf development and hypothesized to mainly promote survival (Dauphinee et al., 2019). Autophagy-related protein 16 (Atg16) and Atg18a were highly expressed in pre-perforation and window stage leaves detected by RNA-Seq. Due to the lack of knowledge behind the role of Atg16 in plant autophagy as well as the role of autophagy in lace plant PCD we sought to characterize Atg16 synthesis during leaf development. Western blotting and quantitative-RT-PCR (qPCR) were used to probe for mRNA and protein levels of Atg16 during different stages of natural leaf development. Additionally, plants were subject to known lace plant autophagy modulators to investigate how perforation formation, anthocyanin and Atg16 levels were affected. As a working hypothesis, we predict that autophagy plays a dual role in PCD and NPCD cells during lace plant leaf development and affects perforation formation through anthocyanin.

CHAPTER 2: TRANSCRIPTOME ANALYSIS OF LACE PLANT LEAF DEVELOPMENT

The work presented in Chapter 2 is published as:

Rowarth, N. M., Curtis, B. A., Einfeldt, A. L., Archibald, J. M., Lacroix, C. R., and Gunawardena, A. H. (2021). RNA-Seq analysis reveals potential regulators of programmed cell death and leaf remodelling in lace plant (*Aponogeton madagascariensis*). *BMC Plant Biol*, *21*(1), 1-20. doi: 10.1186/s12870-021-03066-7.

2.1. ABSTRACT

The lace plant (*Aponogeton madagascariensis*) is an aquatic monocot that develops leaves with uniquely formed perforations through the use of a developmentally regulated process called programmed cell death (PCD). The process of perforation formation in lace plant leaves is subdivided into several developmental stages: pre-perforation, window, perforation formation, perforation expansion and mature. The first three emerging "imperforate leaves" do not form perforations, while all subsequent leaves form perforations via developmentally regulated PCD. PCD is active in cells called "PCD cells" that do not retain the antioxidant anthocyanin in spaces called areoles framed by the leaf veins of window stage leaves. Cells near the veins called "NPCD cells" retain a red pigmentation from anthocyanin and do not undergo PCD. While the cellular changes that occur during PCD are well studied, the gene expression patterns underlying these

changes and driving PCD during leaf morphogenesis are mostly unknown. We sought to characterize differentially expressed genes (DEGs) that mediate lace plant leaf remodelling and PCD. This was achieved performing gene expression analysis using transcriptomics and comparing DEGs among different stages of leaf development and between NPCD and PCD cells isolated by laser capture microdissection. Transcriptomes were assembled from imperforate, pre-perforation, window and mature leaf stages, as well as PCD and NPCD cells isolated from window stage leaves. Differential expression analysis of the data revealed distinct gene expression profiles: pre-perforation and window stage leaves were characterized by higher expression of genes involved in anthocyanin biosynthesis, plant proteases, expansins and autophagy-related genes. Mature and imperforate leaves upregulated genes associated with chlorophyll development, photosynthesis and negative regulators of PCD. PCD cells were found to have a higher expression of genes involved with ethylene biosynthesis, brassinosteroid biosynthesis and hydrolase activity whereas NPCD cells possessed higher expression of auxin transport, auxin signalling, aspartyl proteases, cysteine protease, Bag5 and anthocyanin biosynthesis enzymes. RNA sequencing was used to generate a de novo transcriptome for *A. madagascariensis* leaves and revealed numerous DEGs potentially involved in PCD and leaf remodelling. The data generated from this investigation will be useful for future experiments on lace plant leaf development and PCD in *planta*.

2.2. INTRODUCTION

2.2.1. Programmed cell death

Programmed cell death (PCD) is an essential developmental process that ensures the removal of cells, for tissue remodelling or in response to environmentally induced stress (Kabbage et al., 2017; Kacprzyk et al., 2011, 2016; Van Hautegem et al., 2015). Plant developmental PCD is controlled by internal and external signals resulting in the organization of developing tissues (Daneva et al., 2016; Gunawardena and McCabe, 2015; Kuriyama and Fukuda, 2002). Examples include embryonic suspensor deletion (Bozhkov et al., 2004), aerenchyma formation (Gunawardena et al., 2001a, 2001b) and xylem differentiation (Gunawardena, 2008; Ohashi-Ito et al., 2010). Due to the experimental challenges associated with physically separating PCD destined cells spatially and temporally in plant model systems, there is presently little known about the genetic mechanisms that control developmental PCD. Plant systems with adjacent regions of differing cells fates arising from perforation formation during leaf morphogenesis can provide unique insight into the PCD process (Gunawardena, 2008).

2.2.2. The lace plant model system

The formation of leaf perforations during development is unique and has been studied in plant families such as Araceae and Aponogetonaceae (Gunawardena, 2008). *Monstera obliqua*, *M. deliciosa* and *Aponogeton madagascariensis* utilize PCD to dismantle and

clear designated cells during early shoot development, creating perforations in the leaf blade.

The lace plant *A. madagascariensis* is an aquatic monocot that has recently emerged as a model system for studying PCD, due to the accessibility and predictability of PCD in developing leaves. Lace plant leaves are also thin and translucent, making them ideal for live-cell microscopy. Finally, the sterile propagation of whole lace plants in axenic environments create opportunities for pharmacological studies (Gunawardena, 2008; Kacprzyk et al., 2016).

The lace plant generates leaves with a perforated morphology, wherein specific cells bounded within the vasculature are removed via developmentally regulated PCD (Figure 2.1.A). Leaves of the lace plant emerge in a heteroblastic series from an underground corm and while the first 3–4 leaves (termed imperforate leaves, Figure 2.1.B and C) to emerge do not form perforations at all during development, all successive emerging leaves become perforated (termed adult leaves). Early adult leaves in the "pre-perforation stage" (Figure 2.1.B and D) emerge from the corm in a furled form, with an abundance of anthocyanin and complete vein pattern (Dauphinee et al., 2017; Gunawardena, 2008). As pre-perforation leaves unfurl they transition to the "window stage" (Figure 2.1.B and E). Window stage is reached when cells in the central portion of areoles located between longitudinal and transverse veins undergo PCD, losing their anthocyanin and chlorophyll pigmentation ("PCD cells", Figure 2.1.E). Cells proximal to the veins retain both anthocyanin and chlorophyll pigmentation and do not undergo PCD; and are therefore

called “non-PCD cells” (NPCD cells, Figure 2.1.E). The perforations increase in size until halting 4–5 cell layers from the veins. Once perforation formation is nearly completed, the mesophyll cells at the NPCD cell edge of perforations transdifferentiate into epidermal cells. The leaves reach the mature stage once perforation expansion halts and NPCD cells achieve homeostasis (Figure 2.1.B and F).

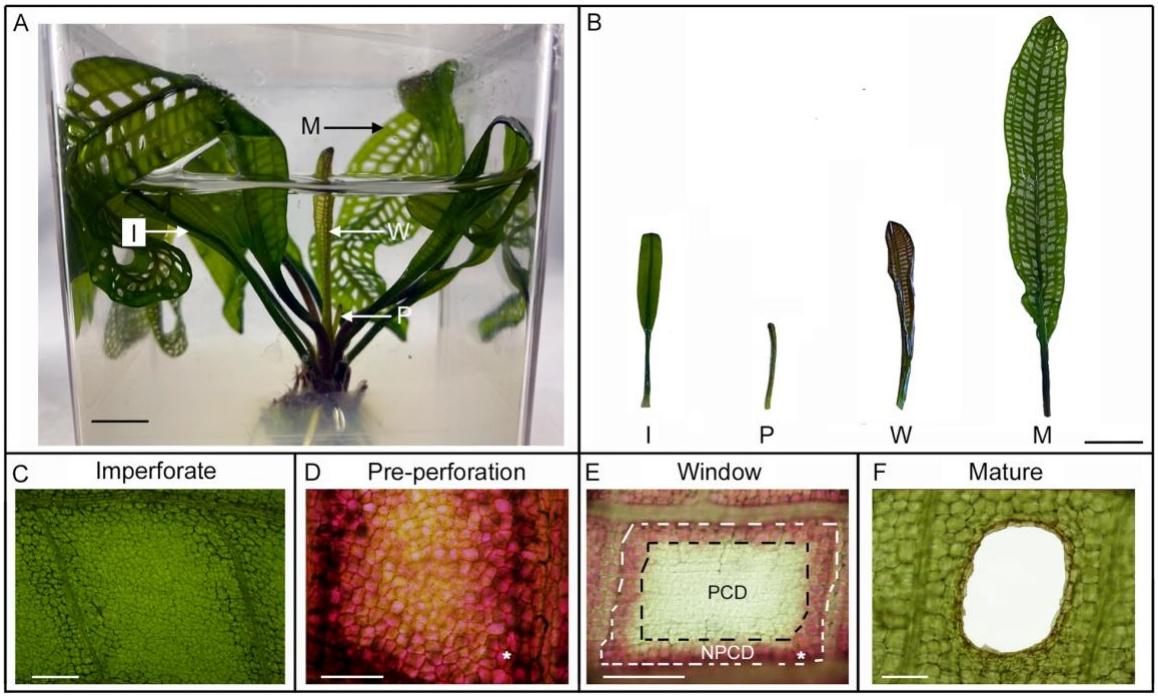

Figure 2.1. The lace plant programmed cell death (PCD) model system. (A-B) Lace plant grown in axenic Magenta box culture with the pre-perforation stage (P), window stage (W), mature stage (M) and imperforate leaves (I). (C) Imperforate leaves emerge from the corm lacking anthocyanin and forming areoles with no perforations. (D) Successive pre-perforation stage leaves emerge from the corm with anthocyanin pigmentation (indicated by *asterisks*). (E) PCD can be seen actively occurring in the window stage of development. Between longitudinal and transverse veins, in spaces known as areoles, a gradient of cell death can be observed. Non-PCD cells (NPCD; bounded by *white dashed lines*) cells persist beyond maturity. PCD cells (bounded by *black dashed lines*) have lost their anthocyanins, are translucent and on the verge of death. PCD and perforation formation is complete in mature stage leaves (F) and anthocyanin pigmentation is visibly reduced and homeostasis for NPCD cells is reached. *Scale bars*: A = 1 cm; B = 2 cm; C = 200 µm.

2.2.3. Lace plant PCD mechanism

The visible gradient of PCD within the areoles of window stage leaves represents a powerful model system for studying cellular changes across this gradient during developmental PCD (Gunawardena, 2008). The cellular changes that occur across the gradient of PCD in the lace plant have been well characterized and some of the early events of lace plant PCD include the reorganization of the actin cytoskeleton, chloroplast ring formation around the nucleus, mitochondrial aggregation and increased number of transvacuolar strands (Gunawardena, 2008). The manner in which organelles are taken up into the vacuole in membrane-bound vesicles suggest the involvement of autophagy (Dauphinee et al., 2019). Further examples of cellular changes include loss of mitochondrial membrane potential, DNA fragmentation and activation of caspase-like proteases before vacuolar collapse, plasma membrane shrinkage and cell wall degradation (Dauphinee et al., 2014; Lord et al., 2013).

In spite of the well characterized progression of PCD, little is known about the molecular mechanisms that control lace plant PCD regulation and execution (Rantong et al., 2016; Rantong and Gunawardena, 2015), in part due to a lack of molecular information for the Aponogetonaceae family. The advancement of comparative RNA sequencing (RNA-Seq) analysis between PCD and NPCD-like cells in other plant models has helped characterize differentially expressed genes (DEGs) that resemble PCD regulators. RNA-Seq analysis of separated embryonal mass and suspensor cells of *Picea abies* has shown that a spruce homolog of bax inhibitor-1 transcript is upregulated in early PCD suspensor cells and

plays a role in regulating vacuolar cell death in suspensor cells (Reza et al., 2018). Moreover, RNA-Seq analysis of early and late cavern forming leaf aerenchyma cells of *Typha angustifolia* revealed expansins, calmodulin-like proteins and pectinases transcripts that were directly related to lysigenous aerenchyma induction (Du et al., 2018).

To date there is only one transcriptome study, Rantong et al. (2016), which investigated lace plant leaf stages using complementary DNA-amplified fragment length polymorphism (cDNA-AFLP). This study identified 79 annotated DEGs which are involved in processes such as photosynthesis, stress responses, pathogen defence and PCD. Importantly, their results suggested that ubiquitin-proteosome machinery may be involved in lace plant PCD. However, as cDNA-AFLP captures only a fraction of the transcriptome, how expression patterns across the entire *A. madagascariensis* genome change during PCD remains unknown.

In this study, we used high-throughput RNA-Seq to compare global transcriptome expression profiles of different stages of lace plant development as well as PCD and NPCD cells. We separated PCD and NPCD cells within window stage leaves by laser capture microdissection, allowing us to identify DEGs that may be involved in PCD and survival. We also identified, through differential expression analyses of RNA-Seq data, genes highly expressed in PCD and NPCD cells that are potential PCD inductors, executors and/or regulators. To identify key regulators of lace plant leaf remodelling, we

additionally tested for genes that are highly expressed among perforating and non-perforating leaves.

Our objectives are to identify and compare DEGs among different leaf developmental stages and between PCD and NPCD cells. We hypothesize that imperforate and mature leaves have significantly higher levels of expression of genes involved in photosynthesis and negative regulation of PCD while pre-perforation and window leaves will have significantly higher levels of expression of genes responsible for anthocyanin biosynthesis, caspase-like activity, cell wall organization and pro-PCD regulation.

2.3. MATERIALS AND METHODS

2.3.1. Plant tissue culturing

Lace plant cultures were propagated aseptically as described in Gunawardena et al. (2006). Lace plant corms were cultured in Magenta GA-7 boxes, embedded in 100 mL of solid MS media made of 1.5% plant tissue culture agar (w/v, Phytotechnology Laboratories) in liquid MS [3% sucrose (w/v), 0.01% Myo-inositol (w/v), 0.215% MS basal salts (w/v, Phytotechnology Laboratories), 0.0025% thiamine-HCl (v/v), pH 5.7] and then submerged under 150 ml of liquid MS. Plant cultures were grown at 24 °C and exposed under light levels of 125 μmol m^{-2} s^{-1} on 12 h light/dark cycles with daylight deluxe fluorescent light bulbs (Philips). *A. madagascariensis* (Mirbel) H. Bruggen corms were originally purchased from The PlantGuy (AB, Canada).

Cultures grew for 30 days or until each plant produced 3 perforated mature leaves. One of each imperforate, pre-perforation, window stage leaves and one of the most recently developed mature leaf were separated from one whole plant culture and washed thoroughly with distilled water before leaf tissue was excised from the midrib and flash frozen for downstream molecular work. Three independent experiments were carried out. For each experiment, one of each leaf stage was taken from one whole plant culture.

2.3.2. PCD and NPCD cell preparations

A Zeiss PALM Laser Capture Microdissection and Imaging System (North York, ON, Canada) was used to separate PCD and NPCD cells. The cells were collected from the areoles of window stage leaves, where each type is visibly distinguishable by colour. Cell type population samples were collected from 4 separate areoles (approximately 2000 cells per areole) per window stage leaf before being flash-frozen. Three independent experiments per cell type were carried out. For each experiment, one window stage leaf was taken from one whole plant culture. A sample diagram of the laser capture and catapult process of the leaf tissue sample is provided in Rowarth et al., (2021); Additional file 2.

2.3.3. RNA extraction and quality control

RNA was extracted from leaves of four developmental stages and from the two different cell types (NPCD and PCD cells). RNA was extracted from 40 mg of flash-frozen, midrib free leaf lamina tissue from one of each imperforate, pre-perforation, window or mature stage leaves from 3 different whole-plant cultures as per instructions for the ReliaPrep RNA Kit (Promega). RNA samples were treated with DNAse I (Thermo Fisher). Eluted RNA quantity was estimated using a Nanodrop spectrophotometer (Thermo Fischer) and a RNA integrity number (RIN) was determined using a Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). Only RNA samples with a RIN $\geq$ 6.5 were approved for cDNA conversion.

2.3.4. cDNA library preparation and Illumina sequencing

cDNA library preparation and sequencing were performed by Génome Québec (Montréal, QC, Canada). Eighteen paired-end RNA-Seq libraries of length 100 bp were generated on an Illumina NovaSeq6000 (CA, USA) using strand-specific Trueseq protocols. The raw read data obtained were deposited to NCBI and are accessible under the SRA. SRA accession IDs: SRR10524134-SR10524151 and BIOPROJECTID: PRJNA591467. Data were first inspected for quality by analysing FastQ files with FastQC (Andrews, 2010). Reads of low quality and containing adapter contaminations were trimmed with Trimmomatic v.0.35 (Bolger et al., 2014) with a k-mer size of 25 and with parameters: ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 SLIDINGWINDOW:5:0 MINLEN:50. The quality of trimmed reads was assessed using FastQC v0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

2.3.5. Transcriptome de novo assembly

High-quality adapter free reads were used to construct a de novo assembly with Trinity v2.3.1 (Grabherr et al., 2011) with default settings. Quality evaluation of assemblies was considered with major bioinformatics indicators such as transcript mean length, GC% and an N50 value (Table 2.1). The Trinity pipeline clusters de novo assembled transcripts into genes and isoforms and we worked only with 'genes' datasets, with the highest expressed isoform chosen as the representative for each gene. This Transcriptome Shotgun Assembly project has been deposited at DDBJ/ENA/GenBank under the accession NO. GJFM00000000. The version described in this paper is the first version, GJFM01000000.

2.3.6. Transcript quantification and identification of differentially expressed genes

The abundance of each gene was calculated by aligning reads from each sample to our de novo transcriptome with RNA-Seq by Expectation–Maximization (RSEM) (Li and Dewey, 2011) for each library. The trimmed mean of *M*-values (TMM) method (Robinson and Oshlack, 2010) was used to calculate the normalization factors (one calculation for NPCD vs PCD cells and one calculation for comparisons among the leaf stages). Using Trinity (Haas et al., 2013), expression normalization was performed using TMM, following fragments per kilobase of exon model per million reads (FPKM) calculations. DEGs among the leaf stage and between the cell type libraries were identified using the Empirical Analysis of Digital Gene Expression (edgeR) statistical package (Robinson et al., 2010)

(http://bioconductor.org/packages/release/bioc/html/edgeR.html) performed with R (v3.3.2; R Core Team 2015). Genes that were more than 2-fold differentially expressed with a False Discovery Rate (FDR) of 1% were defined as differentially expressed (Benjamini and Gavrilov, 2009).

2.3.7. Cluster analysis

Expression patterns of genes among leaf stage samples and between NPCD and PCD cells were separated using cluster analysis of DEGs. Hierarchical clustering of normalized gene expression was achieved using centralized and $\log_2(\text{FPKM} + 1)$ transformation (Haas et al., 2013) and tree cutting at a depth of 40%, with heatmap visualization performed using R and the package Superheat (Barter and Yu, 2018).

2.3.8. Annotation and GO enrichment analysis

To identify the genes and functions associated with each transcript, assembled transcripts were annotated using Trinotate (Bryant et al., 2017) and public genome and functional repositories. We annotated transcripts based on top matching sequence similarity to orthologs in public databases, including GO, the Kyoto Encyclopedia of Genes and Genomes (KEGG), the euKaryotic Ortholog Groups database (KOG), the Swiss Protein Resource (Swiss-Prot) and the Panther Database, using the BLASTX method with a cut-off *E*-value of 10^{-5} (Bateman et al., 2017; Kanehisa, 2000; Tatusov et al., 1997). To eliminate transcripts derived from foreign organisms and lab contaminants, genes of non-

plant origin were removed. Selected annotation data was included with Supplementary files 3 and 5; Rowarth et al. (2021)

GO-enrichment analysis was carried out using the Plant Transcription Factor Database v5.0 (Jin et al., 2017) program based on Fisher's Exact Test with multiple testing correction of FDR = 1%. GO analysis was performed by comparing the GO terms in the test sample to the GO terms in the background reference of the entire lace plant *de novo* transcriptome generated from all samples.

2.3.9. Validation with qRT-PCR

Five selected DEGs (*Bag5*, *expansin A-29*, *aquaporin 4–4*, *anthocyanin regulatory protein C1*, *nuclear transcription factor YC-1* and *α-tubulin*) were used to verify the fold change in gene expression results of RNA-Seq by qRT-PCR using the $2^{-\Delta\Delta CT}$ method. RNA from lace plant leaf stages and isolated cell types were collected and extracted as described above. Single-strand cDNA was synthesized using SuperScript®III First-Strand Synthesis System for qRT-PCR (Invitrogen, Burlington, ON, Canada) and oligo dT_{20} following the manufacturer's instructions. qRT-PCR was conducted on a Rotor-Gene RG-3000 system (Corbett Research, Sydney, NSW, Australia) using 0.5 µl cDNA as a template and 0.4 mM primers for all selected genes (Additional Table A.1.) under the following conditions: 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 54 °C for all chosen genes and 1 min at 72 °C, followed by 72 °C. qPCR was conducted using a QuantiFast® SYBER® Green PCR Kit (Qiagen, Mississauga, ON, Canada). Melt curve

analysis was conducted by Rotor-Gene 6 Software and experiments with at least 90% efficiency were used for analysis (Corbett Research). The experiment was performed in triplicate using three biological replicates of imperforate, pre-perforation, window and mature stage leaves as well as NPCD cells and PCD cells. cDNA copy numbers for five chosen genes (Rowarth et al., 2021; Additional file 4 and 6) were determined from a standard curve of Ct values ($R^2 > 0.99$) and normalized against the *α-tubulin* isoform (Rantong et al., 2015).

2.3.10. Image analysis and processing

Images of leaf layouts were obtained using a Nikon L110 digital camera. Photoshop and Illustrator (Adobe Creative Cloud; Adobe Systems Inc.) were used to prepare images and remove backgrounds of detached leaves for publication. A Nikon AZ100 microscope acquired micrographs of leaf stages. Image adjustments were made evenly within and consistently across figures and included background removal, as well as fine tuning of brightness, contrast and colour balance using Photoshop.

2.3.11. Statistical analysis and data representation

One-way ANOVA followed by a Tukey test was used to identify significant differences among leaf stage means for qRT-PCR validation experiments, and a Student's *t*-test was used to identify significant differences between means of cell types (Rowarth et al., 2021;

Additional file 4). All data are illustrated as the mean ± standard error. Analyses were conducted using GraphPad Prism 5 software (GraphPad Software Inc.).

2.4. RESULTS AND DISCUSSION

2.4.1. RNA-Seq data overview

To identify potential regulators of lace plant developmental PCD and leaf remodelling, we generated a novel lace plant transcriptome and identified DEGs in comparisons of pre-perforation, window, mature and imperforate leaf stages and NPCD and PCD cell types using RNA-Seq. Eighteen paired-end RNA-Seq libraries were generated from three biological replicates of each imperforate, pre-perforation, window, mature leaf stages, NPCD cells and PCD cells. The Illumina (San Diego, CA, USA) NovaSeq6000 sequencing platform was used for paired-end sequencing at Génome Québec (Montréal, QC, Canada), with a 100 bp read length. A total of 1,320,261,351 reads were generated, and data for individual biological libraries were deposited to the NCBI SRA database with the following SRA accession IDs: SRR10524134-SR10524151 and BIOPROJECTID: PRJNA591467. After read filtering, 1,288,318,561 reads remained and 1,102,201,639 reads aligned concordantly (Rowarth et al., 2021; Additional file 1). We assembled 908,119 transcripts with an N50 length of 1,041 bp, and 49.9% GC content from eighteen RNA-Seq libraries. These transcripts translated to 437,825 protein coding genes (Table 2.1.). Gene Ontology (GO) annotated DEGs across leaf stages and cell types accounted for 4,339 of the 106,222 (4.08%) *A. madagascariensis* GO annotated

genes across all leaf stages and cell samples with a 1% adjusted *P*-value cut-off (Figure 2.2.A and B). Of the 10,416 DEGs, 2808, 313, 1541 and 1267 genes were upregulated exclusively in pre-perforation, window, mature and imperforate leaves respectively (Figure 2.2.A). Between cell types, 482 and 166 genes were exclusively upregulated (at least 2-fold) in PCD and NPCD cells, respectively. Remaining DEGs were expressed mutually between different combinations of leaf stages (Figure 2.2.).

Table 2.1. Number of differentially expressed and GO annotated genes across leaf and cell-type samples.

Trinity assembly Data	Total
Number of de novo assembled transcripts	908,119
N50 (bp)	1,041
Median transcript length (bp)	374
Average transcript length (bp)	671.74
Percent GC (%)	49.89
Number of protein coding genes	437,825
Number of GO annotated genes	106,222
Number of DEGs	10,416
Number of GO annotated DEGs	4,339

2.4.2. Transcriptomic profiles of the lace plant developmental leaf stages

BLAST-based comparisons of assembled transcripts yielded 106,222 GO annotated genes that were homologous to sequences in public databases. Of the annotated genes, 30,932 (29.12%) were most similar to *Arabidopsis thaliana*, 6,509 (6.12%) to *Oryza sativa* and 692 (0.65%) to *Zea mays*.

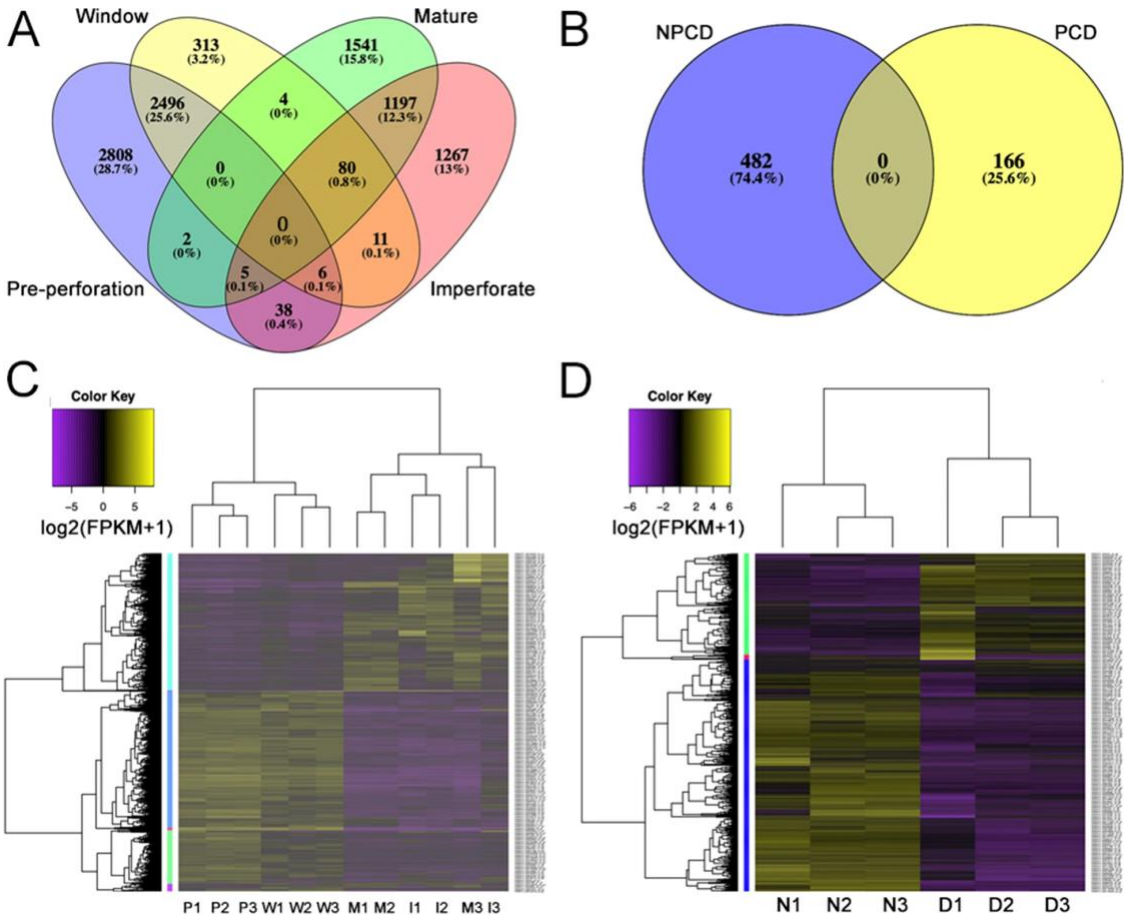

Figure 2.2. Overview of differentially expressed genes in lace plant leaves and cell types. Venn diagram showing the number of mutual and exclusive differentially expressed genes (DEGs) between lace plant leaf stages (A) and between NPCD and PCD cells (B); fold change > 2.0, $P < 0.01$. Heatmaps of gene expression levels of DEGs plotted as log2(FPKM + 1) for indicated samples and biological replicates for leaf stage comparison (C) and comparison of NPCD and PCD cells (D). Low and high gene expression levels are shown in purple and yellow, respectively. Below heatmaps, biological replicates 1–3 are indicated by *P*, pre-perforation; *W*, window; *M*, mature; and *I*, imperforate. Supporting data for gene expression found in Rowarth et al., 2021; Additional files 3 and 5.

A. madagascariensis leaf DEGs were divided into four main clusters generated through the tree cutting method of expression patterns across four leaf developmental leaf stages (Figure 2.3., Rowarth et al., (2021); Additional file 3). RNA-Seq data were divided into the pre-perforation cluster (4544 DEGs), the window cluster (718), the mature cluster (1572) and the imperforate cluster (1387) (Fold change > 2.0, $P < 0.01$, FDR = 1%; Additional file 3). GO enrichment analyses identified biological functions enriched in the four main clusters based on expression patterns across the four stages of leaf development (FDR = 1%, Rowarth et al., (2021); Additional file 3).

The pre-perforation cluster, representing genes that were most highly expressed in the pre-perforation stage leaves and exhibited reduced expression in subsequent stages, included genes encoding for proteins involved in flavonoid biosynthesis, anthocyanin biosynthesis, ethylene-activated signalling pathway, endopeptidase activity, autophagosome formation and regulation of PCD. The window stage leaf cluster represented genes that were most highly expressed in the pre-perforation and window stages and then reduced in subsequent stages. This cluster included genes encoding for proteins involved in ATP-binding, ATPase activity, ion binding, response to auxin, response to oxygen-containing compounds, peroxidase activity and hydrolase activity. Both the mature and imperforate clusters represented genes that increased in expression in later leaf development stages, and both contained genes encoding for proteins involved in photosystem I and II, chlorophyll-binding, light-harvesting complex organization, catalytic activity, ion binding and cell wall biosynthesis.

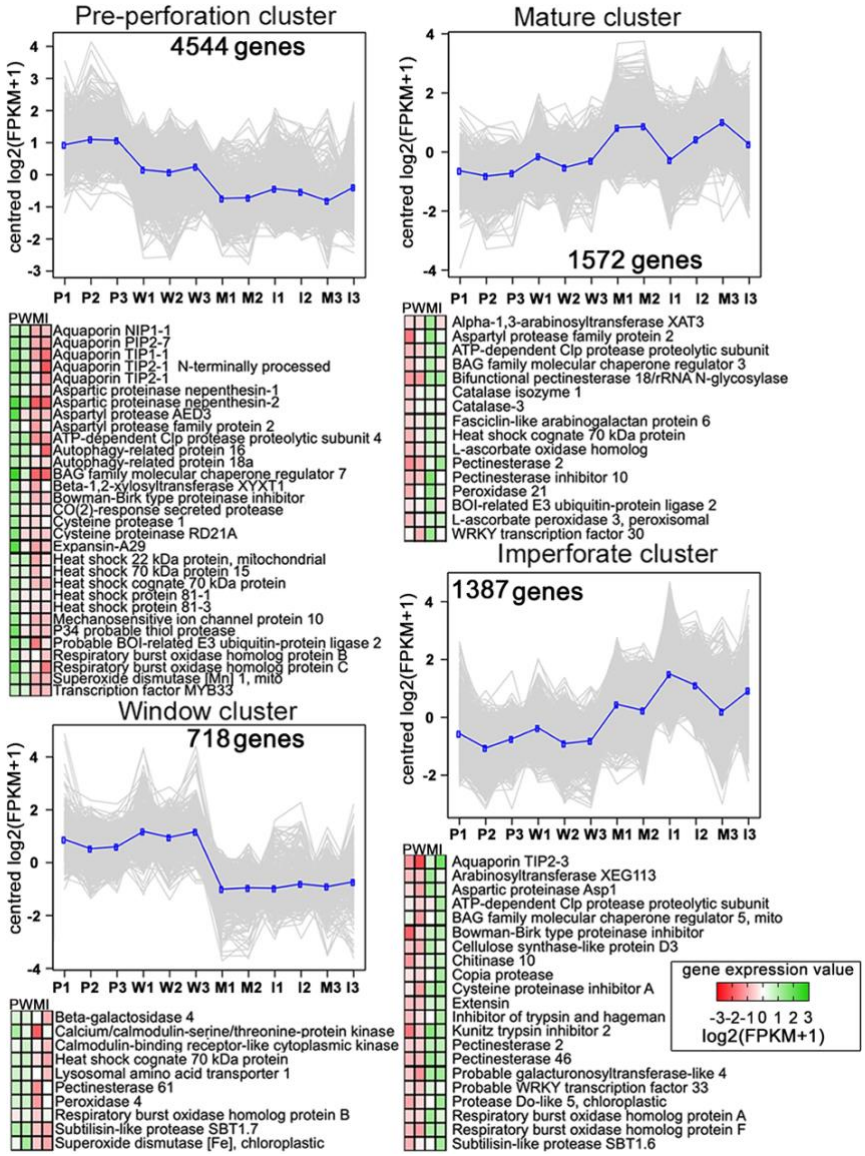

Figure 2.3. Transcriptomic analysis of lace plant leaf developmental stages. Top: Four main clusters grouped by highest expression in respective pre-perforation (P), window (W), mature (M) and imperforate (I) leaf stage biological replicates ($n = 3$). For each cluster, individual DEG expression values (shown as the transformed $\log_2(\text{FPKM} + 1)$ values) are plotted as grey lines and the mean expression profile is highlighted in blue. The total number of DEGs per cluster is shown below each plot ($P < 0.01$, fold change > 2.0). Bottom: Heatmaps of composite gene expression for indicated proteins, with green and red corresponding to high and low gene expression, respectively. Supporting data are found in Rowarth et al., (2021); Additional file 3.

Taken together, clustering data support the hypothesis that growth and organizational processes are enriched in developing pre-perforation and window stage leaves where metabolic processes must occur to fuel development. Many energy-related metabolic processes occur in the mature and imperforate leaves where development is near completion and flavonoid synthesis is reduced (Figure 2.3., Rowarth et al., (2021); Additional file 3). General patterns of gene expression for select biological functions across the leaf clusters showed that genes involved in photosynthesis and negative regulation of PCD are expressed at higher levels in mature and imperforate leaves where PCD is not as active and homeostasis is reached. This suggests that imperforate and mature leaves are the major site of photosynthesis, whereas pre-perforation and window leaves specialize in growth, responding to hormones and executing PCD. All clusters demonstrated high expression of genes involved in cell wall modifying enzymes such as pectinesterases. However, pre-perforation and window leaves possess a greater number of highly expressed expansins, pectinesterases and subtilisin-like proteases than mature and imperforate leaves. Pre-perforation and window stage leaves represent the culmination of many developmental processes such as regulation of PCD, cell wall organization, lignin and stomata development. Genes encoding proteins involved in hormone synthesis and transport were found to be differentially expressed between leaf stages. Pre-perforation and window stage leaves had high expression levels of genes encoding for auxin biosynthesis and transport, abscisic acid (ABA) biosynthesis, brassinosteroid (BR) biosynthesis, cytokinin (CK) biosynthesis, gibberellin (GA) biosynthesis, ethylene biosynthesis, ethylene receptor activity, ethylene signalling pathway, jasmonate biosynthesis and salicylic acid (SA) response. Mature and imperforate leaves expressed

similar levels of ABA transport genes in comparison to pre-perforation and window stage leaves. In total, early developing leaves revealed an expression pattern of leaf development similar to other monocots like *Agave deserti* and *Z. mays* (Li et al., 2010) where expression of most transcription factors (TFs) and hormone biosynthesis tend to be at their highest. Likewise, mature leaves express genes related to photosynthesis (Gross et al., 2013).

2.4.3. Insights from comparative transcriptomics of NPCD and PCD cells

Laser capture microdissection was used to separate NPCD and PCD cells from *A. madagascariensis* window stage leaves to reveal potential regulators of PCD by RNA-Seq analysis. NPCD and PCD transcriptomes were divided into two main clusters based on cluster analysis expression patterns (Figure 2.4.) using the tree cutting method. This cluster analysis identified approximately 431 genes that were differentially expressed in either NPCD (326 DEGs) or PCD (105 DEGs) samples with a minimum of a 2-fold change ($P < 0.01$). In comparison to upregulated genes found in leaf stages; this represents a small fraction of the transcriptome which may be a result of limited biological replicates used in this experiment. We tested for GO enrichment (FDR = 1%; Rowarth et al., (2021); Additional file 5) in each of the NPCD and PCD clusters. The NPCD cluster contained genes encoding for proteins involved in respiratory burst activity, leaf senescence (including negative regulation of senescence), protein autoubiquitination and the ethylene-activated signalling pathway. The PCD cluster

contained genes encoding for proteins involved in ethylene biosynthesis, cell wall modifiers, protease inhibitors, ROS generation and PCD regulation (Figure 2.4.). Comparing the relative GO counts between NPCD and PCD clusters (Figure 2.5.) we found that NPCD cells upregulated more genes encoding for proteins involved in flavonoid biosynthesis, cysteine protease activity, metalloprotease activity, positive regulation of autophagy, PCD regulation and cell wall organization. Conversely, PCD cells upregulated more genes involved in ethylene biosynthesis, photosystem II and I, BR biosynthesis and cutin biosynthesis. NPCD and PCD cells expressed similar numbers of genes in GO categories for homeobox, myeloblastosis (MYB), zinc finger TF families and serine endopeptidase activity, suggesting that these families act as regulatory elements across both cell types during differentiation.

Additionally, five DEGs were selected for fold-change expression validation by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) across leaf stages and cell types (Rowarth et al., (2021); Additional files 4 and 6). The transcripts for *Bag5*, *expansin A-29*, *aquaporin 4–4*, *anthocyanin regulatory protein C1*, *nuclear transcription factor YC-1* and *α-tubulin* were chosen as validation targets. The fold-change expression of the selected genes normalized by *α-tubulin* followed a similar pattern to that seen in the cluster analysis among different stages of leaf development and between cell types (Rowarth et al., (2021); Additional file 4) and were deemed valid.

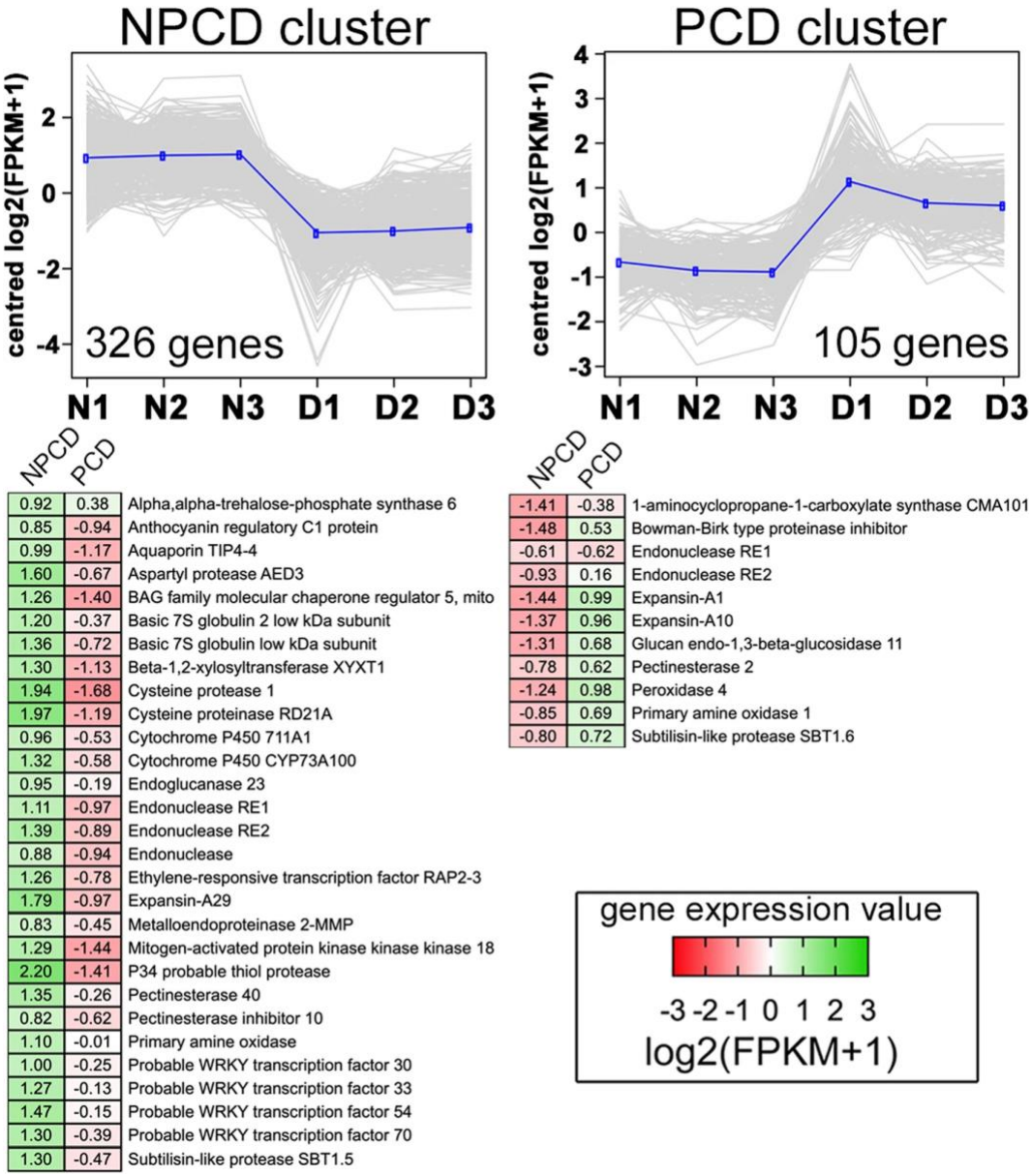

Figure 2.4. Transcriptomic analysis of NPCD vs PCD cells of the lace plant. Top: Two main clusters grouped by highest expression in NPCD and PCD cell biological replicates ($n = 3$). For each cluster, individual DEG expression values (shown as the transformed $\log_2(\text{FPKM} + 1)$ values) are plotted as grey lines and the mean expression profile is highlighted in blue. The total number of DEGs per cluster is shown below each plot ($P < 0.01$, fold change > 2.0). Bottom: Heatmaps of composite gene expression for indicated proteins, with green and red corresponding to high and low gene expression, respectively. Supporting data are found in Rowarth et al. (2021); Additional file 5.

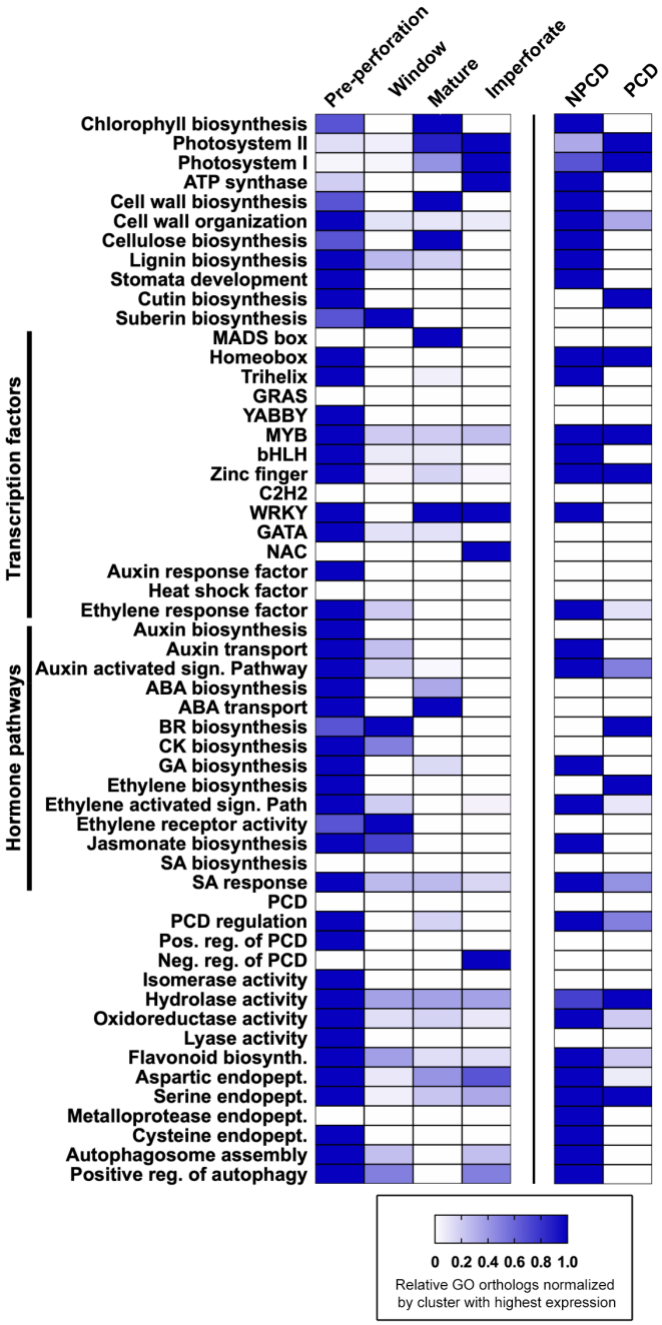

Figure 2.5. Heat map of GO category orthologs differentially expressed in leaf stages, NPCD and PCD cells. Heatmaps show composite select GO term counts normalized by the cluster with highest count for specific biological processes. The number of GO category orthologs were compared among lace plant leaf stages (left) and between NPCD and PCD cells (right). Colour gradient ranges from white (zero genes upregulated) to dark blue (highest number of genes upregulated). Supporting data are found in Rowarth et al., (2021); Additional files 3 and 5.

2.4.4. Transcription factors

Most of the DEGs that encode for TFs were found in early developing leaves (Figure 2.5., Rowarth et al., (2021); Additional file 3). TFs identified in the pre-perforation leaf cluster included YABBY, MYB, bHLH (basic Helix-Loop-Helix), Zn finger, GATA, C2C2 CO (constans), homeobox, DOF (DNA-binding with one finger), TCP (teosinte, branched1, cycloidea and PCF) and trihelix families (Figure 2.5.). These identified families presumably underpin the expression of a broad array of genes important for early leaf development, in particular, the establishment of axial polarity, stomata development and vascular tissue, as seen *in O. sativa* and *Arabidopsis* (Dai et al., 2007; Guo et al., 2010; Husbands et al., 2009; Kaplan-Levy et al., 2012; Kim et al., 2010; Liu et al., 2009a; Reyes et al., 2004; Sawa et al., 1999; Song et al., 2012). Mature and imperforate leaves expressed a greater number of genes than pre-perforation and window leaves that encode for MADS-box and NAC (no apical meristem) family TFs which may underpin the promotion of photosynthetic development, lignin, wax and secondary cell wall development which is enhanced in these later developing leaves.

DEGs encoding for TFs were also detected in NPCD and PCD cell DEG expression profiles (Figure 2.5., Rowarth et al., (2021); Additional file 5). Trihelix and WRKY families were expressed at a high level in NPCD cells. These identified TFs were most likely responsible for transcribing genes involved in stress response and are known to be upregulated in mesophyll cells of developing *O. sativa* leaves (Li et al., 2010). Two constans family TFs were highly expressed in PCD cells. Constans TF families may be

involved in mediating PCD cell collapse or suppressing anti-PCD genes. Both NPCD and PCD cells upregulated an equal number of MYB, Zn finger and homeobox family TFs. There may be MYB TFs in both types of cells but these two groups possess opposite responsibilities in terms of promoting and suppressing flavonoid biosynthesis. The differences in transcriptional regulation of gene expression between perforating and non-perforating lace plant leaves may control the key programming events that result in differential growth.

2.4.5. Plant hormones

We detected higher expression patterns for auxin, ABA, CK, GA, ethylene and jasmonate hormone biosynthesis genes in the pre-perforation cluster (Figure 2.5., Rowarth et al., (2021); Additional file 3). The window stage cluster contained the highest levels of expression for BR biosynthesis and ethylene receptor activity. The mature and pre-perforation stage clusters contained the highest levels of expression for ABA hormone transport (Figure 2.5.). These results support previous findings of several plant-specific hormones being involved in PCD signalling, including SA, jasmonic acid, ABA, GA and ethylene (Gadjev, 2006; Gechev et al., 2006; Petrov et al., 2015). The hormone biosynthesis patterns observed in early lace plant development are similar to the monocot leaves of *A. deserti, A. tequilana* and *Z. mays* (Gross et al., 2013; Li et al., 2010).

To date, several pharmacological whole plant experiments have revealed how lace plant perforation formation is dependent on auxin and ethylene biosynthesis (Dauphinee et al.,

2012; Denbigh et al., 2020; Rantong et al., 2015). Further work is required to disentangle the roles of each plant hormone in mediating lace plant leaf development from perforation formation, outside of ethylene which has been studied extensively. Expression patterns in the NPCD cell cluster included more highly expressed genes related to auxin transport, auxin signalling pathway, GA biosynthesis, ethylene activated signalling pathway, jasmonate biosynthesis and SA response in comparison to the PCD cell cluster (Figure 2.5., Rowarth et al., (2021); Additional file 5). PCD cells upregulated 1 gene encoding for ethylene biosynthesis relative to NPCD cells. Ethylene biosynthesis and ethylene receptors are involved in promoting PCD in cells destined to die in the areoles of lace plant leaves (Dauphinee et al., 2012; Rantong and Gunawardena, 2015). Additionally, PCD cells upregulate more genes associated with BR biosynthesis in comparison to NPCD cells. BRs are believed to mediate the timing of ROS production, and in turn, PCD execution in tapetal cells of *Solanum lycopersicum* (Yan et al., 2020). BRs may also play a similar role in PCD cell triggering, as suggested by the higher expression of genes involved with BR synthesis. NPCD cells upregulated ethylene response factor (ERF) RAP2-3 in comparison to PCD cells. ERF-RAP2-3 has been identified as playing an important role in ethylene mediated hypoxia stress in *Arabidopsis* seedlings (Limami et al., 2014) and may play a role in protecting NPCD cells from PCD cells which accumulate superoxide during PCD execution. Together, our results support the hypothesis that plant hormones are involved in PCD, and targeted approaches are needed to disentangle their specific roles and functions.

2.4.6. Anthocyanin biosynthesis enzymes

Forty-six upregulated genes were categorized as enzymes with flavonoid biosynthesis, isomerase, hydrolase, oxidoreductase or lyase activities in the pre-perforation leaf cluster while < 10 genes for these enzymes were found in each of the window, mature and imperforate leaf clusters (Figure 2.5.). Eight of the upregulated genes in the pre-perforation cluster encoded for the flavonoid biosynthesis pathway. Pre-perforation and window leaves upregulated genes that promote the biosynthesis of early stage and late-stage flavonoids as well as downstream transferase enzymes for promoting the synthesis of anthocyanins, flavonols and anthocyanidins. NPCD cells also upregulated 5 genes that encoded for flavonoid biosynthesis in comparison to PCD cells (Figure 2.5.). Of the genes that are involved in flavonoid biosynthesis only *flavonone 3-dioxygenase 2,*which produces dihydroflavonol (Bu et al., 2020), was upregulated in mature leaves and imperforate leaves as well as PCD cells (Rowarth et al. (2021); Additional files 3 and 5). Upregulated genes that encode for enzymes that promote anthocyanin biosynthesis are summarized in Figure 2.6.

The most notable feature of lace plant leaf development is the visible gradient of anthocyanin pigmentation during PCD in early developing leaves. Previous studies have investigated the role of exogenous ROS and antioxidants on lace plant leaf development, and found that they are key regulators of the establishment of perforation formation in the lace plant model system (Dauphinee et al., 2017). Pre-perforation and window stage leaves have the highest levels of anthocyanin compared to imperforate and mature leaves

(Dauphinee et al., 2017; Rowarth et al., 2020). Our results are consistent with these findings, as pre-perforation leaves, window leaves and NCPD cells expressed genes encoding enzymes that promote the biosynthesis of glucoside constituted anthocyanins (Figure 2.6.). The upregulation of anthocyanidin-3-O-glucosyltransferases (A3OGT), anthocyanidin-5,3-O-glucosyltransferases (A5,3OGT) and anthocyanin-5-aromatic acyltransferase (A5AAT) suggests that anthocyanidin-3-O-glucosides, anthocyanidin-5,3-O-glucosides and anthocyanidin-3-glucoside-6-hydroxycinnamoyl glucosides are being actively synthesized in early-stage leaves, leading to the accumulation of anthocyanins. The presence of pink-red coloured anthocyanin vacuoles in window stage leaves is consistent with the hypothesis that the vacuole has a pH of ~ 5.5 pH which favours the formation of flavylium cations (Kallam et al., 2017).

Recent attention has been drawn to the formation of anthocyanin vacuolar inclusions (AVIs) in window stage leaves, which then dissipate as leaves enter maturity (Gunawardena lab, 2020 unpublished data). Kallam et al. (2017) recently reported that the composition of anthocyanin substituents or decorations determined the solubility of AVIs in tobacco lines. The aromatic acylation of anthocyanidin 3-O-glucoside by anthocyanidin 3-O-glucoside-6″-O-coumaroyltransferase (A3T) promotes the formation of AVIs, while the rhamnosyl decorations decrease the formation of AVIs by competition. The absence of upregulated aromatic rhamnosyltransferases or malonyltransferase DEGs (which inhibit AVI formation) in early leaves suggests a role for AVIs in mediating proper leaf development Kallam et al., (2017). NPCD cells upregulated genes encoding for anthocyanidin 3-O-glucosyltransferases, consistent with

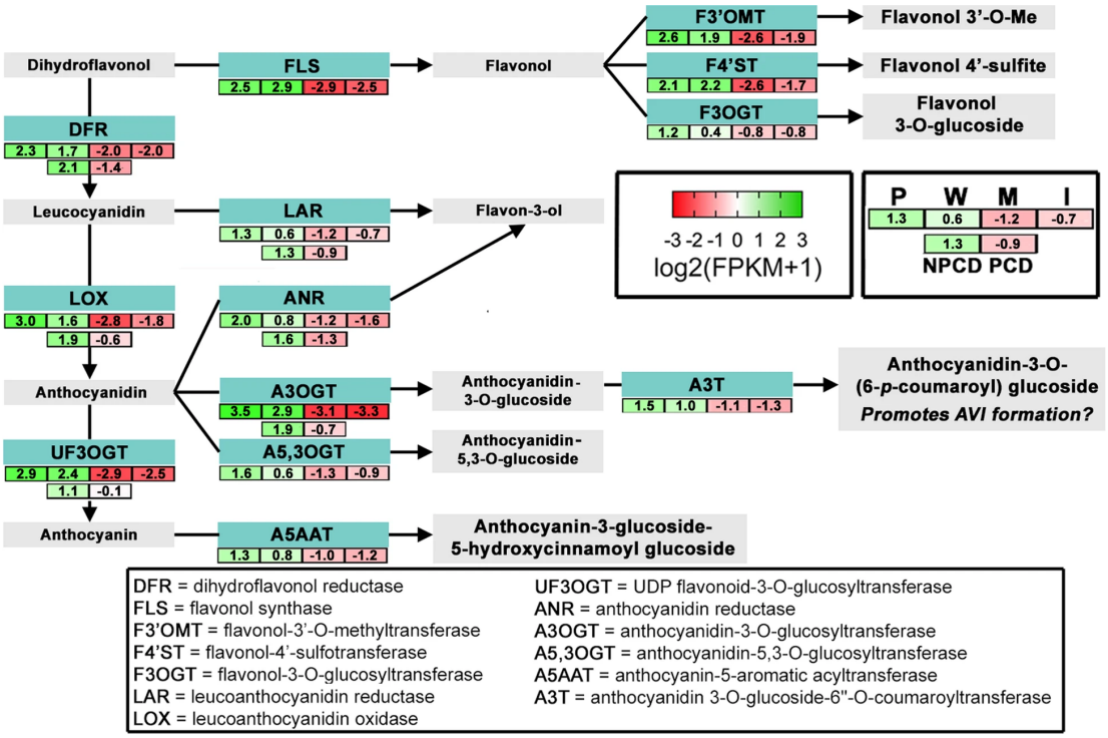

Figure 2.6. DEGs involved in flavonoid biosynthesis. General late-flavonoid biosynthetic pathway (grey boxes) and genes (cyan boxes) expression in pre-perforation (P), window (W), mature (M) and imperforate (I) leaf stages (4-box expression comparison strings) or NPCD and PCD cell samples (2-box expression comparison strings). Detailed gene expression data provided in Rowarth et al., (2021); Additional files 3 and 5. Expression levels displayed in $\log_2$(FPKM + 1) across samples. Green indicates high expression values; red indicates low expression values. Pathway modified from Argout et al. (2008).

the promotion of ROS scavengers and protection from PCD. The identification of anthocyanin compounds across developmental leaf stages and NPCD cells is currently under investigation using global mass spectrometry.

2.4.7. Potential regulators of programmed cell death

2.4.8. Cell wall modification enzymes and aquaporins

Lace plant perforation formation relies on thin cuticle erosion, removal of polysaccharides and degradation of cellulose (Gunawardena et al., 2007) to mediate perforation expansion. The deposition of suberin is imperative to prevent infection and loss of nutrients in NPCD cells while neighbouring PCD cells collapse. We found that the window stage cluster had high expression of the greatest number of suberin biosynthesis-related genes (Figure 2.5.), which is consistent with the hypothesis that suberin deposition is most active during the window stage of development (Gunawardena, 2008). Pre-perforation and window stage clusters contain high expression of 67 and 7 genes, respectively, that were categorized under either cell wall organization or cell wall biosynthesis. Mature and imperforate clusters showed high expression of 9 and 5 genes respectively. The NPCD cluster showed high expression of 4 genes, while the PCD cluster showed high expression of 1 gene (Figure 2.5.). All clusters contained higher expression for orthologs of hydrolases, pectinesterases, glucosidases and xyloglucan glycosyltransferase activities. Mature and imperforate leaves upregulated no genes that encode for expansin enzymes.

The facilitation of cell expansion and cell wall loosening is important for not only the growth of developing lace plant leaves but also the execution of PCD cells and reorganization of NPCD cells. Pre-perforation and window stage leaves upregulated several genes that encode for xyloglucan endotransglucosyltransferases, expansins (*expansin-A29* detected by qRT-PCR, Rowarth et al., (2021); Additional file 3 and 4) and pectinesterases (Figure 2.3.) which are responsible for loosening and reorganizing the cell wall during growth (Franciosini et al., 2017; Landi et al., 2017; Tenhaken, 2015). Mature and imperforate stage leaves followed a similar pattern but did not upregulate genes for expansins (Figure 2.3.). This likely indicates that this activity is no longer needed once leaf maturity is reached.

Aquaporins play an important role in cell expansion by controlling water uptake (Tyerman et al., 2002). Pre-perforation and window stage leaves upregulated several genes encoding for tonoplast intrinsic protein (TIP), nodule intrinsic protein (NIP) and plasma membrane intrinsic protein (PIP) aquaporins. NPCD cell upregulated a TIP4-4 aquaporin gene (Figure 2.4). *TIP4-4* was detected by qRT-PCR (Rowarth et al., (2021); Additional file 4). PCD cells upregulated a TIP2-3 gene and mature and imperforate leaves upregulated both. The expression of aquaporin genes in all samples suggests that cell expansion by vacuole enlargement is needed throughout leaf development, and as well as in differentiating NPCD and PCD cells to control the progression of expansion or cell burst (Tyerman et al., 2002).

2.4.9. Heat shock proteins

Heat shock proteins (Hsps) are synthesized in response to stress to maintain homeostasis by refolding proteins before they become irreversibly denatured (Kaimal et al., 2017; Shin et al., 2005). The pre-perforation leaf cluster contained 25 genes and the window leaf cluster contained 2 genes categorized as protein folding. Mature and imperforate leaf clusters contained 1 and 2 genes under unfolded protein folding, respectively (Rowarth et al. (2021); Additional file 3). The NPCD cell cluster contained 1 gene and the PCD cell cluster contained 0 genes categorized under unfolded protein folding (Rowarth et al., (2021); Additional file 5). ATP-dependent molecular chaperones such as Hsp81-1, Hsp81-3 and Hsp70-15 were upregulated in pre-perforation and window stage leaves.

In *O. sativa* protoplast models, mtHsp70 overexpression prevents ROS burst and PCD in protoplasts under oxidative stress (Qi et al., 2011). In contrast to suppressing stress induced PCD, Hsp70 of *Capsicum annuum* promotes the hypersensitive response in infected *Nicotiana benthamiana* leaves (Kim and Hwang, 2015) by nuclear localization of an effector protein. Hsp70s and their respective Bcl-2-associated athanogene (Bag) proteins can modulate animal PCD and many cellular processes (Kabbage et al., 2017), warranting their investigation in lace plant PCD. Experimental treatment with the Hsp70 inhibitor chlorophenylethynylsulfonamide (PES-Cl) caused a significant decline in the number of perforations, caspase-like activity and anthocyanin levels in window stage leaves (Rowarth et al., 2020), suggesting Hsp70 plays a role in mediating lace plant PCD. Hsp70 proteins are developmentally regulated and significantly higher in pre-perforation

and window stage leaves, which is consistent with the expression pattern of an Hsp70-15 gene (Figure 2.3.). While our results support the hypothesis that Hsp70 activity affects lace plant leaf development at a threshold level, where it localizes is still unknown. Genes for lace plant homologs of *O. sativa* Hsp81-1 and Hsp81-3 were transcriptionally upregulated in pre-perforation stage leaves (Figure 2.3. and Rowarth et al., (2021); Additional file 3). Hsp81s promote salt stress tolerance in *O. sativa* and over-expression experiments in *Arabidopsis* show that it promotes heat tolerance (Dametto et al., 2015; Yabe et al., 1994). Proteomic models predict that AtHsp81 can form a complex with AtHsp70 (Chen et al., 2018) most likely for protein quality control, and our results suggest that both lace plant homologs of Hsp70 (Rowarth et al., 2020) and Hsp81 are being synthesized for maintaining protein homeostasis during early leaf development. Additional pharmacological whole plant experiments are required to improve characterization of Hsps and Bag protein function in lace plant development.

2.4.10. Bag proteins

The Bag protein family has gained recent attention in the field of plant developmental biology for their role in mediating PCD, senescence and autophagy in several plant systems (Chen et al., 2018; Kabbage et al., 2017). Bag proteins are major nucleotide exchange factors for Hsp70 (Kabbage and Dickman, 2008) and help accelerate the Hsp70 protein fold cycle and modulate PCD pathways in animals and plants (Kabbage and Dickman, 2008; Li et al., 2016a). The Hsp70 co-chaperone Bag7 was more highly expressed in pre-perforation and window leaves. Bag3 and mitochondrial (mt) Bag5 were

upregulated in mature and imperforate leaves (*mtBag5* detected by qRT-PCR, Rowarth et al. (2021); Additional file 4). Expression values for mtBag5 were significantly higher in NPCD cells in comparison to PCD cells (Figure 2.4.). In the lace plant, we found that pre-perforation and window stage leaves upregulated genes encoding homologs of *Arabidopsis* for Bag7 (Figure 2.3.). Bag7 is endoplasmic reticulum (ER)-localized in *Arabidopsis* and is involved in the unfolded protein response, and can localize to the nucleus to interact with multiple proteins to modulate PCD pathways (Li et al., 2017; Williams et al., 2010).

Mature and imperforate lace plant leaves upregulated Bag3 and Bag5 homologs relative to pre-perforation and window leaves (Figure 2.3.), whereas NPCD cells upregulated a Bag5 homolog compared to PCD cells (Figure 2.4.). The role of Bag3 involvement in human cargo mediated autophagy has made it a potential cancer therapy target, but its role in plant PCD is unknown (Thanthrige et al., 2021). In *Arabidopsis* leaf systems, Bag5 has been found to bind heat shock cognate 70 (Hsc70) and localize to the mitochondria to promote ROS generation and clearing of chlorophyll while leaves are under senescence (Li et al., 2016a). The fact that Bag5 genes were upregulated in NPCD cells provides an opportunity to investigate the possible role of this gene in separating NPCD cells from PCD cells or preventing mitochondrial burst (Lord et al., 2011). Plant Bag5 plays a role in regulating Ca^{2+} in the mitochondria and possibly the outcomes of mitochondrial stress response in NPCD cells. Bag family proteins seem to play an important regulator role in plant PCD but their precise biochemical roles are still unknown in plants (Thanthrige et al., 2021).

2.4.11. Autophagy-related proteins

Dauphinee et al. (2019) found that autophagy predominately contributes to cell survival and that there is no clear evidence for the direct involvement of autophagy and the induction of PCD during the perforation formation of lace plant leaves (Dauphinee et al., 2019). Only pre-perforation and window stage leaves were found to upregulate genes in the GO category of autophagosome assembly such as homologs of AuTophaGy-related protein 16 (Atg16) and Atg18a (Figure 2.3. and 2.5., Rowarth et al. (2021); Additional files 3 and 5). The pre-perforation cluster contained a SNF1-related protein kinase catalytic subunit alpha KIN10 and a lysosomal amino acid transporter 1, both of which are involved in autophagy regulation. Mature leaves, imperforate leaves and NPCD cells (in comparison to PCD cells, Figure 2.3. and 2.4.) upregulated WRKY33, a TF involved in the positive regulation of autophagy (Xiong et al., 2005). WRKY33 was upregulated in NPCD cells relative to PCD cells, suggesting a requirement for stress resistance in NPCD cells during perforation development. WRKY33 is important for plant resistance to necrotrophic pathogens and interacts with the Atg18a in the nucleus (Lai et al., 2011) in response to biotic stress.

We detected higher expression of genes for Atg18a and Atg16 in pre-perforation and window stage leaves, suggesting changes in the regulation of autophagosome formation and mitophagy occur during plant developmental PCD. There are eight members in the *AtAtg18* gene family (*AtAtg18a–AtAtg18h*) (Xiong et al., 2005); each member has a different expression pattern, and only *AtAtg18a* shows an increased transcript level under

starvation conditions and during senescence in *Arabidopsis*. *AtAtg18a* expression is also upregulated and is required for autophagy during oxidative, salt and osmotic stresses. RNA interference (RNAi) of *AtAtg18a* makes plants autophagy-defective and more sensitive to various stress conditions (Hanaoka et al., 2002; Liu et al., 2009b; Xiong et al., 2005, 2007). Atg16 oligomerizes to form an Atg12-Atg5·Atg16 complex that is essential for autophagy (Yang and Klionsky, 2009).

The *Arabidopsis* protein kinase SNRK KIN10 has been shown to induce several autophagy genes such as Atg8, a protein found to be developmentally regulated in lace plant leaves (Baena-González et al., 2007; Liu and Bassham, 2012). During early leaf development, the level of expression of genes involved in photosynthesis is low in comparison to mature and imperforate leaves. Prolonged stress often causes decreased photosynthesis and increased ROS accumulation, which can trigger PCD pathways (Gechev et al., 2012). Autophagy is used in early leaf development to provide energy while photosynthesis and chlorophyll genes are not upregulated until maturity and optimal photosynthesis activity is reached. It is likely WRKY33 is transcribed to increase autophagosomes formation during early leaf development and to promote survival in stressed NPCD cells.

2.4.12. Regulators of programmed cell death

Genes falling under the GO category “PCD regulation” were differentially expressed across lace plant leaf stages and between NPCD and PCD cells. We found that the pre-

perforation leaf cluster contained homologs for L-type lectin domain kinase IX.I and mechanosensitive ion channel protein 10 (MSL10). MSL10 promotes PCD in response to pathogen invasion, and mechanical stress in *Arabidopsis* (Huang et al., 2013; Maksaev et al., 2018; Wang and Bouwmeester, 2017). Additionally, the pre-perforation cluster contained genes encoding for a BOI-related E3-ubiquitin ligase 2. BOI E3-ubiquitin ligases are capable of inhibiting PCD by limiting α-picolinic acid generation and by ubiquitination of apoptotic inhibitors (Luo et al., 2010; Yang and Li, 2000). The upregulation of MYB33 in pre-perforation and window stage leaves (Figure 2.3.) is of particular interest for further lace plant investigations, due to its role in the promotion of PCD in anthers and seeds of other monocots like barley and rice (Millar and Gubler, 2005).

The mature and imperforate leaf stage clusters contained genes encoding a BOI related E3 ubiquitin ligase 2 and a respiratory burst oxidase homolog-F (Rboh-F) respectively. BOI related E3 ubiquitin ligase 2 genes negatively regulate PCD by suppressing ROS generation (Luo et al., 2010; Yang and Li, 2000) which is consistent with mature and imperforate leaves where perforation formation and superoxide accumulation is less active (Dauphinee et al., 2017; Rowarth et al., 2020). Rboh-F is mostly responsible for ROS generation by ABA signaling in *Arabidopsis* systems (Kwak, 2003) and is implicated in immunity.

We also observed differential expression patterns for PCD regulation genes across NPCD and PCD cells (Figure 2.5., Rowarth et al. (2021); Additional 5). NPCD upregulated

genes encoding for aspartyl protease AED3 and ERF-RAP2-3 which have been previously described as pro-PCD contributors. PCD cells upregulated a gene encoding for primary amine oxidase 1 (PAO1) relative to NPCD cells. PAOs have been shown to play a role in generating ROS in differentiating tissue during organ development and during PCD (Galsurker et al., 2017; Tavladoraki et al., 2016). Pro-PCD genes such as aspartyl protease AED3 upregulated in NPCD was an unexpected result and may potentially be explained by these genes having a function to promote a stress response or senescence in NPCD cells during PCD activation.

2.4.13. Plant proteases and programmed cell death

The pre-perforation, window, mature and imperforate clusters were all found to contain upregulated genes encoding for enzymes with endopeptidase activity. The pre-perforation leaf cluster shows 59 genes encoding for enzymes with aspartic, serine and cysteine endopeptidase activity (Figure 2.5.). NPCD cells upregulated 27 genes encoding for enzymes with aspartic endopeptidase activity (2 for PCD cells), 1 gene with serine activity for both cell types, 4 with cysteine activity (versus for 0 in PCD cells) and 1 with metalloprotease (0 for PCD; Figure 2.4. and 2.5.).

Previous research has pinpointed the roles of caspase-like enzymes in plant development as PCD initiators or executors (Rustgi et al., 2017; Sueldo and van der Hoorn, 2017; Zhang et al., 2014). Subtilisin-like proteases have potential PCD regulation roles with an autocatalysis activity-containing domain that re-enters the cell once the prodomain is

removed to execute PCD, and all lace plant leaf stage clusters upregulated several subtilisin-like proteases. The similar expression of *subtilisin-like proteases* across leaf stages is consistent with a role in leaf remodelling and homeostasis. All leaf stages and NPCD cells upregulated a Bowman-birk serine protease inhibitor. Serine protease inhibition activity may indicate a role for coordinated inhibition of serine endopeptidase activity for proper PCD execution.

Multiple cysteine proteases have documented roles in developmental plant PCD. For example, cysteine protease 1 mediates tapetal PCD in *Arabidopsis* (Zhang et al., 2014) and cysteine protease R2D1A is found to enhance PCD in innate immunity of *Arabidopsis* (Rustgi et al., 2017; Shindo et al., 2012). Cysteine protease activity can be tightly controlled by the activity of serpin and Kunitz protease inhibitors (reversible inhibition (Rustgi et al., 2017), which are upregulated in mature and imperforate leaves in the form of Kunitz protease inhibitor 2 and cysteine protease inhibitor A. Protease cascades may trigger lace plant leaf PCD. Inhibitors such as the Kunitz and cysteine inhibitors in mature leaves could play a role in stopping the effector phase of cysteine protease activity and preventing PCD from becoming active again (Balakireva and Zamyatnin, 2019). We found that both genes for cysteine protease 1 and RD21A previously mentioned are transcriptionally upregulated in NPCD cells. The reason(s) for the accumulation of mRNA for these proteases in cells destined to survive is still uncertain, this could suggest they play an important role in mediating PCD cell collapse.

Aspartyl proteases also regulate plant developmental PCD; specifically, the deletion of reproductive tissues (Gao et al., 2017a; Gao et al., 2017b). In lace plants, pre-perforation, window stage leaves and NPCD cells all had high expression of genes encoding for aspartyl protease AED3, which is involved in PCD (Figure 2.3. and 2.4.). Aspartyl protease AED3 may be upregulated for transdifferentiating NPCD cells into endodermis during PCD progression.

A metalloproteinase 2-MMP gene was upregulated in NPCD cells in comparison to PCD cells (Figure 2.4.). SI2-MMPs have been found to inhibit epidermal cell death in *S. lycopersicum L* and, in contrast, 2-MMP promotes early senescence in *Arabidopsis* (Li et al., 2015; Zimmermann et al., 2016). The upregulation of *2-MMP* in NPCD cells may indicate their role in differentiating NPCD from PCD cells or inhibiting PCD execution. The protease substrate phosphoenolpyruvate carboxykinase 1 (PEPCK1) can be cleaved by *Arabidopsis* metacaspase 9 (MC9) and MC9 in turn promotes the clearance of root xylem tissue (Bollhöner et al., 2013) and possibly gluconeogenesis (Tsiatsiani et al., 2013). A gene encoding an *Arabidopsis* PEPCK1 homolog (Balakireva and Zamyatnin, 2019) was upregulated in our imperforate leaf cluster (Figure 2.3.). The cleavage of PEPCK1 by MC9 may promote gluconeogenesis in imperforate leaves and supports the hypothesis that imperforate leaves serve to generate energy for the lace plant before undergoing rapid senescence.

Interestingly, our RNA-Seq analysis did not reveal differential expression across leaf stages or cell types of any lace plant genes encoding for vacuolar processing enzymes

(VPEs), which are known to play a major role in developmental PCD of lace plant leaves (Dauphinee et al., 2012; Rantong and Gunawardena, 2018). Using qRT-PCR methods, Rantong and Gunawardena (2018) showed that transcript levels of *Am*VPE-1 and -2 are significantly upregulated in early developing leaves and window leaves (normalized to *Am*Actin). Previous work has highlighted the fact that VPE activity is important for vacuolar collapse in lace PCD cells. The absence of this process from results of our study and that of Rantong and Gunawardena (2018) suggests that autoprocessing and post-translational modification of the VPE protein might explain its functional activity, rather than just higher accumulation of mRNA (Rantong and Gunawardena, 2018).

2.5. CONCLUSIONS

The cellular dynamics and chronological events of lace plant leaf PCD are well documented. Here, we investigate the molecular basis of this process by characterizing the transcriptomic profiles of different stages of leaf development and PCD and NPCD cells isolated from window stage leaves. We profiled DEGs to summarize genes controlling the mechanism of developmental PCD and leaf remodelling (Figure 2.7.).

Based on comparative transcriptomics our results support the hypothesis that NPCD and PCD cell differentiation is mediated by a differential balance of plant hormones and TF activities that both promote and limit the PCD pathway. GO enrichment analyses of DEGs suggest that autophagy, cell expansion, protease activity, ROS generation and flavonoid biosynthesis work in concert to ensure promotion of perforation expansion

during lace plant leaf development. The high level of expression of genes involved in these diverse biological functions differed significantly between early and late lace plant leaf developmental stages, indicating their involvement in regulating perforation initiation, execution and leaf growth. The results of our investigation into the lace plant transcriptome and expression patterns reveal a variety of candidate genes with possible involvement in the initiation and progression of lace plant leaf cell death, generating new hypotheses and providing novel insights into plant developmental PCD. Future experiments on candidate DEGs will be required moving forward to characterize and confirm protein functionality in lace plant leaf perforation formation.

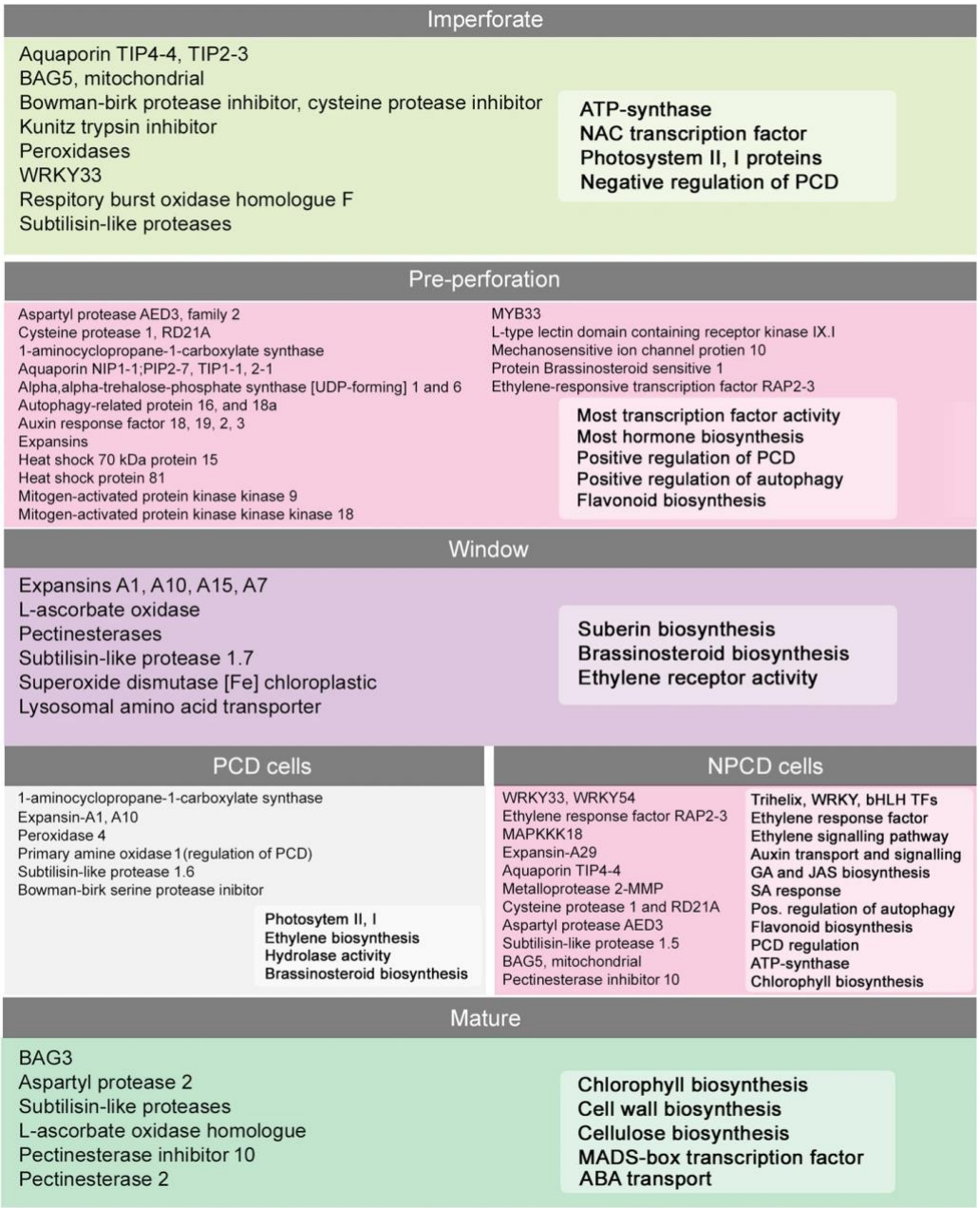

Figure 2.7. Summary of differentially expressed genes involved in lace plant leaf remodelling, based on RNA-Seq data. Summary of transcriptionally upregulated genes involved in lace plant leaf remodelling and differentiation of NPCD and PCD cells. GO terms bounded in white shaded boxes are most highly expressed GO counts in respective sample cluster (Rowarth et al. (2021); Additional files 3 and 5).

All things considered, the *A. madagascariensis* transcriptome data generated and analysed herein exemplifies the power of de novo Illumina-based RNA-Seq. Our transcriptomes serve as both a high-quality gene discovery resource and a framework for the detection of physiological changes through gene expression profiling. Combined with additional transcriptome annotation tools, experimental observations from model plant species will undoubtedly facilitate deeper insights into the biology of PCD and remodelling of lace plant leaves in the future.

2.6. AUTHOR CONTRIBUTIONS

Conceived the study, participated in its design and coordination, secured funding and supervised all experimental work: AHLANG. Carried out all experiments, participated in its design, analysed data, drafted the first manuscript, figures and text: NMR. Help with bioinformatics: BAC. Participated in study design and assisted with bioinformatics: ALE. Contributed to the manuscript revisions: NMR, ALE, BAC, JMA, CRL and AHLANG. All authors reviewed and approved the final manuscript.

2.7. ACKNOWLEDGEMENTS

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CHAPTER 3: THE ROLE OF HSP70 IN LACE PLANT PCD

The work presented in Chapter 3 is published as:

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3.1. ABSTRACT

Lace plant leaves utilize programmed cell death (PCD) to form perforations during development. The role of heat shock proteins (Hsps) in PCD during lace plant leaf development is currently unknown. Hsp70 amounts were measured throughout lace plant leaf development, and the results indicate that it is highest before and during PCD. Increased Hsp70 amounts correlate with raised anthocyanin content and caspase-like protease (CLP) activity. To investigate the effects of Hsp70 on leaf development, whole plants were treated with either of the known regulators of PCD [reactive oxygen species (ROS) or antioxidants] or an Hsp70 inhibitor, chlorophenylethynylsulfonamide (PES-Cl). ROS treatment significantly increased Hsp70 2-fold, anthocyanin and CLP activity in early developing leaves, but no change in the number of perforations formed was observed. Antioxidant treatment significantly decreased Hsp70, anthocyanin and CLP activity in early leaves, resulting in the fewest perforations. PES-Cl (25 μM) treatment

significantly increased Hsp70 4-fold in early leaves, while anthocyanin, superoxide and CLP activity significantly declined, leading to fewer perforations. Results show that significantly increased (4-fold) or decreased Hsp70 amounts lead to lower anthocyanin and CLP activity, inhibiting PCD induction. Our data support the hypothesis that Hsp70 plays a role in regulating PCD at a threshold in lace plant leaf development. Hsp70 affects anthocyanin content and caspase-like protease activity and helps regulate PCD during the remodelling of leaves of lace plant, *Aponogeton madagascariensis*.

3.2. INTRODUCTION

3.2.1. Programmed cell death in plants

Programmed cell death (PCD) is a critical process in plant development that involves the deletion of designated cells to achieve the proper organization of developing tissues (developmental PCD) and the clearance of compromised cells from stress (environmentally induced PCD; Gunawardena, 2008; Huysmans et al., 2017; Kacprzyk et al., 2016; Van Hautegem et al., 2015). Environmental PCD can be triggered by external stressors such as UV (Nawkar et al., 2013), temperature (Vacca, 2006), pathogens (Coll et al., 2011), or an imbalanced accumulation of reactive oxygen species (ROS; Petrov et al., 2015). Developmental PCD is driven by an endogenous signal(s) and is involved in many processes including leaf morphogenesis (Gunawardena et al., 2004), xylem differentiation (Ohashi-Ito et al., 2010), deletion of the embryonic suspensor (Bozhkov et al., 2005) and aerenchyma formation (Gunawardena et al., 2001a, 2001b). Animal PCD

has been well studied, but the identification of molecular mechanisms for plant PCD is still ongoing (Daneva et al., 2016; Huysmans et al., 2017; Kabbage et al., 2017).

3.2.2. Hsp70 in plant PCD

The 70 kDa heat shock protein (Hsp70) is either synthesized in response to stress or constitutively as the major chaperone responsible for maintaining protein homeostasis. This chaperone protein recruits nascent or denatured proteins and either refolds them back to their native state or mediates their destruction by the proteasome pathway (Lüders et al., 2000; Shin et al., 2005).

The structure of Hsp70 consists of a substrate-binding domain that recruits denatured proteins and an ATPase domain which catalyses the conformational change needed to recruit and release proteins rapidly (Couturier et al., 2010; Erbse et al., 2004). In previous plant transgenic studies, it is documented that Hsp70 plays a role in *Arabidopsis* heat stress tolerance (Al-Whaibi, 2011; Li et al., 2014; Montero-Barrientos et al., 2010; Wang et al., 2004), promotes *Nicotiana benthamiana* leaf hypersensitive response (Kanzaki et al., 2003) and participates in proper *Arabidopsis* leaf and root development (Leng et al., 2017; Zhang et al., 2015).

There is ongoing research concerning how Hsps mediate PCD in plant development. The overexpression of mtHsp70 protects *Oryza sativa* protoplasts from DNA laddering under oxidative stress and suppresses ROS burst and PCD (Qi et al., 2011). Additionally, heat-

stressed *N. tabacum* protoplasts synthesize Hsp70 to inhibit salicylic acid-induced apoptosis (Cronjé et al., 2004). Alternatively, Hsp70 of *Capsicum annuum* (*Ca*Hsp70a) helps trigger a hypersensitive response in leaves of *N. benthamiana* under pathogen infection (Kim and Hwang, 2015) by localizing a critical effector protein to the nuclei. One of the major nucleotide exchange factors of Hsp70, the Bcl-2-associated athanogene (Bag) protein family, also plays a role in meditating plant PCD. Li et al. (2016b) showed that Bag6 is cleaved by caspase-like proteases (CLPs) and promotes autophagy and hypersensitive response under pathogen invasion in *Arabidopsis* leaves. During *Arabidopsis* leaf senescence, Bag5 binds Hsc70 and free calmodulin to localize to the mitochondria to promote ROS generation and chlorophyll clearing (Li et al., 2016a). Hsp70s and their respective Bag proteins can modulate animal PCD and many cellular processes (Kabbage et al., 2017), warranting their investigation in lace plant PCD.

Hsp70 inhibitors such as chlorophenylethynylsulfonamide (PES-Cl) inhibit Hsp70's ability to bind proteins and perform its chaperone function (Budina-Kolomets et al., 2014; Endo et al., 2013; Mine et al., 2012; Sherman and Gabai, 2015), which triggers apoptosis in Hsp70-overexpressing cancer cells (Balaburski et al., 2013). The use of these commercial Hsp inhibitors creates an opportunity to investigate the role of Hsps in lace plant leaf development.

3.2.3. Lace plant PCD

The formation of leaf holes or perforations through developmentally regulated PCD has been observed in two plant families: the Araceae and Aponogetonaceae (Gunawardena, 2008). The aquatic lace plant (*Aponogeton madagascariensis*; Figure 3.1.A) is one of the few known plant species to develop a perforated leaf morphology via PCD and has emerged as a novel model system due to: (i) the predictability of perforation formation; (ii) its thin, nearly transparent leaves that are ideal for live cell imaging; and (iii) the availability of sterile cultures for plant propagation and pharmacological experimentation (Gunawardena et al., 2008). By taking advantage of the lace plant model system features, the cellular dynamics of cell death and the time course of lace plant PCD have been characterized (Gunawardena et al., 2004; Wertman et al., 2012).

Lace plant leaves emerge from the corm in a heteroblastic series. The first 3–4 leaves that emerge are referred to as imperforate leaves and do not produce perforations as they mature (arrow 4, Figure 3.1.A). Early-stage adult leaves (pre-perforation; arrow 1, Figure 3.1.A–C) are tightly furled containing pigmentation due to the presence of anthocyanins (indicated by asterisks in Figure 3.1), which are potent antioxidants. As the leaves unfurl, they enter the window stage of development (arrow 2, Figure 3.1.A, D and E), where PCD is actively occurring and a visual gradient of cell death is observed (Figure 3.1.E). Cell death is initiated centrally and then radiates outward within spaces known as areoles, which are located between the longitudinal and transverse veins. Non-PCD (NPCD) cells are replete with anthocyanins and persist beyond the window stage. Early-PCD (EPCD)

cells have lost their anthocyanin pigmentation but still have chlorophyll, late-PCD (LPCD) stage cells are nearly void of anthocyanin and chlorophyll pigmentation and on the verge of collapse (Figure 3.1.E). As the leaves develop into the mature stage (arrow 3, Figure 3.1.A, F and G), their perforations complete, halting 4–5 cell layers from the veins bordering the perforation and the anthocyanin pigmentation fades.

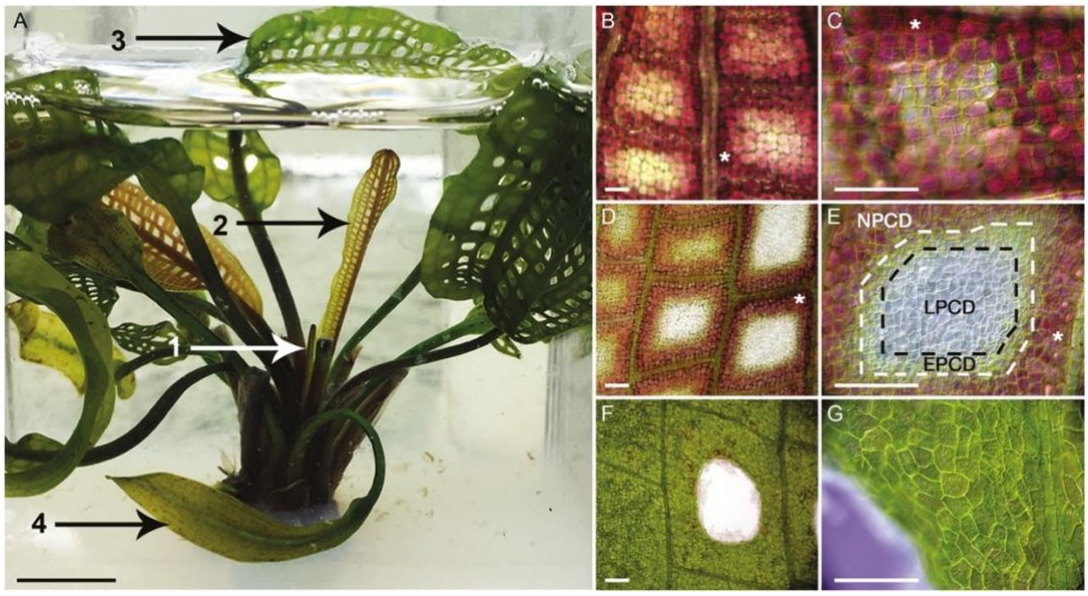

Figure 3.1. The lace plant programmed cell death (PCD) model system. (A) Lace plant grown in axenic Magenta box culture with the pre-perforation stage (1), window stage (2), mature stage (3) and imperforate leaves (4). (B and C) Pre-perforation stage leaves emerge from the corm with anthocyanin pigmentation (indicated by asterisks). (D and E) PCD can be seen actively occurring in the window stage of development. Between longitudinal and transverse veins, in spaces known as areoles, a gradient of cell death can be observed. Non-PCD (NPCD) cells persist beyond maturity, early-PCD (EPCD, bounded by white dashed lines) cells have lost their anthocyanins and are destined to die and late-PCD (LPCD, bounded by black dashed lines) stage cells are nearly transparent and on the verge of death. (F and G) PCD and perforation formation is completed in mature stage leaves. Anthocyanin abundance is visibly reduced, and homeostasis for NPCD cells is reached. Scale bars: A=1 cm; B–G=200 µm.

3.2.4. ROS, anthocyanin and Hsp70 during lace plant leaf development

Accumulation of intracellular ROS can induce the synthesis of antioxidants, Hsps and other ROS scavengers that prevent ROS burst in mitochondria, chloroplasts, or peroxisomes (Maxwell et al., 2002; Van Aken and Van Breusegem, 2015). Observing the conspicuous pattern of anthocyanin disappearance in dying cells led to a study that identified the balance between antioxidants and ROS as a key regulator of lace plant PCD (Dauphinee et al., 2017). Due to the reported involvement of Hsps in other PCD systems and their described antioxidant and cytoprotective behaviour, the role of these proteins warrants investigation in lace plant PCD. In this study, we investigated the role of Hsp70 in PCD during leaf development following exposure to antioxidants, ROS and PES-Cl. Our hypothesis is that manipulating Hsp70 amounts will affect the induction of leaf PCD during leaf development.

3.3. MATERIALS AND METHODS

3.3.1. Plant tissue culturing and treatments

Aponogeton madagascariensis (Mirbel) H. Bruggen cultures were propagated as described in Gunawardena et al. (2006). Plants were cultured in Magenta GA-7 boxes containing 100 ml of solid Murashige and Skoog (MS) medium composed of 1.5% agar (Phytotechnology Laboratories) and 150 ml of liquid MS [3% sucrose (w/v), 0.01% myo-inositol (w/v), 0.215% MS basal salts (w/v), 0.0025% thiamine-HCl (v/v), pH 5.7].

Cultures were grown at 24 °C exposed to 125 µmol m^{-2} s^{-1} on 12 h light/dark cycles with daylight deluxe fluorescent light bulbs (Philips).

3.3.2. Detection of Hsp70 during leaf development

Imperforate (non-perforating), pre-perforation, window and mature leaves from whole plants grown in axenic whole-plant conditions were harvested, and leaf blades were excised from the midrib, blotted dry on filter paper and then flash-frozen. Frozen leaf tissues were homogenized on ice 1:1 (w:v) in a 1% HALT™ protease inhibitor cocktail (Fisher Scientific, #78430) diluted in PIPES buffer solution (100 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.8). Homogenates were centrifuged at 16 000 *g* at 4 °C for 15 min, the supernatants were removed and total protein was quantitated by Bradford assay (Bradford, 1976). Protein samples were diluted 1:1 with 2× Laemmli sample buffer (Bio-Rad) containing 5% β-mercaptoethanol (v/v) and boiled for 5 min. A 10 µg aliquot of each protein sample and a Precision Plus Protein Standard (BioRad) were loaded onto an 8–16% SDS–polyacrylamide Mini-PROTEAN TGX pre-cast gel (Bio-Rad) and resolved at a constant 250 V for 30 min in ice-cold running buffer [0.1% SDS (v/v), 25 mM Tris and 192 mM glycine, pH 8.3]. Proteins were transferred at a constant 100 V for 30 min onto 0.2 µm nitrocellulose membranes (Bio-Rad) in ice-cold transfer buffer [20% methanol (v/v), 25 mM Tris and 192 mM glycine, pH 8.3]. Membranes were stained in Ponceau S stain [0.1% (w/v) Ponceau (Sigma), 5% (v/v) acetic acid] for 2 min and then washed in TBS-T (Tris-buffered saline–Tween: 10 mM Tris, 140 mM NaCl and 0.1% Tween-20, pH 7.4) for 10 s and imaged before being blocked in 5% low fat milk powder

in TBS-T for 1 h at room temperature. Membranes were then incubated at 4 °C overnight in primary rabbit anti-Hsp70 antibody (anti-Hsp70, Agrisera, #AS08 371) diluted 1:10 000 in 4% milk in TBS-T and washed four times in TBS-T for 1, 2, 3 and 4 min, respectively. Membranes were incubated 1:20 000 in TBS-T for 30 min in goat anti-rabbit:horseradish peroxidase (HRP) secondary antibody (Agrisera, #AS10 667) and washed as before followed by an additional 3 min wash in TBS (10 mM Tris, 140 mM NaCl, pH 7.4). After membrane washing, antibody-reactive protein bands were visualized with Clarity ECL Reagent (Bio-Rad) and an MF-ChemiBIS 3.2 gel documentation system (DNR Bio-Imaging Systems). Ponceau S-stained protein lanes and immunoreactive band intensities for Hsp70 were quantitated with Image Studio Lite Software (Li-Cor Biosciences). Hsp70 band intensities were compared between each control and treatment sample. Protein lanes on nitrocellulose stained with Ponceau S served as a loading control. The experiment was performed in triplicate.

3.3.3. Antibody reactivity test

To test for antibody reactivity, 10 µg of lace plant leaf cell-free extracts, 1 µg of purified recombinant *Artemia franciscana* Hsp70 protein (Iryani et al., 2017) and a Precision Plus Protein Standard were resolved in an 8–16% SDS–polyacrylamide Mini-PROTEAN TGX pre-cast gel (Bio-Rad), transferred to nitrocellulose, blocked and probed with anti-Hsp70 prior to washing and imaging as described above.

3.3.4. Measuring anthocyanin content during leaf development

A 20 mg aliquot of tissue from imperforate, pre-perforation, window and mature leaves from axenic whole-plant treatment conditions (described above) were excised and macerated in 200 µl of formic acid/methanol (5:95, v/v). Samples were incubated on ice in the dark for 50 min, followed by 10 min centrifugation at 10 000 *g*. The supernatant was collected, and absorbance immediately read at 520 nm using a SmartSpec Plus Spectrophotometer (Bio-Rad). Results were expressed as cyanidin-3-rutinoside equivalents (C3REs), and standard curves of cyanidin-3-rutinoside (anthocyanin) were used to calibrate concentration. A minimum of three replicates were used for each group.

3.3.5. ROS and antioxidant treatments

Whole-plant cultures were used for experiments if they had at least three perforated mature leaves to synchronize age between replicates. Treatments consisted of: (i) antioxidant (AO) treatments of 400 µg ml^{-1}L-ascorbic acid and 200 µg ml^{-1}L-cysteine (Bio-shop Canada); or (ii) ROS treatment of 2.5 mM H_2O_2 (Fisher Scientific). This antioxidant combination treatment was previously optimized as the most effective treatment to reduce lace plant perforations and H_2O_2 in other plants compared to either of the individual components alone (Dauphinee et al., 2017). Treated plants were grown for 1 week before their leaves were harvested. Leaves were measured for lengths and number of perforations before being processed for downstream molecular work. Controls

consisted of an equal volume of water. A minimum of six replicates were used for each group.

3.3.6. Hsp70 inhibitor treatments

Different concentrations of the Hsp70 inhibitor PES-Cl (Sigma) were dissolved in DMSO and tested to optimize the whole-plant treatment. Concentrations of 1, 5, 10, 15, 20, 25 and 30 μM PES-Cl were tested for 1 week. Leaves were then harvested, and mature leaf length and perforation number were recorded before samples were processed for downstream molecular work. To test the effect of PES-Cl on Hsp70 protein amounts, pre-perforation, window and mature stage leaves were homogenized and probed for Hsp70 by western blotting (described above). A concentration of 25 μM PES-Cl was identified as the optimal treatment that did not cause leaf necrosis or stunt growth. Control whole plants received an equal volume of DMSO. A minimum of three replicates were performed for each group.

3.3.7. Detecting Hsp70 amounts after treatment with ROS, antioxidants and PES-Cl

Pre-perforation, window and mature stage leaves from control, ROS and antioxidant conditions were harvested, their leaf blades were excised from the midrib, blotted dry on filter paper and then flash-frozen. Leaf tissues were homogenized on ice 1:1 (w/v) in a 1% HALT™ protease inhibitor cocktail diluted in PIPES buffer solution. Homogenates were centrifuged at 16 000 *g* at 4 °C for 15 min, the supernatants were removed and total

protein was quantitated by Bradford assay. Protein samples were diluted 1:1 (v/v) with 2× Laemmli sample buffer containing 5% β-mercaptoethanol and boiled for 5 min. A 10 µg aliquot of each protein sample was separated by SDS–PAGE as described above, transferred onto a nitrocellulose membrane and probed with anti-Hsp70 as described above. The experiment was performed in triplicate. Imperforate leaves were not selected for this experiment due to lack of healthy leaves post-treatment.

Pre-perforation, window and mature stage leaves from DMSO control, 15 µM PES-Cl and 25 µM PES-Cl conditions were harvested and their leaf blades were excised from the midrib, homogenized and prepared as mentioned previously. A 10 µg aliquot of each protein sample was separated by SDS–PAGE, transferred onto a nitrocellulose membrane and probed with anti-Hsp70 as described above. The experiment was performed in triplicate.

3.3.8. Measuring anthocyanin content after PES-Cl treatment

A 20 mg aliquot of midrib free tissue was excised from imperforate, pre-perforation, window and mature stage leaves of DMSO control and 25 µM PES-Cl-treated plants and macerated in 200 µl of formic acid/methanol (5:95, v/v). Samples were processed and assayed for anthocyanin content as described above. The experiment was performed in triplicate. Window and mature leaves from ROS and antioxidant treatments had their respective anthocyanin content measured in a previous study by Dauphinee et al. (2017).

3.3.9. Nitro blue tetrazolium (NBT) staining after PES-Cl treatment

Histochemical detection of O_2^- (superoxide) in window and mature stage leaves was performed using a modified protocol from (Grellet Bournonville and Díaz-Ricci, 2011). Leaves from 25 µM PES-Cl treatments and DMSO control groups were cut into 5 mm^2 sections and then incubated in stain solution consisting of 50 mM potassium phosphate buffer (pH 7.8), 10 mM sodium azide and 0.1% NBT (Sigma-Aldrich). The samples were then kept in the dark, vacuum infiltrated at 15 psi for 15 min and incubated at room temperature for 15 min before microscopic observation. A negative control underwent the same procedure with the solution lacking NBT. Post-staining, samples were mounted in distilled water and viewed using a Nikon Eclipse Ti microscope. To confirm the level of staining in leaf stages without the interference of anthocyanin and chlorophyll, samples were placed in 95% ethanol for 3 d. Three replicates were used for all groups.

3.3.10. *In vitro* YVAD peptidase substrate cleavage assay

Because caspase-1 inhibitors have been shown to inhibit perforation formation in lace plant leaves (Lord et al., 2013), the examination of CLP-1 biochemical activity (YVADase) is used to measure lace plant PCD activity. Leaf caspase-1 cleavage activity in both control and treated plants were measured. Midrib free tissue was excised from pre-perforation, window and mature leaves from water control, ROS-, antioxidant-, DMSO control and 25 µM PES-Cl-treated plants, rinsed, blotted dry, weighed and flash-

frozen in liquid nitrogen. Samples were then macerated in assay buffer (100 mM HEPES, 10% sucrose, 0.1% CHAPS, 5 mM DTT, pH 6.5) on ice for 1 min. Leaf tissue homogenate was centrifuged at 16 000 *g* at 4 °C for 15 min, and the supernatant was collected before Bradford assay determined protein concentration. Proteolytic activity was measured in 160 µl of reaction solution containing 30 µg of protein and 50 µM of AFC (antibody-forming cell)-conjugated peptide specific to mammalian caspase-1 (BioVision, #K110-100). Duplicate reactions were incubated at 37 °C for 1.5 h, and fluorescence readings of ice-cold samples were taken at 5 min intervals with excitation of 390/20 nm and an emission of 510/10 nm (Fluoroskan Ascent, Thermo Scientific). Readings were measured and compared against a blank containing assay buffer and the AFC-conjugated peptide, without lace plant protein. The kinetics of substrate hydrolysis were linear during the first hour of the reaction, after which the reaction curve plateaued. AFC standards at 0.005–0.5 µM, diluted in assay buffer, were used to calibrate the amount of fluorochrome released. The YVADase activity was calculated and presented in pmol min^{-1} mg $protein^{-1}$ cleaved. Active caspase-1 (BioVision, # 1081-25) was used as a positive control (data not shown). Each treatment contained an experimental control where leaves were treated with an equal volume of water or DMSO.

3.3.11. Image analysis and processing

Leaf images were captured using a Nikon L110 digital camera. Photoshop and Illustrator (Adobe Creative Cloud; Adobe Systems Inc.) were used to prepare images of leaves and western blots for publication. Images of detached leaves had backgrounds removed using

Photoshop. A Nikon AZ100 microscope acquired micrographs of window and mature stage leaves stained for NBT experiments. Image processing was consistent for all micrograph figures and included background removal, adjustments for brightness, contrast and colour balance.

3.3.12. Statistical analysis and data representation

One-way or Two-way ANOVA followed by a Tukey test was used to identify significant differences among means (α=0.05). All data are presented as the mean ±SE. Analyses were carried out using GraphPad Prism 5 software (GraphPad Software Inc.).

3.4. RESULTS

3.4.1. Anti-Hsp70 immunoreactivity

The anti-Hsp70 antibody reacted with a 70 kDa purified recombinant Hsp70 from *A. franciscana* and with 70 kDa sized bands contained in cell-free protein extracts from pre-perforation, window and mature leaves grown in axenic conditions (see Rowarth et al., 2020; Supplementary Figure S1).

3.4.2. Hsp70 and anthocyanin content during leaf development

Leaves were harvested from whole plants grown in axenic conditions (Figure 3.2.A) and probed for their anthocyanin content (Figure 3.2.B) and Hsp70 amounts (Figure 3.2.C–E). Anthocyanin content was significantly higher in pre-perforation (P) and window stage leaves (W) than in mature (M) and imperforate leaves (I; $P<0.05$). Pre-perforation and window stage leaves contained significantly higher amounts of Hsp70 than mature and imperforate leaves ($P<0.05$). Anthocyanin content and Hsp70 amounts between mature and imperforate leaves were not significantly different ($P>0.05$).

3.4.3. Whole-plant treatments with ROS, antioxidants and PES-Cl

Whole lace plants grown in axenic conditions were harvested to investigate the effects of the treatments on leaf development. These include: control (Figure 3.3.A), ROS (Figure 3.3.B), antioxidants (AO; Figure 3.3.C), as well as 25 µM PES-Cl (Figure 3.3.E) and its respective DMSO control (Figure 3.3.D). Mature leaf length (Figure 3.3.F) and perforation number (Figure 3.3.G) were measured. Control conditions generated mature leaves with an average length of 10.13±0.22 cm and 119.50±15.42 perforations. ROS-treated leaves were not significantly different in length (9.77±0.56 cm) and perforation number (124.17±15.77) compared with controls ($P>0.05$). Antioxidant-treated plants generated mature leaves with significantly fewer perforations (23.17±3.18) compared with the control ($P<0.05$) but were not different in length (9.83±0.72 cm). All PES-Cl treatments generated mature leaves with lengths that were not significantly different from control and DMSO conditions (10.9±0.5 cm), but concentrations of 25 µM PES-Cl significantly decreased perforation numbers (103.89±8.25).

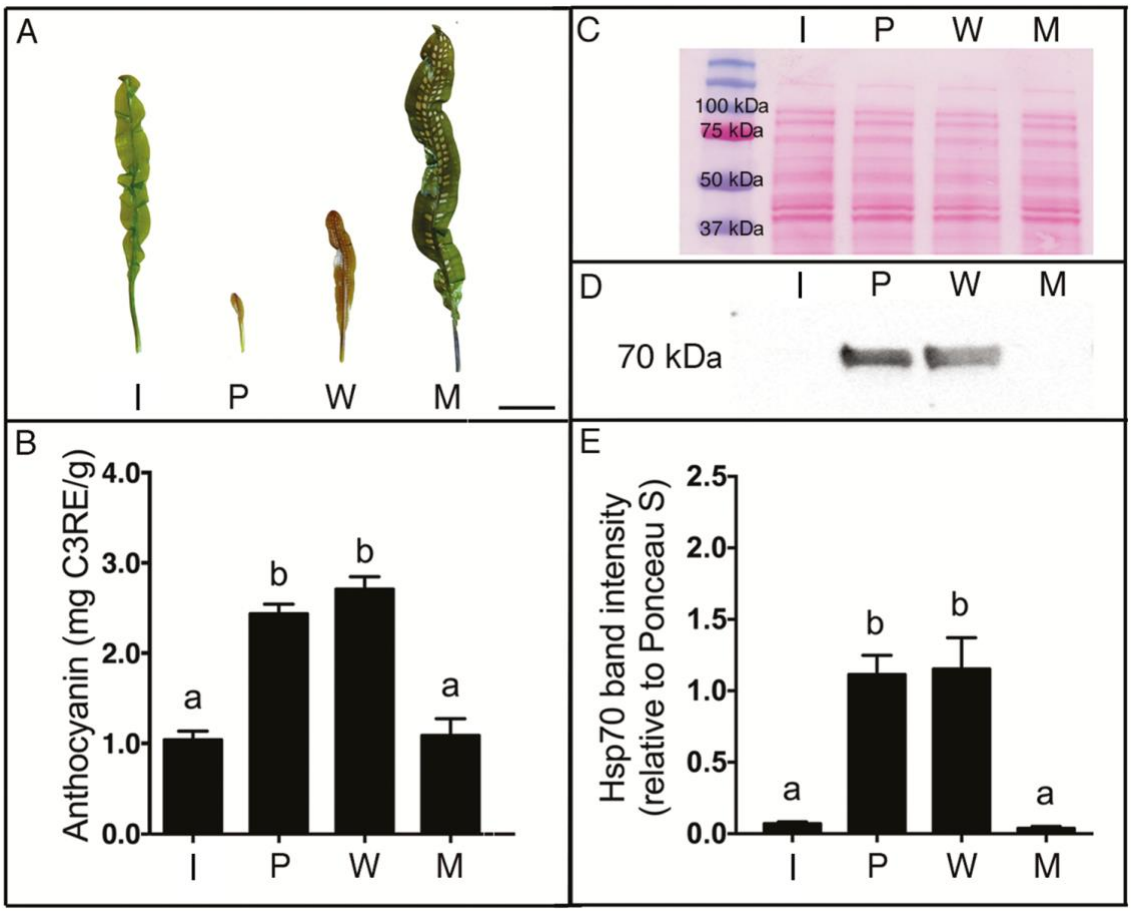

Figure 3.2. Hsp70 and anthocyanin content in lace plant leaf development. (A) Whole leaves harvested from axenic conditions in Magenta boxes: imperforate (I), pre-perforation (P), window (W) and mature (M). (B) Anthocyanin content was quantitated by spectrophotometry for whole-leaf stages, and anthocyanin was plotted as cyaniding-3-rutinoside equivalents (C3REs) and averaged. Equivalent amounts of each whole-leaf protein extract were separated by SDS–polyacrylamide gels, blotted to nitrocellulose and stained with Ponceau S (C) before being probed for Hsp70 protein (D). (E) Immunoreactive protein bands were quantitated, and the ratio of Hsp70 to Ponceau S signal was calculated and averaged. Means not sharing any letter are significantly different. One-way ANOVA, Tukey test ($P<0.05$; $n=3$). Error bars represent the SE. Scale bar: A=2 cm.

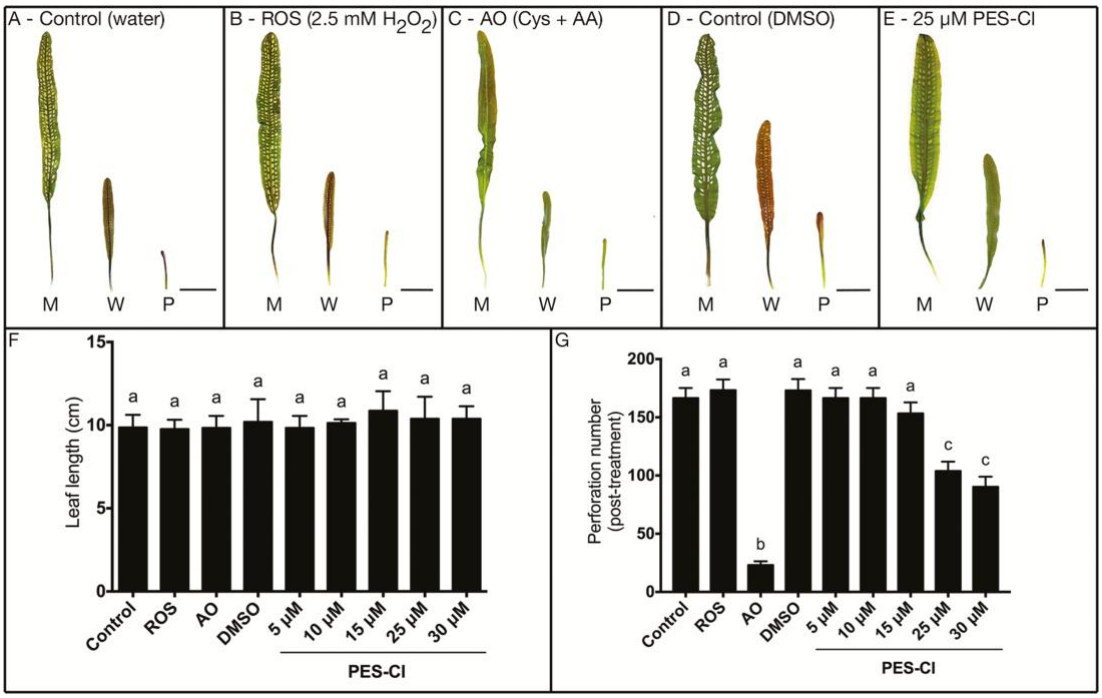

Figure 3.3. ROS, antioxidant and PES-Cl treatment effects on perforation formation. Leaf layouts following week-long treatments of control (A), 2.5 mM H_2O_2 (ROS; B), an antioxidant (AO) combination of 400 µg ml^{-1} ascorbic acid (AA) and 200 µg ml^{-1} L-cysteine (Cys; C), DMSO (D) and 25 µM chlorophenylethynylsulfonamide (PES-Cl; E). Post-treatment mature mean leaf lengths (F) and the mean number of perforations (G) were quantitated. Means not sharing any letter are significantly different. One-way ANOVA, Tukey test (P<0.05, n≥3). Error bars represent the SE. *Scale bars*=2 cm.

3.4.4. ROS, antioxidants and PES-Cl alter Hsp70 amounts in lace plant leaves

Band intensities of Hsp70 protein (normalized to the control pre-perforation mean) were quantitated for pre-perforation, window and mature leaves treated with ROS or antioxidants to investigate if these compounds alter Hsp70 amounts (Figure 3.4.A–C). Under control conditions, pre-perforation and window stage leaves had significantly higher Hsp70 amounts than mature stage leaves (P<0.05, Figure 3.4.C). Pre-perforation

leaves from ROS-treated plants had significantly higher Hsp70 amounts than controls ($P<0.05$), but there was no effect observed in window or mature leaves. Pre-perforation and window leaves from antioxidant-treated plants had significantly lower Hsp70 amounts compared with controls, but there was no effect observed in the mature leaves. Hsp70 amounts were also quantified in leaf stages treated with DMSO, 15 μM PES-Cl and 25 μM PES-Cl to investigate if PES-Cl affected the amounts of Hsp70 (Figure 3.4.D–F). Plants treated with 15 μM and 25 μM PES-Cl had significantly higher Hsp70 amounts in pre-perforation and window leaves compared with DMSO conditions ($P<0.05$). Hsp70 amounts in mature leaves were significantly lower than in pre-perforation and window leaves in plants treated with 15 μM and 25 μM PES-Cl. Hsp70 amounts in mature leaves in plants treated with 15 μM and 25 μM PES-Cl were not significantly different from DMSO controls (Figure 3.4.F).

Hsp70 was increased 4-fold in pre-perforation and window leaves from plants treated with 15 μM and 25 μM PES-Cl. The 25 μM PES-Cl treatment was selected to measure Hsp70 manipulation effects on anthocyanin content, ROS abundance and CLP activity in lace plant leaves due to its ability to significantly reduce perforations formed.

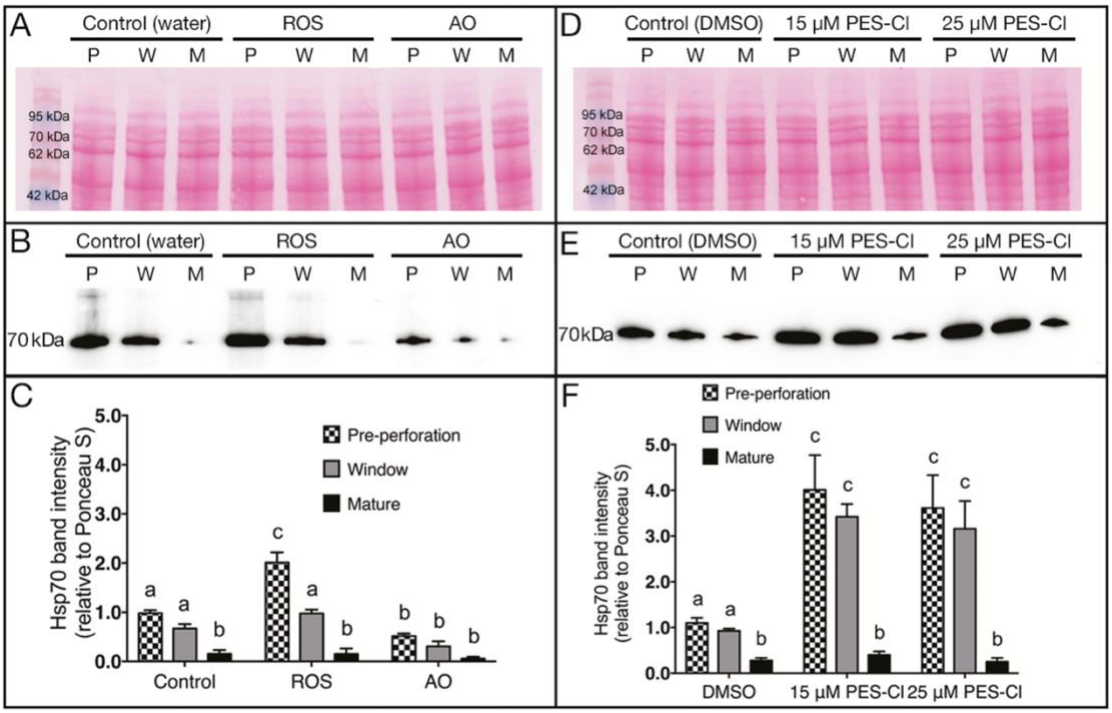

Figure 3.4. Hsp70 amounts of ROS-, antioxidant- and PES-Cl-treated leaves. Protein homogenates of pre-perforation (P), window (W) and mature (M) stage leaves harvested from (A–C) control, ROS, antioxidant (AO), or (D–F) DMSO, 15 µM PES-Cl and 25 µM PES-Cl treatment conditions were resolved by SDS–polyacrylamide gels and blotted to nitrocellulose. Blots were stained in Ponceau S (A and D) before being probed for Hsp70 protein (B and E). (C and F) Immunoreactive protein bands were quantitated, and the ratio of Hsp70 to Ponceau S signal was calculated and averaged. Means not sharing any letter are significantly different. Two-way ANOVA, Tukey test ($P<0.05$; $n=3$). Error bars represent the SE.

3.4.5. Anthocyanin content declined in PES-Cl-treated leaves

The anthocyanin content of imperforate, pre-perforation, window and mature stage leaves from plants treated with 25 μM PES-Cl were compared with DMSO controls using spectrophotometry. Treated mature and imperforate leaves possessed the lowest anthocyanin content compared with pre-perforation and window stage leaves ($P<0.05$) and were not significantly different from DMSO-treated leaves ($P>0.05$). Treated pre-perforation and window leaves possessed the highest anthocyanin content and were significantly lower than DMSO controls (Figure 3.5.). ROS (1 mM H_2O_2) treatment as described in Dauphinee et al. (2017) did not affect anthocyanin content in window or mature leaves. Antioxidant treatment (the same conditions as this study) produced significantly decreased anthocyanin content in window leaves but did not affect mature leaves compared with control (Dauphinee et al., 2017).

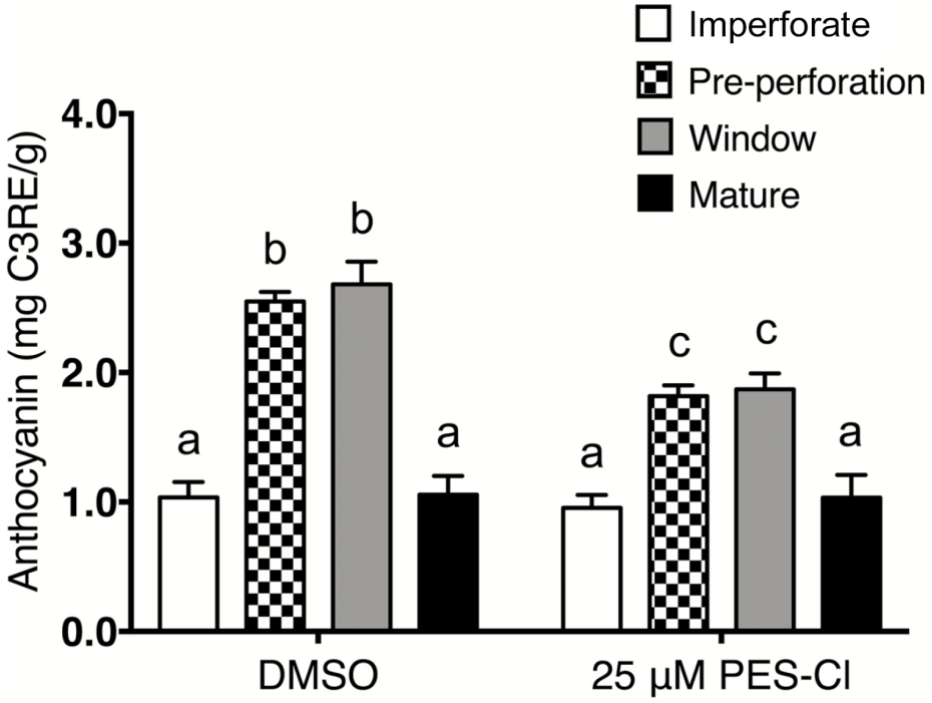

Figure 3.5. Anthocyanin content for PES-Cl-treated leaves. Anthocyanin content measured in imperforate, pre-perforation, window and mature leaves from DMSO and 25 µM PES-Cl treatment conditions and plotted as cyaniding-3-rutinoside equivalents (C3REs). Means not sharing any letter are significantly different. Two-way ANOVA, Tukey test. (P<0.05, n=3). Error bars represent the SE.

3.4.6. ROS abundance declined in PES-Cl-treated leaves

Lace plant leaves exposed to 25 µM PES-Cl treatment were investigated for superoxide production using NBT staining (Figure 3.6.). Window stage leaves treated with 25 µM PES-Cl (Figure 3.6.B) had less NBT staining in the centre of the areole compared with control PCD cells (arrows, Figure 3.6.A). NPCD cells of both control and 25 µM PES-Cl-treated window stage leaves did not stain. Mature leaves treated with 25 µM PES-Cl exhibited little NBT staining throughout areoles, which was similar to controls.

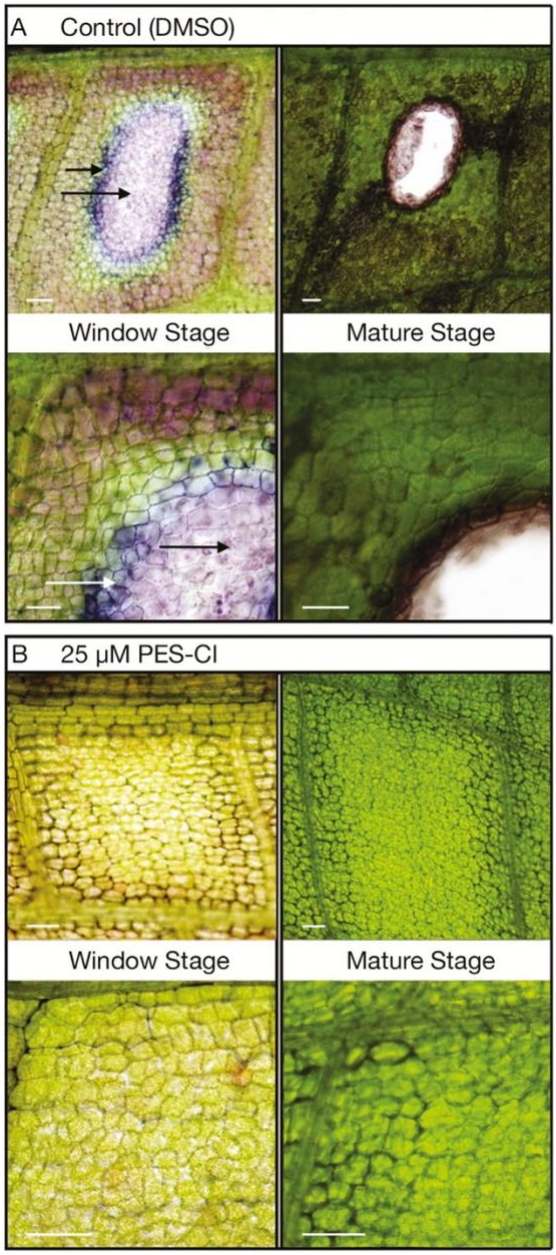

Figure 3.6. Superoxide detection in PES-Cl-treated leaves by nitro blue tetrazolium (NBT). Window and mature leaves harvested from whole plants treated with either DMSO (A) or 25 µM PES-Cl (B) stained with NBT to detect ROS abundance. Cells stained with NBT show superoxide accumulation in leaf tissue (arrows). Samples stained in control solution are not shown. *Scale bars* = 40 µm.

3.4.7. YVADase-like activity declines in PES-Cl-treated leaves

The *in vitro* activity of YVAD–AFC peptide substrates was measured to investigate CLP activity in development of lace plant leaves exposed to ROS, antioxidants and 25 μM PES-Cl. Different leaf stages from whole plants treated with ROS, antioxidant and 25 μM PES-Cl were harvested, and their YVADase activities were compared (Figure 3.7.). Pre-perforation stage leaves had higher YVADase activities than window leaves that possessed significantly higher YVADase activities than mature leaves from plants treated in control conditions or treated with ROS or DMSO ($P<0.05$). ROS-treated pre-perforation leaves had the highest overall YVADase activities. YVADase activities were not significantly different among pre-perforation, window and mature leaves from plants treated with 25 μM PES-Cl or antioxidant conditions ($P>0.05$).

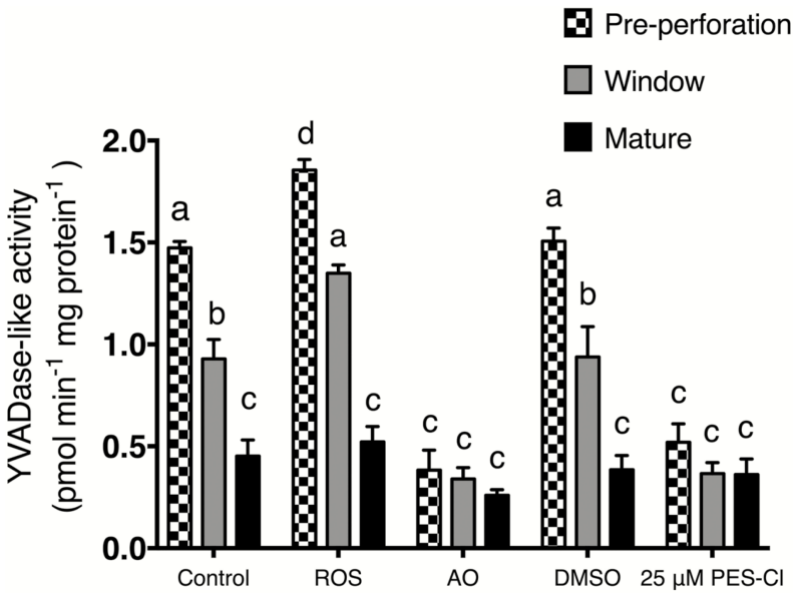

Figure 3.7. YVADase activity for ROS-, antioxidant- and PES-Cl-treated leaves. Kinetics of YVADase activity for pre-perforation, window and mature stage leaves harvested from control, ROS, antioxidant combination (AO), DMSO and 25 µM PES-Cl whole-plant treatment conditions. Means not sharing any letter are significantly different. Two-way ANOVA, Tukey test. (P<0.05, n=3). Error bars represent the SE.

3.5. DISCUSSION

The lace plant has emerged as a model for studying leaf morphogenesis as it produces leaf perforations via developmental PCD. The retention of anthocyanin in NPCD cells and disappearance of anthocyanin and chlorophyll in EPCD and LPCD cells within an areole creates a gradient of PCD, but the molecular mechanisms that control the separation between PCD and NPCD cells are not yet known. PCD also plays an important role in stress tolerance. Hsp70s are synthesized in plants during stress-induced and developmentally regulated PCD (Jiang and Wang, 2004). However, there are no previous studies on Hsp70 and PCD in lace plants or other *Aponogetonaceae* members.

Due to the role of Hsp70 and its co-chaperones in refolding denatured and nascent proteins to ensure proper development in animals and plants (Rowarth and MacRae, 2018a, 2018b; Sable et al., 2018), we investigated its role in the novel lace plant model. We probed for Hsp70 in lace plants and showed that its relative abundance is likely to play a role in lace plant leaf development. There are low amounts of Hsp70 in imperforate and mature lace plant leaves but increased amounts in pre-perforation and window stages where PCD is active (Figure 3.2.). The fact that Hsp70 amounts change during development indicates a differential regulation of Hsp70s throughout leaf development in lace plants. We cannot rule out the synthesis of other Hsps playing a role in lace plant leaf development. Hsp70 amounts declined significantly in mature leaves, implying that Hsp70 functions mainly during early development and when PCD is active, a time when refolding of proteins can influence PCD pathways (Williams et al., 2014).

Lace plant leaves were exposed to ROS and antioxidants to investigate the synthesis of Hsp70 under treatments that promote and inhibit plant PCD, respectively (Dauphinee et al., 2017; Gadjev, 2006; Gechev et al., 2006; Petrov et al., 2015). ROS induced 2-fold higher Hsp70 amounts in pre-perforation leaves, whereas antioxidants lowered Hsp70 amounts in pre-perforation and window stage leaves (Figure 3.4.). Hsp70 has been well documented as a molecular chaperone that counters ROS accumulation under stress conditions such as heat, UV, high light and pathogens (Scarpeci et al., 2008). It is most likely that ROS induce Hsp70; however, it is unclear if antioxidant treatment affects Hsp gene expression or if it is reducing ROS accumulation in lace plant leaves, which indirectly results in lower Hsp70 amounts (Qi et al., 2019).

Whole-plant treatment with 25 µM PES-Cl led to 4-fold increased Hsp70 amounts and decreased numbers of leaf perforations. PES-Cl treatment was similar in nature to the ROS treatment in that it significantly increased Hsp70 amounts, although different in magnitude (Figure 3.4.). In contrast, PES-Cl treatment was different from ROS treatment due to generating significantly fewer perforations, whereas ROS-treated (2.5 mM H_2O_2) leaves did not significantly change numbers of leaf perforations. Antioxidant treatment (400 µg ml^{-1}L-ascorbic acid and 200 µg ml^{-1}L-cysteine) significantly decreased Hsp70 amounts and resulted in fewer perforations, more so than 25 µM PES-Cl treatment. Even though 15 µM PES-Cl treatment increased Hsp70 amounts 4-fold, it did not decrease the number of perforations formed (Figure 3.3.). Due to the unknown specificity of PES-Cl with lace plant Hsp70 isoforms, we speculate that other Hsp70 activities could have been affected in leaves subjected to 25 µM PES-Cl treatment but not to 15 µM PES-Cl treatment. Departure from typical Hsp70 amounts affected lace plant leaf remodelling at a threshold, which was achieved by antioxidant and 25 µM PES-Cl treatment. PES-Cl has been shown to inhibit Hsp70 activity and development in *Gossypium hirsutum* ovules (Sable et al., 2018).

Treatment of lace plants with PES-Cl (25 µM) resulted in lower anthocyanin content in pre-perforation and window stage leaves (Figure 3.5.), consequently decreasing the abundance of superoxide detectable by NBT staining in window stage leaves (Figure 3.6.). This result is comparable with the antioxidant treatment, which also results in lower anthocyanin content and superoxide abundance (Dauphinee et al., 2017). The up-regulation of Hsp70 may stabilize mitochondrial membrane potential and chloroplast

integrity, which normally destabilize during late PCD, where ROS generation is highest (Wright et al., 2009). The lower anthocyanin content in 25 µM PES-Cl-treated leaves is in contrast to ROS treatment, which increased superoxide abundance in window stage leaves (Dauphinee et al., 2017). It is noteworthy that the overexpression of *Arabidopsis* heat shock-related protein (AtHSRP) increases the activity of ROS scavengers such as superoxide dismutase and catalase in *Arabidopsis* and *N. benthamiana* (Yang et al., 2015), two scavengers that are also active in developing window stage leaves (Dauphinee et al., 2017). How and where Hsp70s are active within PCD and NPCD cells will require further immunolocalization studies with more specific antibodies.

Previous studies have suggested that CLPs and vacuolar processing enzymes (VPEs) promote lace plant PCD (Lord et al., 2013; Lord and Gunawardena, 2012; Rantong and Gunawardena, 2018). Treatment with 25 µM PES-Cl also inhibited CLP activity in pre-perforation and window stage leaves but did not affect YVADase activity in mature stage leaves (Figure 3.7.). These results may imply that Hsp70 interacts with proteins that inhibit CLP activity. Leaves with fewer perforations possessed lower CLP activity in pre-perforation and window stages, indicating CLP's possible association with lace plant PCD. These results concur with lace plant leaf treatment with the caspase-1 inhibitor cyclosporin A (CsA; Lord et al., 2013). CsA treatment inhibited mitochondrial collapse and actin breakdown in PCD cells. Increased Hsp70 may also prevent mitochondrial collapse and consequently prevent CLP cleavage that promotes PCD (Qi et al., 2011). Leaves exposed to ROS treatment had up-regulated CLP activity in pre-perforation and window stage leaves, which agrees with the increased rate of PCD in leaves under ROS

exposure (Dauphinee et al., 2017). Antioxidant-treated leaves demonstrated similar CLP activity compared with leaves in the 25 μM PES-Cl treatments. This result indicates that antioxidants and Hsp70 are similar in their ability to affect downstream CLP activity in the lace plant PCD pathway.

Hsp70s bind and protect protein substrates during stress from heat, salinity and oxidation. Newly unfurled lace plant leaves that depart from typical amounts of Hsp70, at a threshold, grew into healthy leaves with fewer perforations. Hsp70 may assist in the translocation of signalling factors or transcription factors to the nuclei (Craig, 2018; Rosenzweig et al., 2019; Wang et al., 2004) that inhibit PCD signalling in PCD cells. Silencing of *CaHsp70* in *N. benthamiana* leaves also inhibited ROS burst, as well as salicylic and jasmonic acid accumulation, indicating a role for Hsp70 during defence signalling and hypersensitive response (Kim and Hwang, 2015). This suggests possible crosstalk between stress hormones such as abscisic acid (ABA), Hsps and ROS that may contribute to antioxidant activity (Li et al., 2014). Shedding light on these interactions within the lace plant will require further investigation.

Antioxidant treatment had more of an inhibitory effect on PCD induction in window stage leaves than 25 μM PES-Cl treatment even though both treatments resulted in a reduction in ROS, anthocyanin and CLP activity. It was noted that these treatments had no significant effect on Hsp70 amounts in mature leaves. Additionally, PES-Cl did not affect the anthocyanin content of imperforate leaves. These results can either be explained by mature and imperforate leaf stages using another isoform of Hsp70 for

development or by these leaf stages being less sensitive to the treatment. It is unknown why PES-Cl does not affect mature stage leaves but may explain the differential roles of Hsp70, anthocyanin, ROS and CLP activity in leaves that are active in PCD and leaves that have reached homeostasis.

Using the observations from previous studies on the lace plant and results from this investigation, we proposed a hypothetical model (Figure 3.8.) that separates PCD from NPCD cells during lace plant perforation formation. Currently we are investigating differentially expressed genes among each of the developmental leaf stages, NPCD and PCD cells using RNA sequencing. The global transcriptomic analysis will provide data that assist in identifying transcripts that promote the initiation and execution of developmental PCD in leaves of the lace plant. Additionally, the transcriptomic analysis will reveal differentially expressed isoforms of Hsps such as Hsp70 found in imperforate and adult leaves that will add to our understanding of its role in lace plant leaf remodelling.

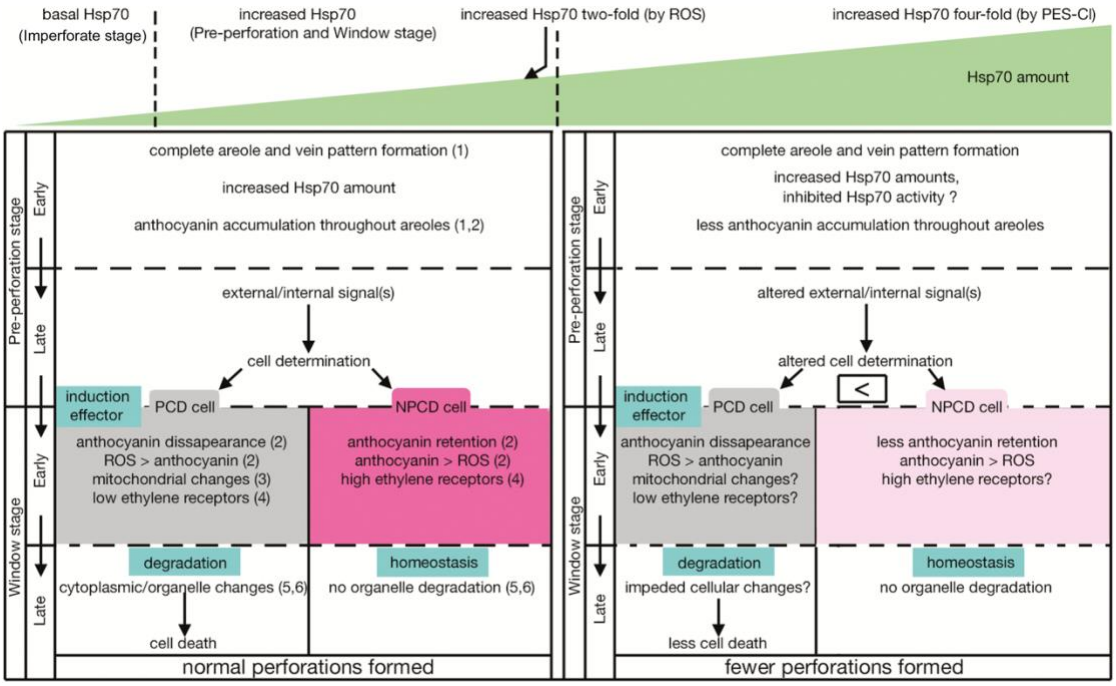

Figure 3.8. A theoretical model for Hsp70's role in lace plant leaf remodelling. Vein and areole formations are completed in early pre-perforation stage leaves where anthocyanin is evenly distributed in between the vasculature. The internal signals that control cell determination for death (PCD cells) or survival (NPCD cells) remain unknown, but intracellular events that occur during PCD are documented in window stage leaves. The effects of Hsp70 on leaf remodelling seem to affect anthocyanin content and ROS abundance in window stage leaves while affecting perforation formation or developmental PCD. How and when Hsp70 affects this theoretical mechanism remains unclear. The departure from typical Hsp70 amounts such as the significant increase (>4-fold) by 25 µM PES-Cl possibly leads to reduced Hsp70 activity and a decrease in the number of perforations formed. ROS, reactive oxygen species; PCD, programmed cell death; NPCD, non-programmed cell death. (1) Gunawardena et al., (2004); (2) Dauphinee et al. (2017); (3) Lord et al. (2013); (4) Rantong et al. (2015); (5) Wertman et al. (2012); (6) Wright et al. (2009).

3.6. CONCLUSIONS

We find that significantly increased (>4-fold) or decreased Hsp70 amounts influenced PCD during leaf development. Departure from typical Hsp70 amounts affected lace plant leaf remodelling at a threshold (lower anthocyanin content, ROS abundance, CLP activity and fewer perforations formed). Our results suggest that Hsp70 plays a role in PCD

during the remodelling of leaves of the lace plant. See Rowarth et al. (2020) Supplementary Figure S2 that summarizes the effects of all treatments used in this study on lace plant leaves.

3.7. AUTHOR CONTRIBUTIONS

NMR carried out all experimentation; AND and GLD contributed to whole-plant treatments, leaf imaging and morphology recording; NMR drafted the first manuscript, prepared figures and revised the final manuscript. AND drafted figures initially and contributed to manuscript revisions. GLD contributed to manuscript revisions; NMR and AHLANG designed and AHLANG supervised all the experiments and helped in manuscript revisions.

3.8. ACKNOWLEDGEMENTS

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CHAPTER 4: THE ROLE OF ATG16 IN LACE PLANT PCD

The work presented in Chapter 4 is currently under review titled as:

Rowarth, N. M., Dauphinee, A. N., Lacroix C. R., and Gunawardena, A. H. L. A. N. (2022). The role of Atg16 in autophagy, anthocyanin biosynthesis and programmed cell death in leaves of the lace plant (*Aponogeton madagascariensis)*. Plos One; submission ID: PONE-D-22-23463.

4.1. ABSTRACT

Aponogeton madagascariensis, commonly known as the lace plant, produces leaves that form perforations by programmed cell death (PCD). Leaf development is divided into several stages beginning with "pre-perforation" furled leaves enriched with red pigmentation from anthocyanins. The leaf blade is characterized by a series of grids known as areoles bounded by veins. As leaves develop into the "window stage", anthocyanins recede from the center of the areole towards the vasculature creating a gradient of pigmentation and cell death. Cells in the middle of the areole that lack anthocyanins undergo PCD (PCD cells) while cells that retain anthocyanins (non-PCD cells) maintain homeostasis and persist in the mature leaf. Autophagy has reported roles in survival or PCD promotion across different plant cell types. However, the direct involvement of autophagy in PCD and anthocyanin levels during lace plant leaf development has not been determined. Previous RNA sequencing analysis revealed the upregulation of autophagy-

related gene *Atg16* transcripts in pre-perforation and window stage leaves, but how Atg16 affects PCD in lace plant leaf development is unknown. In this study, we investigated the levels of Atg16 in lace plant PCD by treating whole plants with either an autophagy promoter rapamycin or inhibitors concanamycin A (ConA) or wortmannin. Following treatments, window and mature stage leaves were harvested and analyzed using microscopy, spectrophotometry and western blotting. Western blotting showed significantly higher Atg16 levels in rapamycin-treated window leaves, coupled with lower anthocyanin levels. Wortmannin-treated leaves had significantly lower Atg16 protein and higher anthocyanin levels compared to the control. Mature leaves from rapamycin-treated plants generated significantly fewer perforations compared to control, while wortmannin had the opposite effect. However, ConA treatment did not significantly change Atg16 levels, nor the number of perforations compared to the control, but anthocyanin levels did increase significantly in window leaves. We propose autophagy plays an important role in maintaining optimal anthocyanin levels leading to both NPCD cell survival and mediating a timely cell death in PCD cells in developing lace plant leaves. How autophagy specifically affects anthocyanin levels remained unexplained.

4.2. INTRODUCTION

4.2.1. Plant Programmed Cell Death

Programmed cell death (PCD) is a highly controlled cellular process that removes compromised cells from environmentally induced stress or designated cells by

developmental regulation to respectively achieve survival or tissue remodeling (Bozhkov and Lam, 2011; Danon et al., 2000; Huysmans et al., 2017). Common examples of developmental PCD in plants used to achieve higher tissue organization include aerenchyma formation and xylem differentiation, suspensor and tapetum cell deletion, the dismantling of rapid growing root tip cells and organ senescence (Du et al., 2018; Filonova et al., 2000; Gunawardena et al., 2001a, 2001b; Kumpf and Nowack, 2015; Ni et al., 2014; Reza et al., 2018; Yan et al., 2020). The molecular pathways that control PCD in animal models are relatively well identified compared to those involved in plant developmental PCD which is less understood (Filonova et al., 2000; Kabbage et al., 2017; Minina et al., 2021).

4.2.2. Plant Autophagy

In many plant systems, developmental and environmentally-induced PCD execution can be influenced by the ubiquitous eukaryotic cellular process of autophagy (Bassham, 2007; Liu and Bassham, 2012; Minina et al., 2013; Üstün et al., 2017). Autophagy is a process that sequesters and reutilizes intracellular contents mediated by double-membrane vesicles called autophagosomes which shuttle contents to the lysosome (animals) or vacuole (plants) for degradation and recycling (Bozhkov, 2018; Dauphinee et al., 2019; Minina et al., 2013). The autophagy phenomenon in plants can play a dual role in promoting or inhibiting PCD making the ability to distinguish the exact function of autophagy in plant PCD difficult (Bozhkov, 2018; Coll et al., 2014; Li et al., 2016b; Thanthrige et al., 2021). To study the core machinery behind autophagosome formation

and fusion to lytic bodies, autophagy-related genes (Atgs) across eukaryotic models have been investigated and characterized. For example, in animal systems, the Atg5-Atg12-Atg16 E3-like complex tethers the Atg8 to the originating phagophore by conjugation to Atg8-PE before the phagophore completes closure (Chung et al., 2010; Feng et al., 2022; Le Bars et al., 2014; Mitou et al., 2009; Yang and Klionsky, 2009, 2010a, 2010b). In plants, the impact of Atg16 on autophagy regulation is not determined compared to Atg8 which has been studied more extensively (Chen et al., 2021;).

4.2.3. The Lace Plant PCD Model System

The aquatic monocot *Aponogeton madagascariensis* (commonly known as the lace plant) is an emerging model system to investigate plant developmental PCD (Dauphinee and Gunawardena, 2015; Gunawardena et al., 2004; Gunawardena, 2008). Leaves of the lace plant form laminar perforations during normal development by PCD (Dauphinee and Gunawardena, 2015; Gunawardena et al., 2004). Leaves emerge from the corm in a heteroblastic series, and leaf development has been defined into several stages. The first 3 to 4 “imperforate” leaves that emerge do not form perforations by maturity. Successive leaves form perforations and go through the following stages of development. Pre-perforation leaves emerge furled and the lamina tissue framed by the vasculature (called areoles) is enriched with red pigment from the accumulation of anthocyanins. As leaves develop into the window stage anthocyanin recedes towards the veins and a gradient of red pigment remains. Cells central to the areole are void of pigment and destined for death (PCD cells). Cells undergoing PCD die off from the center of the areole

progressively towards the veins up until 4-5 living cell layers are left adjacent to the veins. Cells that retain anthocyanin will eventually lose their red pigmentation during development and maintain homeostasis; designated as non-PCD, NPCD cells (Dauphinee et al., 2017; Gunawardena et al., 2021).

The lace plant is an emerging model system (Figure 4.1.) to study plant PCD due to the predictable nature of the spatiotemporal separation of NPCD and PCD cells within areoles. The natural translucency of lace plant leaves is ideal for live-cell microscopy. Additionally, the established protocol for the propagation of whole lace plants in sterile axenic environments allows for pharmacological studies (Dauphinee and Gunawardena, 2015; Gunawardena et al., 2004; Kacprzyk et al., 2016). The chronological order of intra-cellular events in lace plant areoles during PCD is well characterized (Dauphinee et al., 2012, 2014, 2017; Lord et al., 2013; Lord and Gunawardena, 2011; Wright et al., 2009) but the molecular pathways that are critical to lace plant PCD are less well understood.

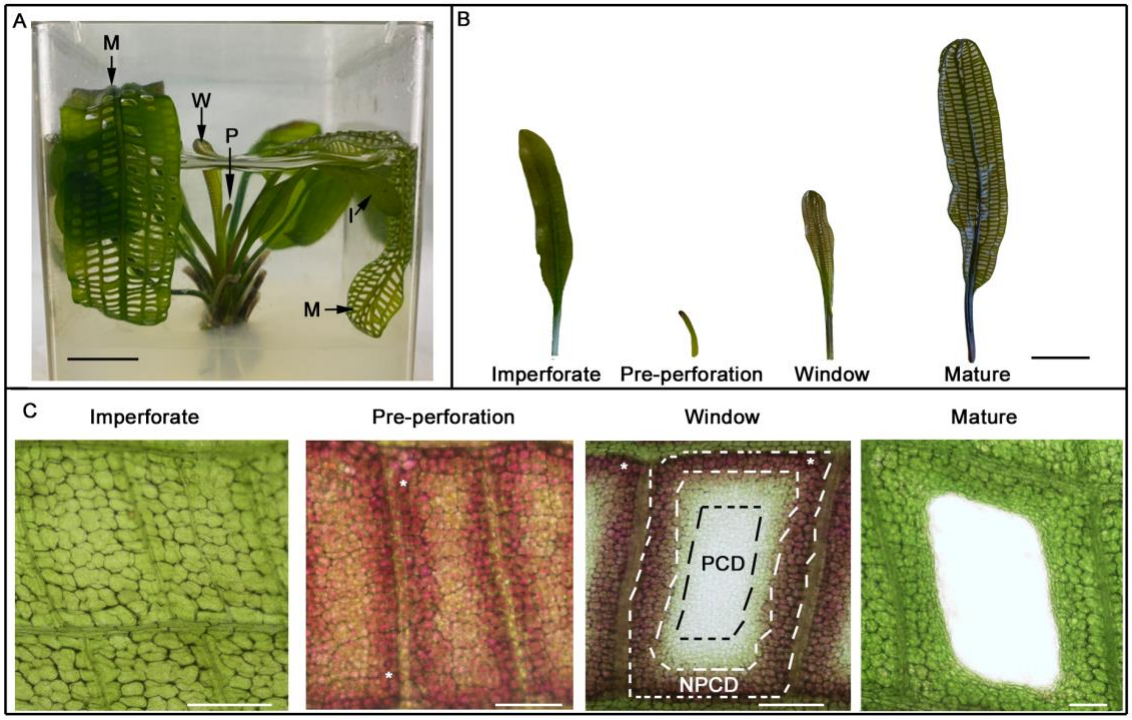

Figure 4.1. The lace plant programmed cell death (PCD) system. (A) Lace plants are grown in axenic Magenta box culture; pre-perforation stage (P), window stage (W), mature stage (M) and imperforate leaves (I). (B) Representative leaves at different stages of development. Imperforate leaves are the first 3-4 leaves to emerge from the corm and do not produce perforations. Pre-perforation stage leaves emerge from the corm with anthocyanin pigmentation. These eventually develop visible areolar "windows" that will eventually become perforated in mature leaves. (C) Details of PCD process. PCD does not take place in imperforate leaves. In pre-perforation leaves, anthocyanin pigmentation is visible, especially at the periphery of the areole (*asterisks*). PCD can be seen actively occurring in the window stage of development as a gradient of cell death. Non-PCD cells (NPCD, bounded by *white dashed lines*) persist beyond maturity, early-PCD cells (EPCD, bounded by inner *white dashed lines* and *black dashed lines*) have lost their anthocyanins and are destined to die and late-PCD cells (LPCD, bounded by *black dashed lines*) are nearly transparent and on the verge of death. Perforation formation is completed in mature stage leaves. Anthocyanin abundance is visibly reduced, and homeostasis for NPCD cells is reached. *Scale bars*: A=1 cm, B=2 cm, C=70 µm.

4.2.4. Investigating The Role of Autophagy in Lace Plant PCD

The autophagy process is important to characterize across plant diversity due to its capability of promoting or inhibiting PCD in plant stress response or development although its precise place in the PCD pathway is uncertain (Minina et al., 2013). Knockdown experiments across different plant tissues have shown individual Atg proteins play a direct role in autophagy and PCD performance during development (Minina et al., 2013). RNA interference (RNAi) of *Atg5* and *Atg6* suppresses vacuolar cell death and promotes uncontrolled necrosis in suspensor cells of developing *Picea abies* embryos (Petersen et al., 2014). Knockout of core autophagy gene transcripts suppresses hypersensitive cell death in *Arabidopsis* and *Nicotiana benthamiana* leaves under pathogen infection (Coll et al., 2014; Hackenberg et al., 2013; Han et al., 2015; Hofius et al., 2009; Lai et al., 2011; Liu et al., 2005). The distinct roles of autophagosome formation and vacuolar breakdown in PCD initiation, execution and inhibition may be different in plant cells destined for death or survival (Minina et al., 2013). The lace plant PCD model system is a uniquely positioned system to further elucidate the mechanisms of plant autophagy. The NPCD/PCD cell 'gradient' of lace plant window leaves provide a tractable platform to investigate autophagy promotion or inhibition in a cell-specific setting (Dauphinee et al., 2019) similar to the *P. abies* embryo-suspensor system (Filonova et al., 2000; Minina et al., 2013; Reza et al., 2018) to better understand the involvement of autophagy in plant PCD.

Dauphinee et al., (2019) utilized commercially available autophagy modulators rapamycin, wortmannin and concanamycin A (ConA) to treat lace plants to determine if autophagic activity helped promote or inhibited lace plant PCD. Their findings showed that the promotion or inhibition of autophagy did not affect the induction of developmental PCD, evidenced by no significant change in the number of perforations formed in mature leaves. However, the promotion of autophagy significantly decreased the rate of cell death events in late-stage PCD cells (Dauphinee et al., 2019) while autophagy inhibitors had the opposite effect. These results indicated that autophagy is active in PCD and NPCD cells during lace plant leaf development but mainly promotes cell survival. However, the authors speculate a possible dual role exists for autophagy occurring between both cell types.

The only Atg protein investigated in lace plant development is Atg8 which is detectable in NPCD and PCD cells by using immunostaining but not differentially expressed between cell types and leaf stages (Dauphinee et al., 2019; Rowarth et al., 2021). Additionally, a transcriptomic analysis of each developmental stage of lace plant leaves revealed that transcripts for *Atg16* and *Atg18a* are significantly up-regulated in pre-perforation leaves and window leaves compared to mature leaves and imperforate leaves (Rowarth et al., 2021) while other detectable *Atgs* genes remained unchanged throughout leaf development. Atg16 has been implicated in the regulation of autophagosome formation in animals and yeast but its specific role in plant autophagy and PCD is not confirmed (Chen et al., 2021; Young et al., 2019).

The goal of this study is to characterize Atg16 levels during lace plant leaf development and to further elucidate the role of plant autophagy and its involvement in lace plant leaf development and PCD. We use previously optimized treatment protocols of known autophagy modulators rapamycin, wortmannin, or ConA on lace plants to compare Atg16 levels, anthocyanin concentration and the formation of perforations in leaves at different stages of development. As a working hypothesis, we propose that autophagy activity promotion by modulation leads to a significant increase in Atg16 levels and a decrease in window stage leaf anthocyanin accumulation which ultimately inhibit PCD in terms of the number of perforations formed in lace plant leaves.

4.3. MATERIALS AND METHODS

4.3.1. Lace plant propagation and treatments

Whole lace plant cultures were aseptically propagated as described in Gunawardena et al. (2006). Newly obtained isolate corms of lace plants [*A. madagascariensis* (Mirbel) H. Bruggen] were obtained from The PlantGuy (Alberta, Canada) and cultured in GA-7 Magenta boxes, embedded fully in solid MS media [100 ml of 1.5% plant tissue culture agar (w/v, Phytotech Laboratories) in liquid MS (3% sucrose (w/v), 0.01% Myo-inositol (w/v), 0.215% MS basal salts (w/v) Phytotech Laboratories), 0.0025% thiamine-HCl (v/v), pH 5.7] and then submerged in 150 ml of liquid MS. Whole plant cultures were grown at 24 °C and exposed to 12 h light: 12 h dark cycles with levels of 125 µmol m^{-2} s^{-1} daylight deluxe fluorescent light bulbs (Philips). Cultured plants were only selected for

pharmacological experimentation after 30 d of growth and the production of 3 perforated mature leaves to control for variation in plant growth.

Plants selected for pharmacological experiments were treated for 1 week with either (i) 5 µM rapamycin (Enzo Scientific, BML-275), (ii) 5 µM wortmannin (Cayman Chemical, 10010591), or (iii) 1 µM ConA (Santa Cruz Biotechnology, sc-202111). Control plants received an equal volume of dimethyl sulfoxide DMSO (<0.1% v/v; BioShop Canada, DMS666). A minimum of six replicates were performed for each group (four groups total).

4.3.2. RNA extraction from lace plant leaf stages

RNA was extracted from individual leaf stages from plants grown for 30 d under normal conditions to measure accumulation of *Atg16* mRNA during lace plant leaf development. Mid-rib free leaf lamina tissue samples were washed with distilled water, blotted dry and flash-frozen. RNA was extracted from 40 mg of frozen tissue from one of each imperforate, pre-perforation, window, or mature stage leaves from 3 different whole-plant cultures and processed as per instructions for the ReliaPrep RNA Kit (Promega). RNA samples were treated with DNAse I (Thermo Fisher). Eluted RNA was quantified using a Nanodrop spectrophotometer (Thermo Fischer) by measuring absorbance at 260 nm.

RNA was extracted from window and mature leaf stages from treated and control plants in the same manner as described above. Leaf lamina tissue samples were obtained from 3 different whole-plant cultures treated with either rapamycin, wortmannin, ConA, or DMSO (control).

4.3.3. Quantification of Atg16 transcripts in lace plant leaves

Transcripts for lace plant *Atg16* were used to verify the synthesis results of Atg16 protein RNA sequencing (RNA-Seq) by calculating the relative fold-change in gene expression of samples by using the $2^{-\Delta\Delta CT}$ method by qRT-PCR (Rowarth et al., 2021). Equal amounts of RNA (0.1 µg) from treated and control leaf stages were used as a template for cDNA conversion. Single-strand cDNA was synthesized using SuperScript®III First-Strand Synthesis System for qRT-PCR (Invitrogen, Canada) and oligo dT_{20} following the manufacturer's instructions. All sample replicate cDNA conversions were performed without reverse transcriptase to verify for contamination of genomic DNA as a control.

qRT-PCR was conducted on a Rotor-Gene RG-3000 system (Corbett Research, Sydney, NSW, Australia) using 0.5 µl cDNA as a template and 0.4 mmol l^{-1} primers for *Atg16* or *α-tubulin* (Online Resource A.5.) under the following conditions: 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 54 °C and 1 min at 72 °C, followed by 72 °C. A QuantiFast® SYBER® Green PCR Kit (Qiagen, Canada) was used for the qPCR procedure. Melt curve analysis was completed using Rotor-Gene 6 Software and experiments with at least 90% efficiency were used for analysis (Corbett Research,

Australia). The experiment was performed in triplicate using three biological replicates of imperforate, pre-perforation, window and mature stage leaves, each preparation was analyzed in duplicate. cDNA copy numbers for *Atg16* were determined from a standard curve of Ct values ($R^2 > 0.99$) and normalized against the lace plant *α-tubulin* isoform as described in (Rowarth et al., 2021). qPCR primers for lace plant *Atg16* and *α-tubulin* were verified for their PCR products on 1.0 % agarose gels (Appendix A, Online Resources A.5 and A.6.). *α-tubulin* was used as an internal control and verified for degenerate expression in lace plant leaves in Rowarth et al., (2021).

Transcript copy numbers for lace plant *Atg16* and *α-tubulin* were determined by qPCR to measure the accumulation of mRNA in window leaves after treatment with autophagy modulators. Equal amounts of RNA (0.1 µg) from treated and control window and mature stage leaf stages were used as a template for cDNA conversion and qRT-PCR was conducted with RNA from biological replicates of the window and mature leaves from plants treated with DMSO control, rapamycin, wortmannin or ConA. The experiment was performed in triplicate using three sets of lace plant leaves from three individual plants respectively.

4.3.4. Detecting Atg16 protein levels in lace plant leaves

Protein extraction and western blotting protein detection were carried out as described in (Rowarth et al., 2020) to measure Atg16 protein amounts in lace plant leaf stages. One of each imperforate, pre-perforation, window and mature leaves from normally grown plants

was harvested. Leaf stages were individually excised from the midrib, rinsed gently with distilled water, blotted dry on filter paper and then flash-frozen. Frozen leaf tissues were homogenized individually on ice 1:1 (w:v) in a 1% HALT™ protease inhibitor cocktail (Fisher Scientific, #78430) diluted in PIPES buffer solution (100 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.8). Homogenates were centrifuged at 16 000 *g* at 4 °C for 15 min, the supernatants were removed and total protein was quantitated using a Bradford assay. Protein samples were diluted 1:1 with 2× Laemmli sample buffer (Bio-Rad) containing 5% β-mercaptoethanol (v/v) and boiled for 5 min. A 10-µg aliquot of each protein sample and a Precision Plus Protein Standard (Bio-Rad) were loaded onto an 8–16% SDS–polyacrylamide Mini-PROTEAN TGX pre-cast gel (Bio-Rad) and resolved at a constant 250 V for 30 min in ice-cold running buffer [0.1% SDS (v/v), 25 mM Tris and 192 mM glycine, pH 8.3]. Proteins were transferred at a constant 100 V for 30 min onto 0.2 µm nitrocellulose membranes (Bio-Rad) in ice-cold transfer buffer [20% methanol (v/v), 25 mM Tris and 192 mM glycine, pH 8.3]. Membranes were stained in Ponceau S stain [0.1% (w/v) Ponceau (Sigma), 5% (v/v) acetic acid] for 2 min and then washed in TBS-T (Tris-buffered saline–Tween: 10 mM Tris, 140 mM NaCl and 0.1% Tween-20, pH 7.4) for 10 s and imaged by the scanner before being blocked in 5% (w/v) low-fat milk powder in TBS-T for 1 h at room temperature. Membranes were then incubated at 4 °C overnight in primary rabbit anti-Atg16 antibody (anti-Atg16, Agrisera, #AS19 4280) diluted 1:10 000 in 4% milk in TBS-T and washed four times in TBS-T for 1, 2, 3 and 4 min, respectively. Membranes were incubated at 1:20 000 in TBS-T for 30 min in goat anti-rabbit: horseradish peroxidase (HRP) secondary antibody (Agrisera, #AS10 667) and washed as before followed by an additional 3 min wash in TBS (10 mM Tris, 140 mM

NaCl, pH 7.4). After membrane washing, antibody-reactive protein bands were visualized using Clarity ECL Reagent (Bio-Rad) and an MF-ChemiBIS 3.2 gel documentation system (DNR Bio-Imaging Systems). Ponceau S-stained protein lanes and immunoreactive band intensities for Atg16 were quantitated with Image Studio Lite Software (Li-Cor Biosciences). Atg16 band intensities were compared between leaf stages. Protein lanes on nitrocellulose stained with Ponceau S served as a loading control. The experiment was performed in triplicate.

Protein extraction for window stage leaves from plants treated with different autophagy modulators was performed as described above. The most recently grown window stage leaf from each of the DMSO control, rapamycin, wortmannin and ConA-treated plants were harvested, with leaf blades excised from the midrib, blotted dry on filter paper and then flash-frozen. Frozen leaf tissues were homogenized individually on ice 1:1 (w:v) in a 1% HALT™ protease inhibitor cocktail diluted in PIPES buffer solution. Homogenates were centrifuged at 16 000 *g* at 4 °C for 15 min, the supernatants were removed and total protein was quantitated using a Bradford assay. Protein samples were diluted 1:1 with 2× Laemmli sample buffer containing 5% β-mercaptoethanol (v/v) and boiled for 5 min. A 10 µg aliquot of each protein sample was separated by SDS-PAGE as described above and transferred to 0.2 µm nitrocellulose membranes and probed and imaged with anti-Atg16 antibody as described before. The experiment was performed in triplicate.

4.3.5. Anti-Atg16 antibody reactivity test

Recombinant lace plant Atg16 protein was cloned and synthesized to test Anti-Atg16 reactivity and specificity by western blotting. For the production of full length recombinant lace plant *Atg16*, cDNA for the protein was generated by PCR using Platinum *Taq* Polymerase (Invitrogen, Canada), 0.2 mM primers containing NcoI and HindIII restriction enzyme sites (Appendix Figure A.5.) and 0.5 µg of cDNA from lace plant pre-perforation leaves as a template using the PCR reaction: 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 53°C and 1 min at 72°C, followed by 10 min at 72°C. The cDNA was digested with NcoI and HindIII (New England BioLabs, USA) at 37°C overnight and purified with a Wizard® SV PCR Clean-Up System (Promega). Digested cDNA product was ligated into the PRSET-C His-tagged prokaryotic expression vector (Invitrogen) that had been digested with NcoI and HindIII and the newly recombinant plasmids were then transformed into *E. coli* BL21(DE3) pLysS (Invitrogen) for purification. Lace plant Atg16 recombinant protein synthesis was induced with 1 mM isopropyl thio-ß-D-galactoside (IPTG, Thermo Fischer) for 6 h at 37°C and the recombinant Atg16 were purified from cell-free extracts of *E. coli* with the MagneHis™ Protein Purification System (Promega).

To test for antibody reactivity, 0.1 µg of recombinant lace plant Atg16 protein, 20 µg of lace plant pre-perforation leaf, 20 µg window stage leaf cell-free extracts and a Precision Plus Protein Standard were resolved in an 8–16% SDS–polyacrylamide Mini-PROTEAN TGX pre-cast gel (Bio-Rad), transferred to nitrocellulose, blocked and probed with anti-

Atg16 prior to washing and imaging as described above. This protocol was adapted from Rowarth and MacRae 2018a (Appendix A, Online Resource A.7.).

4.3.6. Anthocyanin content quantification of treated leaves

Quantification of anthocyanin in leaves harvested from treatment and control conditions was performed as described in Dauphinee et al. (2017) and Rowarth et al. (2020) to test if lace plant leaf anthocyanin concentration is affected by autophagy modulators. Twenty mg of excised leaf tissue from the window and mature leaves from axenic whole-plant treatment conditions (described above) were excised and macerated in 200 µl of formic acid/methanol (5:95, v/v). Samples were incubated on ice in the dark for 50 min, followed by 10 min centrifugation at 10 000 *g*. The supernatant was collected, and absorbance was read at 520 nm using a SmartSpec Plus Spectrophotometer (Bio-Rad). Results were expressed as cyanidin-3-rutinoside equivalents (C3REs), and standard curves of cyanidin-3-rutinoside (anthocyanin) were used to calibrate concentration. A minimum of three replicates were used for each group.

4.3.7. Image analysis and processing

Leaf images were captured using a Nikon L110 digital camera. Photoshop and Illustrator (Adobe Creative Cloud; Adobe Systems Inc.) were used to prepare images of leaves and western blots for publication. Images of detached leaves had backgrounds removed using Photoshop. A Nikon AZ100 microscope acquired micrographs of window leaf areoles

post-treatments. Image processing was consistent for all micrograph figures and included background removal, adjustments for brightness, contrast and color balance.

4.3.8. Statistical analysis and data representation

One-way ANOVA or Two-way ANOVA followed by a Tukey test was used to identify significant differences among means for all treated mature leaf perforation counts, mature leaf lengths, window stage leaf anthocyanin content, leaf *Atg16* transcript copy numbers and leaf protein Atg16 protein band intensity experimental comparisons. All data are presented as the mean ± SE (α=0.05). Analyses were carried out using GraphPad Prism 5 software (GraphPad Software Inc.).

4.4. RESULTS

4.4.1. Anti-Atg16 was immunoreactive with recombinant lace plant Atg16

The anti-Atg16 antibody reacted to purified recombinant lace plant Atg16 alongside of pre-perforation and window stage leaf protein extracts all generating bands ~56 kDa in size (see Appendix A, Online Resource A.7.).

4.4.2. Atg16 is upregulated in pre-perforation and window leaves

Atg16 mRNA and protein in individual imperforate, pre-perforation, window and mature leaves were measured to characterize their accumulation across leaf development. Imperforate and mature leaf Atg16 mRNA levels were not significantly different (P = 0.9985) and both were significantly lower than pre-perforation and window stage leaves ($P < 0.05$; Figure 4.2.A). Pre-perforation and window leaf *Atg16* mRNA levels were not significantly different (P = 0.9997).

Protein detection by western blotting was used to investigate Atg16 protein levels in leaves at different developmental stages (Figure 4.2.B-D). Imperforate and mature leaf Atg16 protein levels were not significantly different (P = 0.9996) and both were significantly lower than pre-perforation and window stage leaves ($P < 0.0001$). Pre-perforation and window leaf Atg16 protein levels were not significantly different from one another (P = 0.4996; Figure 4.2.D, normalized to the Ponceau S-stained protein lane signal).

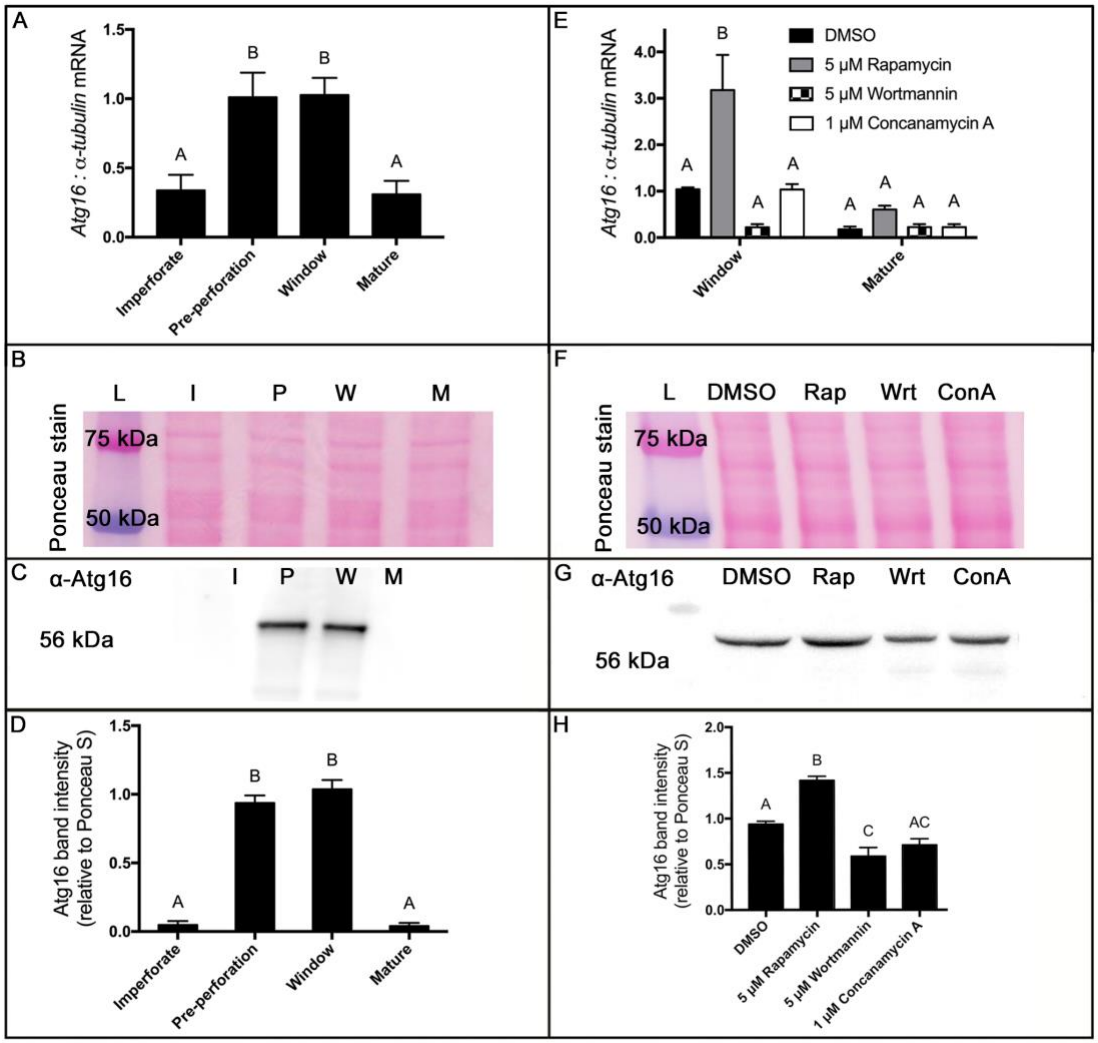

Figure 4.2. Detection of lace plant Atg16 in lace plant leaves. RNA and protein were extracted and probed for Atg16 from imperforate (I), pre-perforation (P), window (W) or mature (M) lace plant leaf stages (A-D); or only window (E-H) and mature (E) leaves from plants treated with either 5 μM rapamycin (Rap), 1 μM wortmannin (Wrt) and 1 μM concanamycin A (ConA) compared to DMSO control. (A and E) The mean levels of *Atg16* mRNA in different lace plant leaf stages were determined by qRT-PCR and normalized to lace plant *α-tubulin* levels as a control. Protein extracts and a molecular protein standard (L) were resolved by SDS–polyacrylamide gels and blotted to nitrocellulose membranes. (B and F) Membranes and proteins lanes were stained with Ponceau-S to serve as a loading control before subsequent detection of presence or absence of ~56 kDa sized Atg16 protein bands detected by anti-Atg16 antibodies (C and G). (D and H) Immunoreactive protein bands were quantitated, and the ratio of Atg16 band intensity to the Ponceau lane signal was averaged. The experiments were performed in triplicate. Means not sharing any letter are significantly different. One-way ANOVA, Tukey test (A, D and H), or Two-way ANOVA, Tukey test (E, $P < 0.05$; $n = 3$). *Error bars* represent the SE.

4.4.3. Autophagy modulators affected Atg16 levels in window stage leaves

The level of *Atg16* mRNA in window leaves from plants treated with rapamycin was significantly higher (approximately 3-fold) than in DMSO controls ($P = 0.0010$; Figure 4.2.E). *Atg16* levels in wortmannin and ConA-treated plants were not significantly different compared to control ($P > 0.05$). No autophagy modulator treatment significantly changed *Atg16* levels in mature leaves compared to control mature leaves or control window leaves ($P > 0.05$).

Protein detection by western blotting was used to investigate if autophagy modulators affected Atg16 protein levels in window stage leaves of treated leaves. Atg16 protein levels in mature leaves were not analyzed (Figure 4.2.F-H). Band intensities of Atg16 protein were normalized to the first replicate of window stage leaves from control plants and quantified for window stage leaves from plants treated with rapamycin, wortmannin and ConA (Figure 4.2.H). Window stage leaves of rapamycin-treated plants had significantly higher Atg16 levels (normalized to Ponceau S-stained protein lanes) compared to window stage control leaves ($P = 0.0047$). Atg16 levels were significantly lower in window stage leaves of wortmannin-treated plants than controls ($P = 0.0320$). Levels of Atg16 were not significantly different between window stage leaves of ConA-treated plants and wortmannin-treated leaves ($P = 0.7201$).

4.4.4. Autophagy modulators affected mature leaf perforations

The number of perforations formed in the most recently developed mature leaves was recorded after one week of treatment with either DMSO control, rapamycin, wortmannin, or ConA (Figure 4.3.). Mature leaves from DMSO control plants produced 156.19 ± 6.61 perforations. Mature leaves from rapamycin plants produced significantly fewer perforations (94.18 ± 6.43 perforations, $P = 0.0002$) compared to the control. Conversely, weeklong treatment with wortmannin produced mature leaves with significantly more perforations compared to the control (235.56 ± 8.71, $P < 0.0001$). Week-long treatment with ConA did not significantly alter the number of perforations formed (Figure 4.3.B; 144.56 ± 11.41, $P = 0.9447$) compared to control. There was no significant difference in leaf length between treatments and control (Figure 4.3.C; $P > 0.05$).

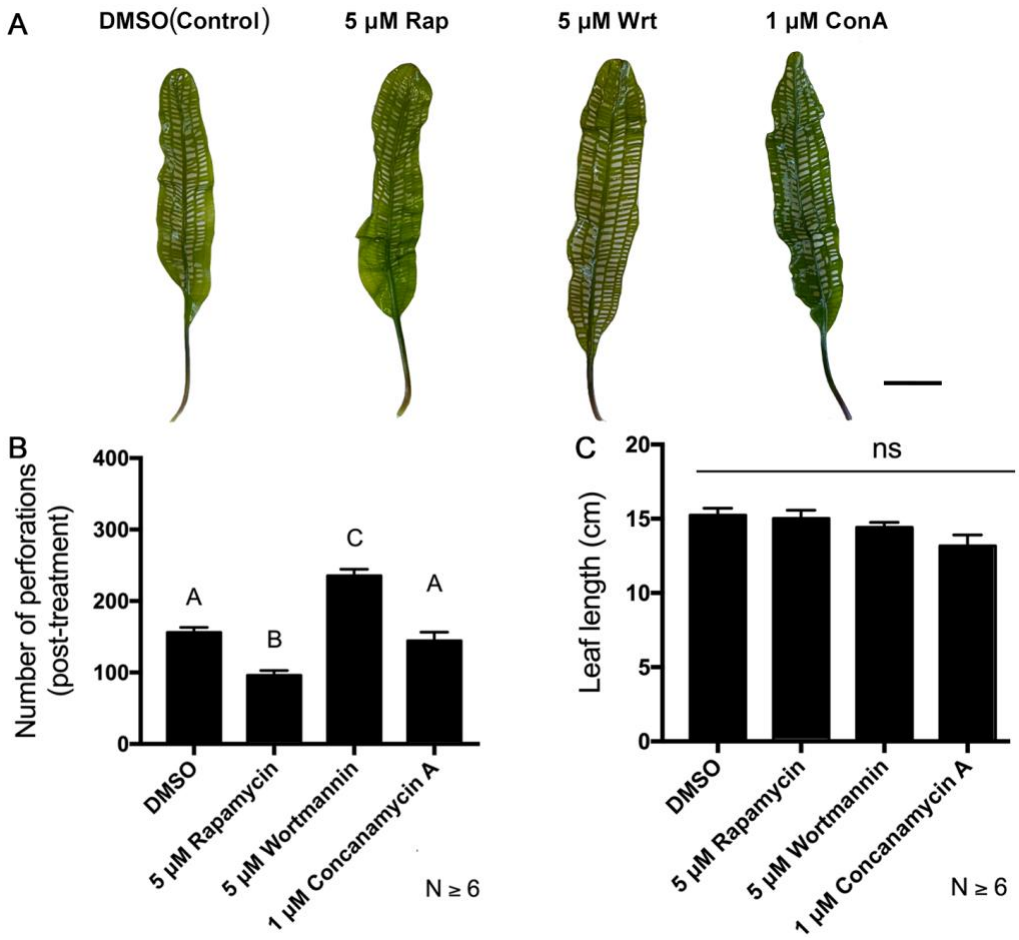

Figure 4.3. Effects of autophagy modulation treatment on mature leaf perforations. (A) Representative leaves from plants treated with 5 μM rapamycin (Rap), 1 μM wortmannin (Wrt) and 1 μM concanamycin A (ConA) compared to DMSO control. (B) Mean number of perforations formed in mature leaves post-treatment. (C) Mean leaf lengths of mature leaves post-treatment. (B-C) Means not sharing any letter are significantly different. One-way ANOVA, Tukey test (*ns* = non-significant, $P > 0.05$, $n \geq 6$). *Error bars* represent the SE. *Scale bars*=2 cm.

4.4.5. Autophagy modulators affected window leaf anthocyanin levels

The anthocyanin content of window and mature stage leaves from plants treated with autophagy modulators was quantified and compared to DMSO controls (Figure 4.4.). Window stage leaves from control plants had a mean anthocyanin content of 2.5 mg ± 0.07 C3RE/g which was significantly higher than the anthocyanin content from control mature leaves (Figure 4.4.D; 0.98 ± 0.062 C3RE/g, $P < 0.0001$). Rapamycin treatment resulted in significantly lower anthocyanin in window stage leaves (1.05 ± 0.25 C3RE/g, $P < 0.0001$) than control window stage leaves but did not significantly differ from control mature leaves ($P = 0.9993$). The concentration of anthocyanin in window stage leaves from wortmannin-treated plants did not differ significantly from control window leaves (3.13 ± 0.21 C3RE/g, $P = 0.0369$) but was significantly higher than rapamycin-treated window leaves ($P < 0.0001$). Window stage leaves from ConA-treated plants produced significantly higher amounts of anthocyanin (3.32 ± 0.07 C3RE/g, $P = 0.0018$) compared to control window leaves but did not differ significantly from wortmannin-treated leaves ($P > 0.05$). The amount of anthocyanin in mature leaves regardless of treatment was not significantly different from control mature leaves ($P = 0.9543$).

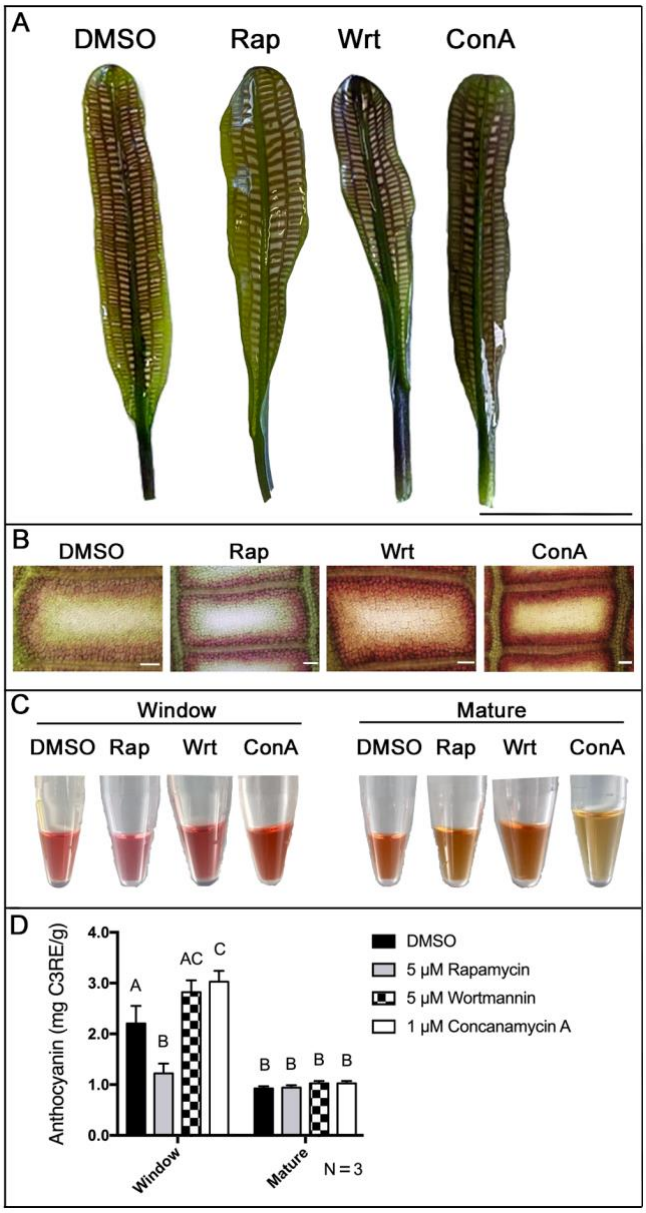

Figure 4.4. Anthocyanin concentration of autophagy modulator treated window or mature stage leaves. (A) Window stage leaves from plants treated with either DMSO (control), 5 µM rapamycin (Rap), 5 µM wortmannin (Wrt), or 1 µM concanamycin A (ConA) and (B) corresponding micrographs of representative areoles. (C) Anthocyanin extracted from the window and mature leaves from treated plants and (D) absorbance values of window and mature leaves from plants treated with either DMSO (control), 5 µM rapamycin (Rap), 1 µM wortmannin (Wrt), or 1 µM concanamycin A (ConA). Mean anthocyanin concentrations were plotted as standard equivalents of cyaniding-3-rutinoside (C3REs). Means not sharing any individual letters are significantly different. Two-way ANOVA, Tukey test. (*ns* = non-significant, $P > 0.05$, $n = 3$). *Error bars* represent the SE. *Scale bars*: A=2 cm; B=50 µm.

4.5. DISCUSSION

Previous investigations using the lace plant model have elucidated the effects of autophagy modulators on Atg8 levels, NPCD cells and PCD cells during leaf development (Dauphinee et al., 2019). Even though there was no direct involvement of Atg8 protein in the PCD process of perforation formation, autophagy played a key role in leaf development by mediating proper cell death rates in PCD cells and survival in NPCD cells. However, there is still a need for investigating the effects of other autophagy-related proteins, Atg16 and autophagy modulators on lace plant leaf anthocyanin content and perforation formation during development. Since autophagy is implicated in developmental PCD in other systems like xylem formation in *Arabidopsis* (Kwon et al., 2010), suspensor deletion in Norway spruce embryos (Minina et al., 2013), differentiation of proximal root cap cells (Feng et al., 2022; Goh et al., 2022) and anthocyanin modulation (Chanoca et al., 2015; Pourcel et al., 2010) we set out to further elucidate this mechanism by investigating the involvement of another key protein, Atg16, and the effects of autophagy on lace plant leaf anthocyanin and development.

4.5.1. Atg16 was developmentally regulated and levels affected by autophagy modulators

We measured Atg16 levels by qPCR and western blotting to test its synthesis during lace plant leaf development. Both Atg16 mRNA and protein levels were significantly higher in pre-perforation and window stage leaves compared to mature and imperforate leaves. These levels are correlated with early stages of lace plant leaf development when PCD

and anthocyanin accumulation is active (Figure 4.2.A and D) and are also consistent with the RNA-Seq data reported in (Rowarth et al., 2021). Pre-perforation and window stage leaves have been shown to up-regulate transcripts encoded for genes involved in autophagy promotion such as *Atg16*, *Atg18a* and *SNF1-related protein kinase KIN10* (Rowarth et al., 2021; Soto-Burgos et al., 2018) suggesting an increased need for Atg16, autophagosome formation and cell maintenance during early lace plant developmental PCD.

Lace plants were treated with autophagy modulators rapamycin, wortmannin, or ConA to test the effects autophagy activity has on Atg16 levels in window stage leaves and leaf development in general. Rapamycin caused Atg16 levels to significantly increase in window leaves, and wortmannin had the opposite effect (Figure 4.2.E and H). AZD-8055 (another TOR suppressor) treatment has previously shown promotion of autophagosome-like bodies and Atg8 puncta in lace plant cells (Mishra et al., 2017) similar to rapamycin treatment, indicating multiple autophagy promoters induce higher autophagic activity and Atg8 levels in lace plants (Dauphinee et al., 2019). Our results show that Atg16 levels are correlated with autophagic activity in window stage leaves, but future experiments with other autophagy promoters like AZD-8055 or nutrient deprivation should be performed to confirm autophagic effects on Atg protein levels. ConA treatment did not affect Atg16 levels (Figure 4.2.E and H) but was shown to increase autophagic body build up in lace plants (Mishra et al., 2017). This suggests that prevention of autophagic vacuolar breakdown either does not provide feedback to affect, or only causes subtle changes to, Atg16 biosynthesis in lace plants.

Knockdown experiments in *Dictyostelium discoideum* have shown that Atg16 is needed for autophagosome formation and is important for crosstalk between autophagy and the ubiquitin proteosome system (Xiong et al., 2018). *Atg16* is detected in early roots and leaves of *Oryza sativa* seedlings and its one homolog is slightly inducible by salt, cold, desiccation and dark treatment (Xia et al., 2011). The lace plant Atg proteins along with Atg16 likely work in concert to ensure that proper balance of leaf growth and nutrient requirements are met during early leaf development and down-regulated in maturity and imperforate leaves when Atg16 is no longer required. Homologs of *Atg16* have been sequenced and characterized in several other plant species but it is not determined if Atg16 plays a critical requirement in activating plant autophagy (Chen et al., 2021; Xia et al., 2011; Yoshimoto, 2012). The advancement of functional-transformational studies showing how plants develop without Atg16 will help elucidate its role in plant PCD.

4.5.2. Autophagy modulators affected lace plant leaf anthocyanin levels and PCD

Plants treated with rapamycin generated window stage leaves with significantly lower anthocyanin concentration (Figure 4.4.D) and led to fewer perforations formed in mature leaves (Figure 4.3.B). This result is similar to other lace plant leaf pharmacological treatments with exogenous antioxidants (Dauphinee et al., 2017; Rowarth et al., 2020), auxin transport inhibitor NPA (Denbigh et al., 2020) and Hsp70 inhibitor PES-Cl (Rowarth et al., 2020) which all lowered anthocyanin production, superoxide generation in PCD cells and total perforations formed. Autophagy, Hsp70 and antioxidants are known to mitigate ROS generation by degrading deleterious protein aggregates and ROS-

generating organelles through chlorophagy and mitophagy (Broda et al., 2018; Izumi et al., 2017; Nakamura et al., 2021; Nillegoda and Bukau, 2015). Higher activity of autophagy and suppression of ROS generation in PCD cells as well as lower anthocyanin concentration in NPCD cells may be the lead cause of lower cell death rates under rapamycin treatment (Dauphinee et al., 2019). These results, taken together with previous pharmacological experiments, indicate that departure from the basal imbalance of the ROS-anthocyanin gradient threshold within areoles leads to irregular or inhibited lace plant PCD. We consistently have observed that ROS accumulation in PCD cells is correlated to anthocyanin accumulation in NPCD cells (Dauphinee et al., 2017; Denbigh et al., 2020; Rowarth et al., 2020). Our observations that rapamycin inhibited the formation of perforations (this study) and cell death rates (Dauphinee et al., 2019) suggests an inhibition of ROS accumulation in PCD cells as well. Therefore, using the cell death assay and measuring the number of perforations formed is a suitable proxy to determine ROS (and ultimately PCD) is inhibited when autophagy is promoted by rapamycin.

ConA-treated leaves raised anthocyanin levels similar to wortmannin but generated mature leaves with fewer perforations compared to wortmannin. These results show that both of these autophagy inhibitors increased the likelihood of stress in NPCD cells, visible as a higher concentration of anthocyanin (Figure 4.4.B) but differentially affected the number of perforations formed (Figure 4.3.B), a measure of the extent of PCD. Wortmannin treated lace plant leaves increases the number of vesicles containing organelles in NPCD cells, inhibits Atg8 synthesis and reduces autophagosome-like

bodies in lace plant leaves (Dauphinee et al., 2019; Mishra et al., 2017) indicating inhibiton of autophagic activity.

The exact mechanism that causes wortmannin and ConA to generate lace plant leaves with similar raised anthocyanin levels but with a different number of perforations is unknown. We speculate these different effects are caused from the different ways these modulators affect autophagic activity. ConA increases the number vacuolar aggregates and Atg8 puncta accumulated in the vacuole of *P. abies* suspensor cells (Minina et al., 2013) and lace plant leaf cells (Mishra et al., 2017). ConA inhibits vacuolar H^+-ATPases (Ishida et al., 2008) preventing the degradation of accumulated autophagic bodies in the vacuole by raising vacuolar pH and preventing the activity of hydrolases (Bassham et al., 2006). It is likely that late-phase autophagy inhibition by ConA causes a compounding of stress in NPCD cells such as loss of hydrolase activity and recycled nutrients which leads to the higher accumulation of anthocyanins and then increased cell death rates in PCD cells (Dauphinee et al., 2019). The up-stream autophagy inhibition by wortmannin may cause even higher rates of cell death in PCD cells. While the down-stream inhibition of autophagy coupled with the possible pleiotropic effects of ConA may still cause enough stress to raise anthocyanin levels but not enough to significantly affect the formation of perforations.

We cannot rule out the limitations of the autophagy modulators used to study autophagy in the lace plant model system. Plants are known to be less sensitive to rapamycin which can affect metabolism and growth. However, it has been shown that separately the lace

plant (Dauphinee et al., 2019) and *Arabidopsis* in liquid MS medium can partially recover this sensitivity (Deng et al., 2016). Since the lace plant is an aquatic plant, this might represent a path for aquatic plant species to be more sensitive to rapamycin treatment. The phosphatidylinositol 3-kinase (PI3K) inhibitors wortmannin and 3-methyladenine (3-MA) can affect endosomal and vacuolar trafficking while 3-MA has been documented to both promote and inhibit autophagy in a time dependent manner (Wu et al., 2010). 3-MA has been previously tested in lace plant experiments but showed a poor response intracellularly compared to wortmannin and ConA and thus was not used in these experiments (unpublished thesis work; Dauphinee, 2017). As well, autophagy modulation has the capabilities to modify gene expression of other plant and algae *Atgs* (Li et al., 2022b; Minina et al., 2013; Pérez-Pérez et al., 2010). Future experiments should be used to separate which Atgs along with Atg16 are influenced specifically by developmentally-, treatment- and nutrient deprived-induced autophagy.

Dauphinee et al. (2019) previously reported that week-long autophagy modulator treatments did not significantly change the number of mature leaf perforations. However, the previous investigation used a different *A. madagascariensis* cultivar that naturally produced thinner mature leaves with fewer rows of perforations, which we no longer have access to. The present cultivar used in this study provides a larger sample size of potential perforations per leaf to survey post-treatment which may explain the differences in results between the two investigations.

How plant autophagy affects anthocyanin biosynthesis and accumulation is not fully understood. Autophagy is at least partially involved in anthocyanin shuttling to vacuoles in plants (Floyd et al., 2012; Pourcel et al., 2010; Zientara-Rytter and Sirko, 2016). Mutant *Arabidopsis* seedlings for *Atg5*, *Atg9* and *Atg10* accumulate fewer anthocyanin vacuolar inclusions (AVIs) and corresponding changes in anthocyanin profiles compared to wild-type (Pourcel et al., 2010). Anthocyanin transport to the vacuole is believed to be controlled by novel selective autophagy mechanisms (Floyd et al., 2012). Atgs and other autophagosome interacting proteins like Hsp70, NBR1 and Exo70B1 may help selectively guide anthocyanin cargo to the vacuole for accumulation as shown in *Arabidopsis* (Gamerdinger et al., 2011; Kulich et al., 2013; Zhang and Chen, 2020; Zhou et al., 2014). Future experiments investigating anthocyanin profiles between NPCD and PCD cells are ongoing to elucidate and resolve the roles of different anthocyanins in cell differentiation and survival.

4.6. CONCLUSIONS

Cell type-specific approaches are important to resolve the roles of autophagy in different contexts like stress or development (Feng et al., 2022; Wada et al., 2009), making the lace plant PCD gradient a suitable model to study the relationship between autophagy and plant PCD. Taken together, our results show that higher levels of Atg16 in lace plant window leaves were induced by rapamycin and were inversely correlated to anthocyanin concentration and the number of perforations formed in mature leaves; the opposite effect was observed with wortmannin. The link between autophagic activity, monitored through

Atg16 levels, and anthocyanin levels as well as perforation formation, points to a role for autophagy in mediating the amplitude of lace plant PCD.

More recent studies like Feng et al. (2022) have shown that PCD and corpse clearance dependency on autophagy is cell-type specific during *Arabidopsis* lateral root cap and columella development. Possibly, a similar scenario is in place in lace plant leaf morphogenesis where cell-specific differences between NPCD and PCD cells occur. Future lace plant experiments should utilize live-cell imaging and lace plant *Atg* knockout lines to measure autophagic activity more accurately within the 48-h window of cell death (Wertman et al., 2012).

We summarize the involvements of autophagy, Atg16, anthocyanin, ROS and a variety of other genes in lace plant leaf development (Figure 4.5.). This shows that disruptions in the autophagic machinery can affect the potency of PCD response in lace plant leaf development. The results from this investigation infer a role for autophagy and other up-regulated *Atg* genes in window stage leaves to help optimize anthocyanin accumulation and promote survival in NPCD cells and mediate a timely cell death in PCD cells.

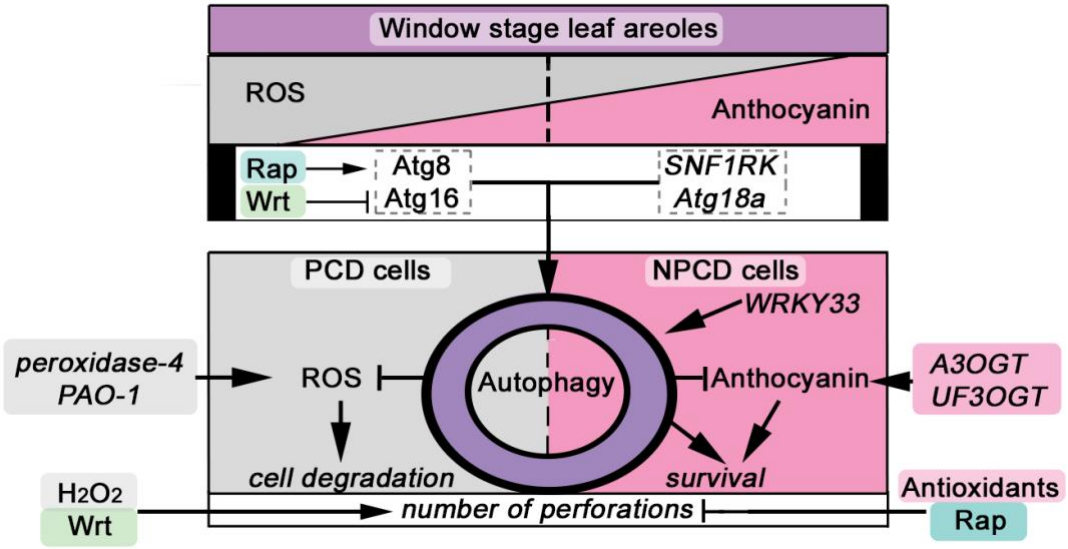

Figure 4.5. Diagram of potential interactions between lace plant PCD, ROS, anthocyanin, autophagy and pertinent genes. An unknown induction signal(s) during window leaf development initiates the differentiation of PCD from NPCD cells within the areole, visible by an imbalance between reactive oxygen species (ROS) and anthocyanin (1). RNA-Seq analysis has shown that window leaves up-regulate genes such as *Atg18a and SNF1RK* that promote autophagy (2). During window stage of leaf development Atg8 (3) and Atg16 protein levels are high and can be manipulated by autophagy modulators (this study, 4). PCD cells up-regulate ROS (1) and ROS generating genes *peroxidase-4* and *primary amine oxidase-1* (PAO-1) while NPCD cells up-regulate *anthocyanidin* (*A3OGT*) and *UDP flavonoid-3-O-glucosyltransferases* (*UF3OGT*) that promote the production of anthocyanins and a *WRKY33* transcription factor that promotes autophagy (2). Wortmannin (Wrt) and exogenous ROS in the form of H_2O_2 have similar effects on window stage leaves possibly accentuating cell death and the ROS-anthocyanin gradient imbalance (this study, 1). On the other hand, rapamycin (Rap) and exogenous antioxidants inhibit anthocyanin accumulation (this study, 1, 5), cell death (3) and perforation formation (this study, 5). When the ROS-anthocyanin imbalance threshold is not reached this consistently leads to inhibited lace plant PCD (1, 5, 6). Under normal conditions autophagy can mediate ROS (7) and anthocyanin levels (this study). How autophagic activity, the ROS-anthocyanin 'gradient' and associated molecular targets all mediate PCD and intracellular communication between PCD and NPCD cells requires further investigation. (1) Dauphinee et al. (2017); (2) Rowarth et al. (2021); (3) Dauphinee et al. (2019); (4) Mishra et al. (2017); (5) Rowarth et al. (2020); (6) Denbigh et al. (2020); (7) Pérez-Pérez et al. (2012).

4.7. DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher. Publicly available datasets were analyzed in Rowarth et al. (2021; doi: 10.1186/s12870-021-03066-7) and discussed in this paper. This data can be found here: NCBI, PRJNA591467.

4.8. AUTHOR CONTRIBUTIONS

Conceived the study, participated in its design and coordination, secured funding and supervised all experimental work: AHLANG. Carried out all experiments, participated in its design, analyzed data and drafted the first manuscript: NMR. Participated in study design: AND. Contributed to the manuscript revisions: NMR, AND, CRL and AHLANG. All authors reviewed and approved the final manuscript.

4.9. ACKNOWLEDGEMENTS

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CHAPTER 5: DISCUSSION

5.1. ESTABLISHING THE LACE PLANT AS A MODEL TO STUDY PCD

PCD is an essential eukaryotic process for the organized removal of compromised cells from stress or achieving higher tissue remodelling (Green 2011; Bozhkov and Lam 2011; Kabbage et al. 2017). Plant PCD is paramount in plant reproduction, immunity, development and stress tolerance. Entering a new decade of NGS, plant-specific PCD knowledge is ever increasing and uncovering what is and is not conserved in terms of molecular programming (Davena et al., 2016). Even with recent advances in uncovering PCD key regulators across plant species, many fundamental questions remain along with current technological hurdles. Nevertheless, the range of model and non-model plants used for plant PCD investigations and their molecular regulations are increasing yearly toward uncovering more PCD element commonalities and classifications. This new knowledge will build toward more agriculturally important plants with greater longevity and stress-tolerance in preparation for upcoming threats of climate change (Avin-Wittenberg et al., 2018; Dauphinee and Gunawardena, 2015; Thanthrige et al., 2021).

With this, the lace plant and its ability to form perforations through its leaves by developmental PCD can be identified as a potential model system to easily access and study PCD in a predictable manner. Furthermore, lace plant leaf developmental PCD is a unique feature in the plant kingdom but shares intracellular changes with PCD processes in other plants such as *Arabidopsis* leaf senescence and Norway spruce embryogenesis

(Filonova et al., 2000; Iakimova and Woltering, 2017; Lim et al., 2007; Ni et al., 2014; Reza et al., 2018; Wertman et al., 2012). The accessibility to select and observe cellular changes within PCD cells simultaneously next to surviving control NPCD cells in lace plant areoles is a sound system compared to other plant models. However, the lace plant PCD system is still in the development phase of becoming a model plant. Chapter 1 of this thesis discussed the practical methods to develop the lace plant as an established model plant to study PCD. The untapped potential of applications that comes with a better understanding of plant developmental PCD is also highlighted. The long-term goal of lace plant research is to improve our understanding of PCD with the ultimate goal of manipulating the process for applications in medicine and agriculture. The sequencing of the lace plant genome using short-read and long-read technology is a priority moving forward to assess the size and completion of the lace plant gene space. Furthermore, once the lace plant genome is better annotated, a suitable gene editing protocol developed for lace plant leaves is needed. A CRISPR/Cas9 or a particle bombardment method tailored for aquatic leaves (Kruse et al., 2002; Muranaka et al., 2015) will allow the manipulation of lace plant PCD candidate genes involved in developmental regulation.

The body of work of this thesis utilized RNA-Seq to improve our understanding of the lace plant transcriptome and attempted to identify essential genes that play a role in lace plant PCD. The lace plant transcriptome has provided important information on the molecular mechanisms and genetics underpinning developmental PCD. The *de novo* transcriptomic profiles of lace plant leaf developmental stages, NPCD cells and PCD cells within leaf areoles were annotated and characterized. DEGs found among leaves

and cells where PCD is active helped identify potential candidate genes for future confirmation of leaf developmental roles. In addition, the part of Hsp70, and the process of autophagy (detected through Atg16), a key PCD regulator, was investigated through pharmacological treatment. This final chapter revisits the findings of these investigations and summarizes how the body of lace plant PCD knowledge has been improved.

5.2. TRANSCRIPTOME ANALYSIS GIVES INSIGHT TO LACE PLANT PCD

Chapter 2 presents the first successful protocol for sequencing, assembling and annotating the *A. madagascariensis* leaf *de novo* transcriptome. The transcriptomes of several lace plant leaf stages and individual NPCD and PCD cell populations offer a new foundation to study plant developmental PCD from this submerged aquatic monocot. Comparing *Aponogeton de novo* transcriptome and proteomic profiles will help elucidate key gene expressions that make lace plant leaves perforate apart from other *Aponogeton* species. However, the transcriptome alone is not enough to tell the story of how its perforations evolved in lace plant leaves. A better understanding of PCD in lace plant leaves will require future experiments to uncover the regulation of PCD genes in response to environmental stressors and different growth conditions. Additionally, functional studies are a priority moving forward to understand which genes are critical for mediating lace plant PCD.

This *de novo* transcriptome is the first modern omics study of *Aponogeton* outside of the plastome sequencing of several other *Aponogeton* species (Mwanzia et al., 2020). The

visible 'gradient' of PCD progression and control NPCD cells allows one to separate PCD and NPCD cells to identify cell death and survival-specific DEGs. Modern proteomic approaches come with challenges when attempting to profile the vast array of post-translational modifications and isoforms for protein profiles (Lai et al., 2021). Still, future work profiling the PCD versus NPCD cell proteome and metabolome will shed light on molecular compositions that may play functional roles in PCD execution. In addition, a more resolved single cell-specific transcriptome project will better profile mRNA changes throughout lace plant areole differentiation.

We also profiled transcriptional differences between imperforate and adult perforating leaves (pre-perforation, window and mature) through RNA-Seq. The imperforate and mature stages represent a time of leaf development where PCD does not occur or has been completed. Mature and imperforate leaves are enriched in biological activities relating to wall organization, photosystems I and II and negative regulation of PCD. These highly expressed genes are consistent with the developmental state of fully developed leaves where PCD is complete, anthocyanins levels are low and chlorophyll pigment are abundant. The low expression pattern for genes involved in hormone biosynthesis and transcriptional activity in mature and imperforate leaves was consistent with leaf development in other monocots such as *A. deserti*, *A. tequilana* and *Z. mays* (Gross et al., 2013; Li et al., 2010).

What genes regulate the consistent emergence of imperforate leaves before perforating leaves from the corm remain unknown. Stored nutrients from the corm and

photosynthesis from imperforate leaves likely provide a minimum threshold of energy and hormones needed to develop successive perforating leaves. Imperforate leaves up-regulated genes involved in senescence regulation, including *mtBag5* and *NAC* transcription factor (Li et al., 2016a; Melo et al., 2021). The high expression of genes involved in photosynthesis and the observed early senescence in imperforate leaves support the notion that these leaves quickly grow first to increase light capture efficiency and support the growth of successive adult leaves as detailed in other heteroblastic plants (Guzmán, 2015). The ability to induce perforation formation into lace plant imperforate leaves has not been successful (assuming the genetic foundation exists). Auxin and ethylene are plant hormones that regulate perforation formation in adult leaves, and the homeostasis of imperforate leaves may influence the performance of subsequent adult leaf development.

Pre-perforation and window leaves represent an early stage of development where leaves are unfurling, growth is taking place, anthocyanin is maximized and PCD is preparing or underway. Cluster analysis revealed that pre-perforation and window stage leaves up-regulate a similar subset of genes involved in flavonoid and anthocyanin biosynthesis, ethylene signalling, protease activity, autophagy and regulation of PCD compared to mature and imperforate leaves. Compared to the other leaf stages, the window stage leaves up-regulated genes involved in ATP-binding, auxin response, ATPase, hydrolase and peroxidase activity. Indicating the highest expression is needed to promote development from a furled state and prepare the leaf for PCD. Window leaves possess the

most PCD activity, where genes involved in peroxidase and hydrolase activity may promote this toward PCD cells (Lord et al., 2013; Lord and Gunawardena, 2012).

Cysteine protease R2D1A and aspartyl protease AED3 play pro-cell death roles in other plant PCD processes (Gao et al., 2017a; Gao et al., 2017b; Rustgi et al., 2017; Shindo et al., 2012) and their homologs are up-regulated in pre-perforation, window leaves and NCPD cells. These proteases are candidate genes to investigate *in vivo* in the lace plant moving forward to elucidate if their role is to serve remodelling during leaf growth or specifically mediate PCD. Additionally, the up-regulation of anthocyanin biosynthesis genes *A3T*, *A5AAT and A5,3OGT* in pre-perforation and window leaves indicate that the promotion of different anthocyanin compounds is needed to mediate a protective response from the cell death cascade during lace plant leaf remodelling. As the presence and disappearance of anthocyanin pigment is the first indication of the start of lace plant PCD, identifying and quantifying the anthocyanin composition during leaf development and between NPCD and PCD cells is crucial and currently under investigation. After the loss of anthocyanin pigment in the centre of areoles, ROS then accumulates in PCD cells destined for death. NPCD cells up-regulate *A3OGT* compared to PCD cells which agree with the notion that anthocyanins are retained in these cells next to the veins to potentially mitigate damage from superoxide and other ROS from PCD cells (Dauphinee et al., 2017; Gadjev, 2006; Gadjev et al., 2008; Van Breusegem et al., 2001). It is still unknown if ROS accumulation in PCD cells directly results from this loss of anthocyanin or another mechanism, but it is known to be partly from ethylene promotion (Dauphinee et al., 2012).

The principal objective of this investigation was to identify DEGs among different leaf developmental stages to identify critical regulators of PCD during lace plant leaf remodelling. Comparing *de novo* lace plant leaf transcriptomes and assemblies to future *Aponogeton* genomes will indicate the completeness and accuracy of this transcriptome (Gross et al., 2013). In addition, the lace plant transcriptomes generated and the protocols developed here will prove helpful with future transcriptomic investigations to reveal new expression profiles with leaves under different environmental stressors (Dauphinee et al., 2014), PCD inhibitors (Lord et al., 2011) and hormone modulation (Dauphinee et al., 2012; Denbigh et al., 2020).

5.3. THE ROLE OF HSP70 IN LACE PLANT PCD

Chapter 3 examines the role of Hsp70 and its effects on leaf anthocyanin levels, caspase-like protease activity and perforation formation through pharmacological experimentation. Plant Hps70s have been shown to play essential roles in stress tolerance and development by maintaining proteostasis or chaperoning intracellular localization of protein signals (Al-Whaibi, 2011; Cho and Choi, 2009; Cho and Hong, 2006; Leng et al., 2017). Furthermore, Hsp70 possesses dual roles in promoting (Kanzaki et al., 2003; Kim and Hwang, 2015) and inhibiting cell death in different PCD processes (Hoang et al., 2015; Li et al., 2016a; Qi et al., 2011), supporting the need to investigate their role in lace plant PCD.

There was a threshold effect when Hsp70 levels were increased above 4-fold, which inhibited perforation formation. Furthermore, by exogenous treatment with antioxidants and H_2O_2, we showed that ROS caused Hsp70 levels to increase 2-fold along with effects of promoted PCD, caspase-like protease activity and anthocyanin levels in response to mitigate ROS, while antioxidants had the opposite effect. In contrast, PES-Cl had a dose-dependent impact on whole plants, producing window leaves with Hsp70 levels that were raised significantly higher than ROS-treated plants and interestingly inhibited PCD, ROS accumulation, caspase-like activity and anthocyanin. The results showed that disruption of basal Hsp70 amounts affected lace plant leaf remodelling, inferring that Hsp70 plays a role in lace plant PCD.

How and when exactly Hsp70 influences lace plant PCD signalling beyond mediating ROS accumulation is yet to be determined. Hsp70 can play a pro-cell death role in *N. benthamiana* and *Capsicum annuum* hypersensitive responses (Kanzaki et al., 2003; Kim and Hwang, 2015), localizing signals either to the cytoplasm or the ER. We performed Hsp70 immunoprobing in fixed lace plant leaf tissue from different leaf stages, NPCD and PCD cells (Appendix A, Online Resources A.8. and A.9.). Hsp70 puncta were detected throughout the PCD gradient, but the resolution was insufficient to determine if Hsp70 localized to specific organelles through PCD. Overexpression of *N. tabacum Hsp70-8* in stressed leaves was localized to the ER and reduced the expression of an auxin efflux carrier *NtPIN*, increasing their antioxidant capacity and drought tolerance (Song et al., 2021). Furthermore, polar auxin transport has been disrupted in lace plant leaves and significantly inhibited proper perforation formation (Denbigh et al., 2020).

Hsp70 plays a role in influencing hormone balance, limiting sources of ROS from intracellular compartments (Marshall et al., 1990; Xu et al., 2013; Zhang et al., 2009). Future experiments, including disruption of known lace plant PCD regulators auxin transport or ethylene signalling factors (Denbigh et al., 2020; Rantong et al., 2015), will elucidate how these hormones affect Hsp70 levels while influencing perforation formation.

The ER plays a role in mediating cell death in plants and animals through the unfolded protein response (UPR; Williams et al., 2010, 2014). Hsp70s and their co-chaperone Bag proteins, as described in Chapter 2, can mediate ER stress-induced cell death and autophagy pathways (Williams et al., 2014). Therefore, the possible localization of Hsp70s and interactive Bag proteins between NPCD and PCD cells should be investigated for a potential role in cell death-decision making during lace plant developmental PCD.

5.4. ATG16 AND AUTOPHAGY

The eukaryotic autophagy ("self-eating") catabolic pathway is utilized to sequester, break down and recycle cytoplasmic contents (Bassham, 2007; Bassham et al., 2006; Bozhkov, 2018). In plant PCD processes, autophagy can play varying roles in promoting or inhibiting cell death in a tissue or species-specific manner (Chen et al., 2021; Hofius et al., 2009; Minina et al., 2013; Minina et al., 2014a; 2014b). With the PCD field searching to understand how to manipulate plant autophagy and its influence on PCD, stress

tolerance and nutrient remobilization (Bozhkov, 2018; Dauphinee et al., 2019; Mishra et al., 2017), the role of autophagy on lace plant PCD has been investigated (Dauphinee et al., 2019) but did not investigate how anthocyanin is affected by autophagy modulation.

It was previously shown that one of the major machinery proteins Atg8 and acidic vesicles are detectable across the lace plant PCD gradient and determined to be highest in PCD cells (Dauphinee et al., 2019; Mishra et al., 2017), suggesting a role for autophagy in promoting cell survival in lace plant leaves as rapamycin slowed the rate of cell death. The increased expression of autophagy-related *Atg16* and *Atg18a* genes detectable by RNA-Seq suggests that autophagic activity is needed during lace plant PCD, warranting their investigation. We revisited lace plant treatments with known autophagy modulators and found that the promotion of autophagy with rapamycin increased levels of Atg16 protein, decreased anthocyanin concentration and perforation formation. Conversely, treatment with autophagy inhibitors wortmannin and ConA had the opposite effects. We suggest a role for autophagy (monitored through Atg16 levels) in mediating optimal anthocyanin levels and PCD during lace plant leaf development. Autophagy activity is documented to be up-regulated in lace plant PCD cells compared to NPCD cells (Dauphinee et al., 2019). We theorize that autophagy plays an important role in maintaining optimal anthocyanin levels which plays a part in promoting survival in NPCD cells and mediating a timely cell death in PCD cells.

Atg16's role in Atg12-Atg5 and Atg8 mediated pre-autophagosome formation is documented in yeast and animals but not evident in plants (Xiong et al., 2018; Yang and

Klionsky, 2010a). Changes in plant autophagic flux can be assessed using GFP-Atg8 reporters, as detectable free GFP puncta indicate Atg8 has been cleaved and lipidated to Atg8-PE. This method has been used in *Arabidopsis* to show that Atg5 and Atg7 are rate-limiting steps for Atg8-PE formation and autophagy performance (Minina et al., 2018). Future experiments should utilize the knockdown or overexpression of Atg16 protein in transformed GFP-Atg8 lace plant leaves and measure how Atg16 and autophagic flux affects lace plant cell death rates.

The results of Atg16 levels being correlated to lace plant leaf development, anthocyanin concentration and PCD is but one step toward unravelling the synthesis of other core autophagy proteins in plant PCD. In other plant systems, autophagy plays a role in PCD in a tissue-specific and time-specific manner (Feng et al., 2022; Goh et al., 2022), likely limiting ROS damage in NPCD cells and preventing uncontrollable cell death in PCD cells (Minina et al., 2013). With this, the lace plant PCD gradient will prove to be a tractable and valuable model system to study the role of autophagic flux on PCD compared to NPCD cells.

Plants also induce autophagy when under nutrient starvation (Li et al., 2022a; Sun et al., 2018). Therefore, lace plants were also subjected to 2-weeks of growth in nitrogen-free MS media to investigate if N_2 deprivation significantly altered autophagic activity and Atg16 levels. This experiment compared different means of autophagy manipulation to our previous autophagy modulation experiments. Nitrogen-deprived lace plants produced window stage leaves with higher anthocyanin levels, Atg16 levels and inhibited

perforation formation. However, more replicates are required to verify these preliminary results.

5.5. BUILDING ON POTENTIAL INTERACTIONS WITHIN LACE PLANT PCD

Lace plant PCD is developmentally regulated; while the signals that initiate the process of perforation formation are unknown, the network is starting to come into focus. The expression patterns discussed in Chapters 2-4 show a wide range of candidate transcription factors, hormones and PCD regulators, coupled with Hsp70 and autophagy activity that work in concert to mediate lace plant PCD. Finally, we summarize and put forth a model for lace plant PCD based on previous investigations (Dauphinee et al., 2017; Lord et al., 2013; Rantong and Gunawardena, 2018) and findings from this thesis (Figure 5.1.).

Once leaves are unfurled, the receding of anthocyanin pigment in the centre of window stage areoles is highly predictable. Still, the original developmental signal(s) that start the initiation phase of PCD in this gradient is unknown. With the change in anthocyanin comes an ethylene production that can produce more intracellular ethylene and ROS in a positive feedback loop manner (Dauphinee et al., 2012, 2017; Rantong et al., 2015), both concentrated within the centre of the areole in a gradient-like fashion. PCD cells are sensitive to ethylene reception indicated by low levels of receptors, while the opposite is the case for NPCD cells (Rantong et al., 2015). Auxin is likely transported from the leaf veins (Denbigh et al., 2020) and plays a role in mediating ROS accumulation and cyt c

releases in a gradient-like fashion. Cross talk between hormones auxin and ethylene exists in aerenchyma formation and is worth investigating moving forward in the lace plant as their interplay potentially regulates the initiation of PCD (Kacprzyk et al., 2022; Muday et al., 2012).

Hsp70 is detectable across the PCD gradient and can maintain homeostasis by refolding denatured proteins rapidly and also keeping the integrity of organelles that are capable of generating more ROS under stress (Kim and An, 2013; Nillegoda and Bukau, 2015; Rosenzweig et al., 2019; Stankiewicz et al., 2005). Hsp70 levels were inducible by H_2O_2, while PCD was promoted by intracellular ROS and inhibited by exogenous antioxidants. Treatment with 25 µM PES-Cl induced a higher 4-fold level of Hsp70 that likely suppresses ROS and the anthocyanin gradient, inhibiting perforation formation.

Autophagy is active across the PCD gradient, potentially playing a differential role in promoting survival in NPCD cells and controlling the rate of PCD in dying cells (Dauphinee et al., 2019). Autophagy machinery can be promoted by ethylene (Zhu et al., 2018b), auxin (Pu et al., 2017) and their respective response factors (Li et al., 2020b; Schepetilnikov et al., 2013; Wang et al., 2020; Zhu et al., 2018b), but the role of phytohormones in regulating lace plant autophagy has not been investigated. Hormones such as ethylene, auxin, ABA and SA, among others, regulate autophagic activity across

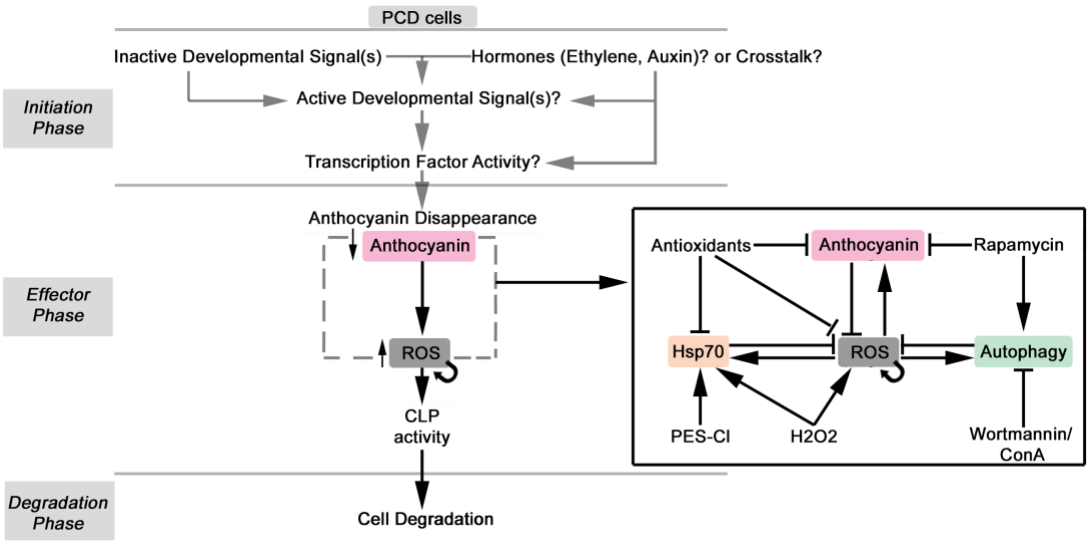


Figure 5.1. A generalized model for lace plant PCD cells. *Initiation phase*: an unknown developmental signal(s) activates independently or from the cross talk of phytohormones ethylene, auxin, or others (*gray faded arrows*). Potential transcription factor(s) then cause the disappearance of anthocyanin in PCD cells. *Effector phase*: Loss of anthocyanin is followed by internal ROS accumulation in PCD cells. ROS promotes higher CLP-1 activity in PCD cells (VPEs; Rantong et al., 2018). Ethylene production promotes ROS and CLP-1 activity, leading to more perforations. *Degradation phase*: autophagic and CLP-1 activity leads to tonoplast rupture and clearing of cell contents, leading to cell death. Findings from this work and previous work (*right box*) have determined that exogenous H_2O_2 promotes internal ROS, increasing anthocyanin and inducing Hsp70 levels in window leaves (Dauphinee et al., 2017; Rowarth et al., 2020). Exogenous antioxidants inhibit internal ROS, anthocyanin and Hsp70 levels. Treatment with inhibitor PES-Cl causes induction of 4-fold Hsp70, inhibition of ROS, anthocyanin, CLP-1 activity and cell death (Rowarth et al., 2020). Macroautophagy (autophagy) is induced by ROS and acts to limit further ROS production (Signorelli et al., 2019). Rapamycin (Rap) promotes lace plant autophagy (Mishra et al., 2017; Dauphinee et al., 2019) and inhibits anthocyanin and cell death rates. Wortmannin (Wrt) and concanamycin A (ConA) inhibit autophagic activity in lace plants and cause an increase in leaf anthocyanin levels and promote cell death. Changes in auxin flux and ethylene influence ROS, anthocyanin and cell death (Denbigh et al., 2019), but how phytohormones regulate autophagy, Hsps and anthocyanin loss is not determined. Plant anthocyanins are regulated by autophagic machinery, but how exactly in lace plants is not specified (Masclaux-Daubresse et al., 2014).

different PCD processes (Feng et al., 2021; Liao et al., 2022; Pu et al., 2017; Signorelli et al., 2019). Specifically, auxin promotion represses *Arabidopsis* TOR-dependent induced autophagy pathways (Pu et al., 2017). Additionally, *WRKY33* and *SnRK1* are up-regulated in NPCD cells and promote *Arabidopsis* autophagy in a TOR pathway-independent manner (Li et al., 2020b). The possibility of autophagy regulation outside of the TOR pathway may explain different regulation pathways of autophagy between PCD and NPCD cells (Pu et al., 2017). Autophagy clears harmful contents such as damaged proteins or organelles from ROS (Goh et al., 2022; Izumi et al., 2017; Zhu et al., 2018b), likely promoting cell survival in NPCD cells. Plant autophagy can also be highly selective in targeting specific compartments and proteins to regulate cellular processes (Avin-Wittenberg et al., 2018; Michaeli et al., 2016). Plant Atg8 isoforms have been shown to select different cargo receptors and target substrates for degradation and may play a role in differentiating what autophagy mediates to the vacuole for degradation between NCPD and PCD cells (Eickhorst et al., 2020; Ran et al., 2020).

Rapamycin promotes autophagic activity across the PCD gradient (Mishra et al., 2017), inhibiting anthocyanin levels (Chapter 4) and cell death rates (Dauphinee et al., 2019). Wortmannin and ConA inhibit different molecular activities of autophagy and increase anthocyanin levels and cell death rates, most likely due to increasing ROS from cell stress. *Atg5* mutants cannot properly up-regulate anthocyanins in *Arabidopsis* under nutrient starvation (Masclaux-Daubresse et al., 2014). With auxin flux playing a prominent role in lace plant perforation formation (Denbigh et al., 2020), how

phytohormones and lace plant Atgs regulate autophagic activity and anthocyanin biosynthesis should be investigated moving forward.

Plant VPEs exhibit caspase-like protease-1 activity that plays a role in executing plant PCD via tonoplast rupture in leaf senescence, lateral root formation PCD and embryogenesis (Minina et al., 2013; Rantong and Gunawardena, 2018; Yamada et al., 2005). During lace plant PCD, *VPE* and caspase-like protease is highest in PCD cells (Lord et al., 2013; Rantong and Gunawardena, 2018) and are regulated by ethylene (Rantong and Gunawardena, 2018). An interplay of protease cascades may exist between VPEs and other proteases that provide a gain or loss of function, which leads to PCD execution in PCD cells (Balakireva and Zamyatnin, 2019). VPE activity can execute cell death by promoting rupture of the tonoplast in many plant PCD processes (Hara-Nishimura et al., 2005; Yamada et al., 2005). Both autophagy and Hsp70 activities may halt caspase-like protease activity by negating ROS or slowing the execution of proteases to avoid uncontrolled cell death (Li et al., 2016a; 2016b; Minina et al., 2013; Minina et al., 2014b; Ribeil et al., 2007).

There is evidence that under abiotic stress, *Arabidopsis* Bag4 binds to Hsp70 to suppress cell death and is needed to promote autophagy and proper flowering and root development (Doukhanina et al., 2006; Thanthrige et al., 2020). Autophagy and Hsp70 may separately play roles in lace plant PCD cells to timely promote cell death, but the exact mechanism is unknown. Hsp70s can mediate cell signalling to promote plant cell death alone or with the co-chaperone activity of Bag proteins (Cho and Choi, 2009; Li et

al., 2016a). Metacaspase activity can promote autophagy in Norway spruce suspensor and hypersensitive cell death (Bozhkov et al., 2005; Minina et al., 2014b). The relationship between autophagic machinery regulation and VPE activity should be elucidated in lace plant PCD to understand better the role of autophagy in lace plant PCD cells.

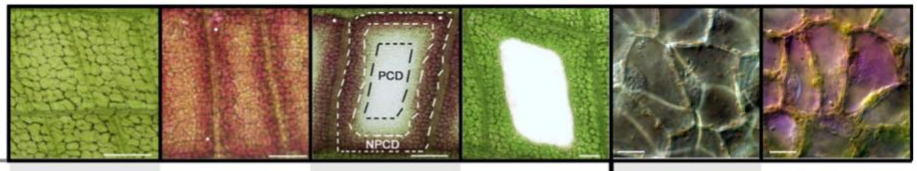
5.6. CONCLUSIONS

The formation of leaf perforations in the lace plant is a rare phenomenon in plant development. However, despite the uniqueness of lace plant PCD, we have unraveled common signaling mechanisms in other plant PCD systems. Therefore, the lace plant is an intriguing model for fundamental PCD research with great potential for broader applicability. As identifying signalling molecules that differentiate NPCD and PCD cells is a priority for lace plant PCD research, we sought to utilize RNA-Seq technologies to profile transcriptomes across leaf development and find key DEGs that could control PCD regulation.

Additionally, two candidate genes (Hsp70 and Atg16) were detected as up-regulated when PCD is active during leaf development. Hsp70 and Atg16 were characterized as developmentally regulated during leaf development and Hsp70 plays a role during lace plant PCD. The results from modulating autophagy and Atg16 levels in window stage leaves infer that autophagy helps optimize anthocyanin accumulation, promotes survival in NPCD cells and mediates timely cell death in PCD cells. The results from the previous chapters highlight that Hsp70 and autophagy mediate a balance of anthocyanin and ROS.

Although the exact mechanisms need further investigations, Hsp70, Atg16 and autophagy play a role in PCD cell differentiation during lace plant development. Combining previous findings and work from this thesis, a summary figure (Figure 5.2.) illustrates the relative levels of the lace plant PCD regulators detected between leaf stages and cell types.

This thesis generated the first successful protocol for analysis of *de novo A. madagascariensis* leaf transcriptomes by Illumina-based RNA-Seq. The profiling results yielded various candidate genes involved in cell death initiation, mediation and execution, providing new insights into lace plant PCD. Future experiments using transcriptomic analysis will help further elucidate which genes control intracellular changes during PCD caused by different environmental cell death inducers (Dauphinee et al., 2014). The combination of future transcriptome analytical tools and experimental treatments will provide further insights into lace plant leaf remodelling and plant PCD. Most plant PCD understanding has been heavily weighted towards model plants, specifically *Arabidopsis.* Utilization of the PCD gradient of the lace plant system will provide a better understanding of the molecular mechanisms controlling plant PCD. Findings will expand the ability to manipulate critical pathways and potentially improve agricultural performance and post-harvest losses (Feng et al., 2021).



Molecules and associated genes	Imperforate	Pre-perforation	Window	Mature	PCD	NPCD
Anthocyanin	Low	High	High	Low	Low	High
DEGs		*A3OGT, A35OGT, A5AAT, A3T*	*A3OGT, A35OGT, A5AAT ,A3T*			*A3OGT*
ROS (1)	ND	Low	High	Low	High	Low
DEGs	*Peroxdidases*		*Ascorbate oxid. SOD [Fe] Chl.*		*Peroxidase-4 PAO-1*	
Hsp70	Low	High	High	Low	High	Low
DEGs		*Hsp70-15*	*Hsp70-15*			
Autophagy (2)	ND	ND	Basal	ND	High	Basal
Atg8 (2)	ND	ND	Basal	ND	High	Basal
Atg16	Low	High	High	Low	ND	ND
DEGs	*WRKY33*	*Atg16, 18a SNFR1K*	*Atg16, 18a SNFR1K*			*WRKY33*
CLP-1 activity	ND	Highest	High	Low	High (3)	Low (3)
DEGs		*CP1-R21A, VPE (3)*	*CP1-R21A, VPE (3)*		*VPE (3)*	*CP1-R21A*

Figure 5.2. Summary of findings. Summarized molecular experiments showing relative levels of probed molecules among lace plant leaf stages (left of the *black vertical line*) and between cell types (right of the *black vertical line*). In *gray rows* are described relative levels of anthocyanin, ROS, Hsp70, autophagy, Atg8, Atg16 and caspase-like protease-1 (CLP-1) activity. Potentially associated DEGs detected during RNA-Seq analysis (Rowarth et al., 2021; Supplementary files 3 and 5) are listed in *italics in white rows*. Previous lace plant findings not determined in this thesis are referenced by (1); Dauphinee et al. (2017), (2); Dauphinee et al. (2019) and (3) Rantong and Gunawardena (2018). *ND*; not determined.

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APPENDIX A ONLINE RESOURCES

Online Resource A.1. Live-cell video of early-PCD (EPCD) cells from lace plant window leaf stage. Cells have lost anthocyanin pigment, retain pigmentation from chlorophyll and are fated to die. Additionally, there is an increase in transvacuolar strands and some organelles are visible in the vacuole. Scale bars: 100 μM, ~50 X playback speed.

Online Resource A.2. Live-cell video of late-PCD (LPCD) cells from lace plant window leaf stage. Cells have lost all pigmentation and are on the verge of collapsing. Additionally, aggregates form in the vacuole becoming visible and undergo Brownian motion. Scale bars: 100 μM, ~50 X playback speed.

Online Resource A.3. Live-cell video of non-PCD (NPCD) cells from lace plant window leaf stage. As PCD progresses, NPCD cells retain pigment from anthocyanin and chloroplasts and do not undergo PCD. Scale bars: 100 μM, ~50 X playback speed.

Online Resource A.4. Zeiss laser capture microdissection and catapult procedure. An NPCD cell population separated by laser from tissue and catapulted by pressurized air into desired containment vessel in under 1-min.

Online Resource A.5. Primers used for Atg16 PCR experiments.

Online Resource A.6. Atg16 and tubulin PCR products. cDNA from imperforate (I), pre-perforation (P), window (W) and mature (M) leaves were probed with qPCR primers for lace plant Atg16 (top gel) and α-tubulin (bottom gel; see Online Resource A.5. above for primer information) for 30 cycles at 54°C. PCR products were then resolved in a 1.0% agarose gel alongside a Gene Ladder (Thermo Scientific). Primers for lace plant Atg16 produced a 227 bp product and primers for lace plant α-tubulin produced a 197 bp fragment.

Online Resource A.7. Anti-Atg16 reactivity. Protein extract from lace plant leaves and recombinant Atg16 were resolved in SDS polyacrylamide gels, transferred to nitrocellulose and probed with anti-Atg16 antibody. Lane 1, protein standard ladder; 2, 0.1 μg of recombinant Atg16 protein; 3, 20 μg of protein extract from lace plant pre-perforation leaf stage; 4, 20 μg of protein extract from lace plant window leaf stage.

Online Resource A.8. Two different replicates (A and B) of Hsp70 immunoprobing of lace plant pre-perforation, window and mature leaf stages. Arrows indicate detection of +Hsp70 puncta in different leaf stages.

Online Resource A.9. Hsp70 immunoprobing within window leaf NPCD, EPCD and LPCD cells.

APPENDIX B COPYRIGHT RELEASE LETTERS

B.1. COPYRIGHT RELEASE FOR CHAPTER 2

SPRINGER NATURE

RNA-Seq analysis reveals potential regulators of programmed cell death and leaf remodelling in lace plant (Aponogeton madagascariensis)

Author: Nathan M. Rowarth et al

Publication: BMC Plant Biology

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B.2. COPYRIGHT RELEASE FOR CHAPTER 3

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