Investigation of Molecular Mechanisms Involved in Developmentally Regulated Programmed Cell Death in the Lace Plant (*Aponogeton madagascariensis*)

by

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Dedicated to my parents and grandmother, for being the best role models.

Table of Contents

List of Tables

List of Figures

Abstract

Programmed cell death (PCD) is essential for the development and survival of eukaryotes. PCD is well understood in animals and relatively less is known in plants. PCD occurs throughout a plant's life cycle, from the fertilization of ovule to the death of the whole plant. One of the fascinating examples of PCD in plant development is perforations formation in the leaves of lace plant (*Aponogeton madagascariensis*). PCD begins in the center of an areole of young leaves and develops towards the veins and stops approximately 4-5 cell layers from the vein, and these cells (NPCD cells) do not undergo PCD during perforation formation. Lace plant is an excellent system to work on developmentally regulated PCD because of the accessibility and predictability of perforation formation. Extensive morphological studies have been performed in the lace plant; however, less is known about the molecular mechanisms that drive lace plant PCD. The emphasis of this dissertation was to provide insights into the molecular mechanisms of developmentally regulated PCD in lace plant. We investigated the role of ethylene and ethylene receptors in lace plant PCD. Results suggested that ethylene is involved in lace plant PCD, in a climacteric-like pattern. Three lace plant ethylene receptors were isolated and their transcript expression pattern was studied throughout leaf development and between PCD and non-PCD (NPCD) cells. Based on the results, a newly proposed model in which ethylene and ethylene receptors regulate PCD and perforation formation was illustrated. The role of vacuolar processing enzymes (VPEs), in lace plant PCD was also investigated. Two lace plant VPEs were isolated and their transcript levels during leaf development and PCD were investigated. Results suggested that both VPEs are involved in lace plant PCD, but at different stages of leaf development. VPE activity analysis also suggested that VPE activity is higher in PCD compared to NPCD cells. Further, we investigated the effect of ethylene on the transcript expression pattern of VPEs. It was determined that ethylene plays a role in stimulating VPE transcriptional upregulation during lace plant PCD.

List of Abbreviations and Symbols Used

ACC	1-aminocyclopropane-1-carboxylic acid
AP-1	Activator protein 1
Apaf-1	Apoptotic protease-activating factor-1
Arg	arginine
AS-1	ASYMMETRIC LEAVES 1
Asp	aspartate
AtANP1	*Arabidopsis thaliana* ANP1
AtBI-1	Arabidopsis BI-1
ATP	adenosine triphosphate
AVG	aminoethoxyvinylglycine
BAK1	Brassinosteroid Insensitive 1 (BRI1)-associated receptor Kinase 1
Bcl-2	B-cell lymphoma 2
BI-1	Bax inhibitor 1
bp	base pairs
CAD	caspase-activated deoxyribonuclease
CARD	caspase recruitment domain
cDNA	complementary DNA
CRKs	cysteine-rich RLKs
CTPP	C-terminal inhibitory pro-peptide
CTR1	CONSTITUTIVE TRIPLE RESPONSE 1
Cyt c	cytochrome c

DED	death effector domain
DISC	death-inducing signaling complex
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
DR	Death Receptor
DREBa	dehydration responsive element binding protein a
DTT	dithiothreitol
EBF	EIN3 BINDING F-box
EDTA	ethylenediaminetetraacetic acid
EIN	ethylene insensitive protein
ER	endoplasmic reticulum
ERF	ethylene-response factors
ERS	ethylene response sensor protein
EREBP	ethylene-responsive element binding protein
ETR	ETHYLENE RESPONSE 1
FADD	Fas-associated death domain
FAS	fatty acid synthetase
FLS2	FLAGELLIN SENSITIVE2
g	gravity
GADPH	glyceraldehyde-3-phosphate dehydrogenase
HSF	heat shock transcription factor
HR	hypersensitive response
IAP	Inhibitors of Apoptosis

ICAD	inhibitor of CAD
iVPE	intermediate vacuolar processing enzyme
JAZ	Jasmonic Acid Zim domain proteins
kb	kilobases
M-MuLV RT	Moloney Murine Leukemia Virus Reverse Transcriptase
MAMPs	microbe associated molecular patterns
MAP	mitogen activated protein
Mcl-1	myeloid cell leukemia 1
mg	milligrams
ml	millilitre
mM	millimolar
mRNA	messenger RNA
MS	Murashige Skoog
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NO	nitric oxide
NPCD	non-PCD
NTPP	N-terminal pro-peptide
PCD	programmed cell death
PCR	polymerase chain reaction
PDF1.2	PLANT DEFENSIN1.2
ppVPE	proprotein precursor vacuolar processing enzyme
PR	pathogenesis related

PRRs	pattern recognition receptors
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
rpm	revolutions per minute
RLKs	receptor-like/Pelle kinases
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SA	salicylic acid
SEM	standard error of means
SP	signal peptide
TMV	tobacco mosaic virus
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TSN	Tudor staphylococcal nuclease
U	unit
UTR	untranslated region
UV	ultraviolet
VPE	vacuolar processing enzyme
ºC	degree Celsius
µM	micromolar
µl	microlitre
µg	microgram

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Chapter 1 Introduction

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In addition, an introduction to the lace plant and objectives are included in this Chapter.

1.1 Programmed Cell Death in Plants and Animals

Programmed cell death (PCD) is a genetically regulated process responsible for the elimination of undesirable cells in eukaryotes; it is necessary for development and survival. It is also essential for response and adaptation to the environment (Lam 2004; Gechev et al. 2006). In plants, PCD is involved from formation of embryos until death of the whole plant (Thomas and Franklin-Tong 2004; Gechev et al. 2006). There are at least two broad categories of PCD in plants; developmentally regulated and environmentally induced PCD (Gunawardena et al. 2004). During plant development, PCD is involved in processes such as embryonic suspensor deletion (Giuliani 2002; Rogers 2005), aerenchyma formation (reviewed by Evans 2003; Seago et al. 2005), tracheary element differentiation (Mittler and Lam 1995; Fukuda 2000), root cap shedding (Wang et al. 1996), leaf and flower abscission (Bar-Dror et al. 2011), self-incompatibility in pollen (Thomas and Franklin-Tong 2004; Rogers 2005; Kacprzyk et al. 2011), leaf re-modelling (Gunawardena et al. 2004, 2005, 2006, 2007; Wertman et al. 2012), and leaf senescence (Hadfield and Bennett 1997; Yen and Yang 1998). Environmentally induced PCD occurs in response to external stimuli such as heat (Lord and Gunawardena 2011), ultraviolet (UV) light (Nawkar et al. 2013), hydrogen peroxide (H_2O_2; Gechev et al. 2006), and pathogen attack (Greenberg 1997). Though initiated by intrinsic and extrinsic stimuli respectively, developmentally regulated and environmentally induced PCD seem to share similar PCD mechanisms. Also, these two types of PCD share some of the genes involved in their regulation and execution.

In animals, cell death can be divided into three main categories based on morphological characteristics: apoptosis, autophagic PCD and necrosis (Kroemer et al.

2009). Apoptosis is characterized by formation of apoptotic bodies, blebbing, and engulfment of the apoptotic bodies by neighbouring cells (Kroemer et al. 2009; Ravichandran 2010; van Doorn 2011). Autophagy involves an increase in the formation of vesicles such as autophagosomes, lytic vacuoles and autolysosomes (Kroemer et al. 2009; Ravichandran 2010; van Doorn 2011). In plants, autophagosomes and lytic vacuoles have also been observed during PCD (Liu et al. 2005; Borén et al. 2006; Minina et al. 2013; Minina et al. 2014). Necrosis is more rapid and characterized by general cell lysis and spilling of cytoplasmic contents into the extracellular fluid: causing inflammation (Kung et al. 2012). Morphological characterization of plant PCD is still ambiguous and not well defined. At the molecular level, this chapter focuses on comparing plant PCD with mammalian apoptosis since its molecular pathways are well elucidated.

This chapter serves as an overview of the genetic regulation of plant PCD in general, focusing on the genes involved in signaling, regulation and execution. It also serves as a comparison of what is known about the plant PCD mechanism thus far, with its animal PCD counterpart (mammalian apoptosis) in terms of genes involved. This chapter is timely considering that progress has been made in recent years in terms of providing more insight in the molecular processes involved in mammalian apoptosis and plant PCD.

1.2 Signaling During Apoptosis and Plant PCD

During normal cell function, signals that perpetuate cell life are transmitted. There has to be a stimulus that offsets the normal function and initiates cell death. This stimulus can be extrinsic or intrinsic. PCD signals may also be activated by default if a proper

survival signal is lacking (Raff 1992; Lam 2004). Mammalian apoptosis initiated by extrinsic signals (the extrinsic pathway; Figure 1.1), is triggered by extracellular death ligands such as FasL, TNF-α, Apo3L, and Apo2L, which interact with a tumor necrosis factor (TNF) subfamily of death receptors such as FasR, TNFR1, DR3, DR4 and DR5 (Reviewed in Elmore 2007). Upon this interaction, adaptor molecules such as Fas-associated protein with death domain (FADD), TNF receptor type 1-associated protein death domain TRADD, and receptor interacting protein (RIP) are recruited to bind to the receptors (Elmore 2007). Procaspase 8 also binds to the adaptor through its DED: forming a death-inducing signaling complex (DISC). An active initiator caspase, caspase 8, is produced in the process. Initiator caspase precursors are usually activated through dimerization instead of cleavage. The caspase 8 will go on to activate precursors of executioner caspases 3 and 7. Executioner caspases will then cleave death substrates, which include other proteases and nucleases (such as CAD), which are responsible for the biochemical and morphological changes observed in cells undergoing apoptosis.

The extrinsic pathway sometimes recruits the intrinsic pathway to amplify the death signal (Kantari and Walczak 2011). In this case, instead of directly cleaving executioner caspases, the active caspase 8 will cleave BH3 interacting-domain death agonist (Bid) to trigger the intrinsic apoptotic pathway (McIlwain et al. 2013; Marino et al. 2014). The pro-apoptotic Bcl-2 family protein Bid is converted to tBid, which translocates to the outer mitochondrial membrane where it interacts with other pro-apoptotic Bcl-2 family proteins such as Bax and Bak. The intrinsic pathway can also be triggered by stimuli that do not involve the use of death receptors, such as radiation, hypoxia, hyperthermia and developmental hormones (Elmore 2007; McIlwain et al.

2013). These stimuli and tBid compromise the integrity of the mitochondrial inner membrane and allows for release of pro-apoptotic proteins such as cytochrome c (Cyt c) into the cytosol (Reviewed in Elmore 2007). In the cytosol, the Cyt c will then form a complex with procaspase 9, apoptotic protease-activating factor-1 (Apaf-1), and adenosine triphosphate (ATP). This complex, called an apoptosome, allows procaspase 9 to cluster and get activated (Shiozaki et al. 2002). The initiator caspase 9 will activate executioner caspases, after which the extrinsic and intrinsic pathways converge.

While the initiation of PCD in animals is well understood, the process is still relatively less studied in plants. We know that inducers like reactive oxygen species (ROS), such as hydrogen peroxide are key modulators of PCD in plants (Gechev et al. 2006). Hydrogen peroxide induces PCD in Arabidopsis and sets of hydrogen peroxide-responsive genes are regulated in a similar fashion during different types of plant PCD (Gechev et al. 2005). It also induces PCD in a concentration dependent manner in cultured tobacco cells (Houot et al. 2001). Specific receptors and sensors related to PCD initiators such ROS are either not known or less understood.

1.2.1 Receptor-like or Pelle Kinases Involved in Signaling During Plant PCD

A large gene family of Receptor-like/Pelle kinases (RLKs), (Shiu and Bleecker 2001a, 2001b, 2003; Shiu et al. 2004), have been shown to play a vital role in plant responses to stress. Like protein kinases in all organisms, RLKs are responsible for passing on information from signal perception down to effector genes. They are serine-threonine protein kinases that possess an extracellular ligand-binding domain that perceives signals (Shiu and Bleecker 2003). Even though the RLK gene family has about

600 members, only a few of them have been functionally characterized. Among the functionally characterized are *flagellin-sensitive-2* (FLS2), *arabidopsis ethylene responsive element binding factor* (ERF) and *rice Xa21*; which are leucine-rich repeat RLKs important in pathogen recognition and plant immunity (Figure 1.2; Zipfel et al., 2006; Chinchilla et al. 2007; Heese et al. 2007; Park and Ronald 2012). FLS2 recognizes microbe associated molecular patterns, or MAMPs (e.g. bacterial flg22 protein), and interacts with co-receptors such as Brassinosteroid insensitive 1-associated receptor kinase 1 (BAK1) and Botrytis induced Kinase 1 (BIK1)/PBS1-like (PBL) kinases (Yadeta et al. 2013). Calcium dependent protein kinases (CDPKs), RBOHD, SYP121, and H^+-ATPase are also involved in signaling during microbial attack (Nühse et al. 2007; Benschop et al. 2007; Yadeta et al. 2013). Another group of RLKs important in plant PCD are Cysteine-rich RLKs (CRKs). In Arabidopsis, there are at least 42 CRKs and their transcription is induced in response to cell death-causing stimuli such as oxidative stress, pathogen attack and salicylic acid (SA, Czernic et al. 1999; Chen 2001; Chen et al. 2003, 2004). CRK5 is involved in signaling during hypersensitive response (HR) to *Pseudomonas syringae* in tomato, and when over expressed, CRK4, CRK5, CRK19 and CRK20 caused cell death (Czernic et al. 1999; Chen 2001; Chen et al. 2003, 2004). CRK13 is also involved in the modulation of plant PCD in response to pathogen attack, but it requires high SA levels (Acharya et al. 2007).

CRK transcription regulation responds differently depending on the type of stimuli. O_3 induced ROS production increases the transcription of most members of the CRK family (Czernic et al. 1999), while most abiotic factors like heat, cold, salt and drought result in decreased transcription of CRKs (Lehti-Shiu et al. 2009). Even though a

few abiotic stresses like osmotic stress, wounding and UV-B exposure also result in increased transcription of CRKs, it is evident that there are differences in CRK transcriptional response during PCD caused by biotic versus abiotic stimuli. While most of the biotic stresses result in increased CRK transcription, most of the abiotic stresses decrease CRK transcription (Lehti-Shiu et al. 2009; Wrzaczek et al. 2010).

1.2.2 Role of Reactive Oxygen Species in Signaling

ROS are another type of signaling molecule conserved across kingdoms (Overmyer et al. 2003). In animals, by affecting the levels of pro-apoptotic proteins such as caspases and anti-apoptotic proteins such as Bcl-2, ROS can regulate apoptosis (Azad and Iyer 2014). Overproduction of ROS in response to various stimuli may lead to apoptosis (Circu and Aw 2010; Azad and Iyer 2014). It stimulates the intrinsic pathway by allowing for Cyt c and apoptosis-inducing factor (AIF) release in to the cytosol through fostering mitochondrial outer membrane permeabilization (Circu and Aw 2010). ROS may also induce the extrinsic apoptotic pathway through induction of death receptor clustering, formation of signaling platforms involving lipid rafts and activation of death receptors (Zhang et al. 2006, 2007; Circu and Aw 2010).

The plant molecular signaling pathway, involving ROS (Figure 1.2) is not yet well understood, but it is known that in response to specific stimuli, ROS production is spatially regulated (Apel and Hirt 2004). For example, ROS are known to play an important role in plant defense against pathogens (Mendoza 2011); plant cells will display increased extracellular ROS production through NADPH oxidases (in the plasma membrane; Bolwell and Wojtaszek 1997), peroxidases (in the cell wall; Kawano 2003) and amine oxidases (Allan and Fluhr 1997). Through diffusion, this extracellular ROS,

nitric oxide and SA are thought to enter through cell aquaporins and activate response pathways including PCD (Figure 1.3; Gadjev et al. 2008). The SA and nitric acid will down regulate ascorbate peroxidase and catalase, which are involved in scavenging of cellular ROS. As a result, the cell will produce and accumulate more ROS: leading to the activation of PCD. ROS signal transduction leading to PCD involves unknown ROS sensors, mitogen-activated protein (MAP) kinase kinase kinases (NPK1 and AtANP1), MAPKs (AtMPK3/6, and Ntp46MAPK) and calmodulin (Kovtun et al. 2000; Samuel et al. 2000). Transcription factors such as WRKYs, ethylene-responsive element binding protein (EREBP), dehydration-responsive element-binding 2A-like (DREBA), Myeloblast (MYB), AP-1 and AS-1 and heat shock factor (HSF) are also involved downstream, before activation of the response to the pathogen, including PCD (Figure 1.2 and 1.3; Mittler 2002; Ogawa et al. 2005; Gadjev et al. 2008). Elevated ROS levels may also result in increased Ca^{2+} levels in the cytoplasm. The Ca^{2+} will enter the mitochondria, resulting in high mitochondrial Ca^{2+} concentration. This causes opening of mitochondrial permeability transition pore and release of Cyt c, which will induce transcription of PCD genes (Figure 1.3; Virolainen et al. 2002; Lin et al. 2005; Martínez-Fábregas et al. 2013, 2014b).

It has been shown that under normal conditions most of the Cyt c is retained in mitochondrial inter membrane space, and is released into the cytoplasm and nucleus during PCD (Vianello et al. 2007; Li and Xing 2010; Martínez-Fábregas et al. 2014b). As in mammalian apoptosis, during plant PCD the Cyt c will then interact with various anti-apoptotic and pro-survival proteins in the cytoplasm and nucleus (Martínez-Fábregas et al. 2014a). In plants, it is not known how the release of Cyt c from mitochondria upon

death stimuli results in transcription of PCD genes, but Martínez-Fábregas et al. (2013) and Martínez-Fábregas et al. (2014a) identified proteins that interact with it in Arabidopsis. These proteins include transcriptional coactivator-like protein (TCL) which is involved in mRNA metabolism, and nucleosome assembly protein 1-related protein 1 (NRP1), which is involved during DNA damage. As in human cells, the other Cyt c interacting proteins they discovered are involved in energetic metabolism, oxidative stress, translational regulation, protein folding, and cell death (Martínez-Fábregas et al. 2013, 2014). Therefore, the PCD-related function of Cyt c seems to be conserved to an extent between plants and mammals.

In addition, some of these Cyt c interacting proteins functionally resemble those that interact with human Cyt c during apoptosis (Martínez-Fábregas et al. 2013, 2014a). For example, Cyt c interacts with SET in human cells and NRP1 in plant cells, which are both involved in DNA damage. It also interacts with components of the eukaryotic initiation factor 2 (eIF2a in humans and eIF2g in plants) involved in protein synthesis and enzymes involved in energy metabolism (ALDOA in humans and GAPC1 in plants). Plant BiP1 and BiP2, involved in protein folding, also interact with the Cyt c; they resemble HSPA5 which interacts with Cyt c in the human apoptotic system and is also involved in protein folding (Martínez-Fábregas et al. 2013, 2014a). In plants, the Cyt c also interacts with the cysteine proteinase RD21 and an oxidative stress related protein GLY2, both of which play key roles in PCD (Martínez-Fábregas et al. 2014a). Martínez-Fábregas et al. (2014a) reported that all but one (ALDOA and GAPC1) of the above mentioned comparable pairs of Cyt c interacting proteins between humans and plants impact trimerization of the protein synthesis factor eIF2, therefore mediating cell

responses during PCD or survival. Martínez-Fábregas et al. (2014a) claim that the shared mode of interaction involving Cyt c and eIF2 trimerization mediating proteins during PCD signaling in some plant PCD mechanisms is the first essential indicator of an evolutionarily conserved core PCD mechanism between distantly related human and plant cells. They also demonstrated that in addition to mediating PCD through pro-apoptotic proteins, Cyt c could also enhance PCD by inhibiting cellular pro-survival proteins (Martínez-Fábregas et al. 2014a). These new discoveries are essential since they highlight that despite vast differences in signaling evident so far and differences in metabolic pathways, plant PCD and mammalian apoptosis systems may share many similarities that need to be examined.

1.3 Regulation of Plant PCD

Regulation of PCD in plants seems to be similar to apoptosis regulation in some ways: such as the involvement of Bcl-2/Bcl-2-like proteins, defender against apoptotic cell death (DAD1) and Bax inhibitor 1 (BI-1). Since more is known about the role of this family of proteins in animal apoptosis than in plant PCD, their role in apoptosis is discussed and compared to what is known so far about their role in plant PCD. Plants also possess unique regulators in the form of plant hormones; SA, jasmonic acid (JA) and ethylene. Their regulatory role in plant PCD is also discussed.

1.3.1 Bcl-2 family

1.3.1.1 Bax

Bax is a Bcl-2 family protein and its role in Bax induced PCD is well elucidated in mammals but less understood in plants. In mammalian apoptosis, Bax forms pores on

the outer mitochondrial membrane, allowing Cyt c release and activation of caspases (Danial and Korsmeyer 2004). Plant genomes lack a Bax homologue, but expression of Bax in transgenic plants results in Bax localization on the mitochondrial membrane, increased ROS production and triggers PCD (Lacomme and Cruz 1999; Yoshinaga et al. 2005). In tobacco, Bax induced PCD was found to resemble hypersensitive response (HR) and lead to the accumulation of pathogenesis-related protein 1 (PR1) (Lacomme and Cruz 1999). It is likely that plants possess functional homologues of Bax; their genomes have a Bax inhibitor, which is highly conserved between plants and animals (Reviewed in Ishikawa et al. 2011). The plant functional homologue needs to be identified and if it exists it could be playing a similar role as in animals, considering that Cyt c is also released from the mitochondria during plant PCD. In cucumber cotyledons, heat treatment was observed to induce Cyt c release from mitochondira and PCD (Balk et al. 1999). Studying the relationship between expression of Bax in transgenic plants, Cyt c release and activity of caspase-like enzymes would provide insights into whether the plant PCD system may be following a similar mechanism, involving some form of a Bax homologue.

1.3.1.2 Bax Inhibitor

BI-1 is involved in inhibition of PCD in Metazoa and plants (Hückelhoven et al. 2003; Watanabe and Lam 2009; Ishikawa et al. 2011). Arabidopsis BI-1 has been shown to slow down progression of PCD induced by fungal toxin and heat stress (Watanabe and Lam 2006). BI-1 is employed in stress responses that cause an increase in ROS production; these include pathogen attack, heat, high salinity and chemical induced oxidative stress (Hückelhoven et al. 2003; Isbat et al. 2009). BI-1 genes have been

identified in Arabidopsis, rice and pepper (Kawai et al. 1999). Over expressing the Arabidopsis BI-1 (AtBI-1), inhibited PCD induced by mammalian Bax, which is known to promote PCD in transgenic plants (Kawai-Yamada et al. 2001) and yeast (Kawai et al. 1999). This demonstrates how highly conserved the genes are and also suggests the presence of a common PCD mechanism within yeast, mammals and plants involving BI-1. BI-1 is localized in the endoplasmic reticulum and works by blocking PCD after Bax induced mitochondrial membrane alterations, but before Cyt c release (Reviewed in Ishikawa et al. 2011). Exactly how BI-1 blocks plant PCD is not known, but it may be inhibiting Cyt c release from the mitochondria and prohibiting the Cyt c interactions with pro-apoptotic and pro-survival proteins: an interaction which is essential for plant PCD.

1.3.1.3 Bcl-2

Dion et al. (1997) used western blot and immunochemistry to identify a plant epitope of Bcl-2. It was identified in a broad range of plants; in species such as *Zea mays*, *Brassica napus, Nicotiana tabacum* and in green algae (Dion et al. 1997; Danon et al. 2000). The molecular mass (28-29 kDa) of this protein falls within range of known animal Bcl-2 proteins. In mammalian cells, it is found in the endoplasmic reticulum (ER), mitochondrial outer membrane, nuclear envelope and chromatin (Monaghan et al. 1992; Krajewski et al. 1993; Akao et al. 1994; Givol et al. 1994; Hickish et al. 1994; Lu et al. 1994; Chan et al. 1995; Riparbelli et al. 1995; Dion et al. 1997). In plants, it is found mainly in the mitochondria and nuclei, but it is also found in plastids and chloroplasts (Dion et al. 1997). The similar primary localization of the Bcl-2 protein in animals and the epitope in plant cells suggest they play similar roles. In mammals, Bcl-2 promotes cell survival by negatively regulating adapters required to activate caspases, which are

key executors of PCD in mammals (Adams and Cory 1998).

In plants, it is likely playing a similar role by inhibiting plant caspase-like enzymes activating adaptors. Deng et al. (2011) showed that over-expressing the human Bcl-2 in transgenic rice suppressed H_2O_2 induced cell death and also inhibited H_2O_2 induced transcription of rice caspase-like enzymes OsVPE2 and OsVPE3. Similar results were also obtained using salt as the stress inducer (Deng et al. 2011). This evidence suggests that plant epitope most likely inhibits the adapters involved in activation of plant caspase-like enzymes, such as; vacuolar processing enzymes (VPEs). These adaptors have not been identified in plants. Even though the sequence of this epitope was not reported, new studies also hint towards conservation of Bcl-2 in other kingdoms as well.

Bcl-2-associated athanogene (BAG) proteins, a family of proteins known to regulate various physiological processes in animals, including apoptosis, and interact with Bcl-2 were discovered in plants (Doukhanina et al. 2006; Rana et al. 2012). These proteins were identified in Arabidopsis and rice and the domain organization in most of the plant BAG protein resembles that of animal homologues. Similar to animal BAG proteins, the plant homologues were also shown to be involved in stress responses (Rana et al. 2012). They may also have a role in plant PCD, and elucidating more of their functions may uncover new insight into whether they interact with a functional homologue of Bcl-2 in plants. Other researchers (Yang et al. 2012; Wang and Bayles 2013) have also identified a plant homologue of a bacterial protein (Cid/Lrg) evolutionarily associated with Bcl-2 family proteins and involved in plant PCD. Plant and bacterial Cid/Lrg proteins as well as mammalian Bcl-2 proteins are thought to be

functional holins in a model developed by Wang and Bayles (2013). Collectively, this evidence suggests the presence of plant proteins that carry Bcl-2-like properties essential for PCD.

1.3.2 Defender Against Apoptotic Cell Death

Defender against apoptotic Cell Death 1 (DAD1) is a highly conserved suppressor of PCD. Its involvement in PCD has been demonstrated in several plant species such as Arabidopsis (Gallois et al. 1997), pea (Orzáez and Granell 1997), rice (Tanaka et al. 1997), apple (Dong et al. 1998), barley (Lindholm et al. 2000), and tomato (Hoeberichts and Woltering 2001). DAD1 is both evolutionarily and functionally conserved; it inhibits plant PCD during senescence (Gallois et al. 1997; Dong et al. 1998; van der Kop et al. 2003), UV-C overdose (Danon et al. 2004), and maize seed development (Shun-bin et al. 2001). However, there is counterevidence that suggests DAD1 may not be involved in at least some of the plant processes that employ PCD (Dong et al. 1998; Moriguchi et al. 2000; Hoeberichts and Woltering 2001).

During apple fruit ripening and petal senescence, DAD1 expression increases (Dong et al. 1998). However, DAD1 expression did not show any significant changes during fruit over-ripening involving PCD in tomato and citrus (Moriguchi et al. 2000; Hoeberichts and Woltering 2001). The counter evidence nevertheless does not entirely eliminate a possible role of DAD1 in PCD during these processes. Both ripening and senescence are long processes, and PCD occurs during the late stages of these processes. Therefore, despite the constant or increased DAD1 expression reported during the earlier stages of these processes, the expression levels may eventually subside during the PCD

stage. The exact biochemical function of DAD1 is still elusive, but evidence suggests that its biochemical action is conserved between nematodes, mammals and plants. Plant DAD-1 genes are able to inhibit PCD in mutant hamster cell line tsBN7 (Gallois et al. 1997; Tanaka et al. 1997), indicating that DAD1 is functionally conserved. Sequence analysis of different DAD1 homologues also suggests that it is evolutionarily conserved (Sugimoto et al. 1995; Tanaka et al. 1997): leading to the possibility that it may be playing a similar role during PCD in plants and animal apoptosis (van der Kop et al. 2003).

In animal cells, DAD1 interacts with an anti-apoptotic Bcl-2 family protein Mcl-1; which interacts with pro-apoptotic Bcl-2 members like BAK1 (Leu et al. 2004), Noxa (Chen et al. 2005; Willis et al. 2005), Bcl-2-associated death promoter (Bae et al. 2001; Chen et al. 2005), BCL2L11 (Hsu et al. 1998; Bae et al. 2000; Chen et al. 2005), and BH3 interacting domain death agonist (Chen et al. 2005; Weng et al. 2005) during PCD. How DAD1 interacts with Mcl-1 protein is not completely clear. In mammals, DAD1 forms a subunit of an ER localized key enzyme, oligosaccharyltransferase (OST) complex, involved in *N*-linked protein glycosylation (Kelleher and Gilmore 1997; Makishima et al. 1997). *N*-linked glycosylation plays a major role in cell function; it is essential for protein transport (Silberstein and Gilmore 1996; Kelleher and Gilmore 1997; Makishima et al. 1997; Ceriotti et al. 1998). Deactivating DAD1 in animal cells not only causes cell death, it also decreases N-linked glycosylation (Makishima et al. 1997). Decreased *N*-linked glycosylation could lead to excessive accumulation of proteins in the ER, causing ER stress and leading to PCD.

N-glycosylated proteins like phytepsin, a vacuolar aspartic proteinase, were detected during the onset of DNA fragmentation in barley scutella (Lindholm et al. 2000). Lindholm et al. (2000) also showed that expression of OST increased before the beginning of DNA fragmentation. Phytepsin is also highly expressed during PCD in tracheary element differentiation (Runeberg-Roos and Saarma 1998). Phytepsin shares similar primary and secondary structure, substrates and localization within the cell with animal cathepsin D; which moderates PCD in the human cell line HeLa (Deiss et al. 1996). It is likely that they play a similar role during PCD, and both depend on N-glycosylation through the DAD1 containing OST complex for their translocation to the vacuole, where they carry out their PCD roles. Shun-bin et al. (2001) revealed the expression of DAD1 in highly metabolizing cells including cotyledon and root tip, dividing cells in the endosperm and maize female germ cells, providing evidence for its broader function outside of plant PCD.

1.3.3 Plant Hormones

Plant defense against pathogens is orchestrated by SA or JA/ethylene pathways (Figure 1.2 and 1.3; Cao et al. 1994; Berrocal-Lobo et al. 2002; Ton et al. 2002; Mur et al. 2008; Reviewed in Mur et al. 2013). Both pathways involve nitric oxide (NO) as a key inducer or suppressor signal (Mur et al. 2013). SA mainly regulates HR by inducing a large number of defense genes such as pathogenesis related (PR) proteins (e.g. PR1) (Cao et al. 1994; Mur et al. 2013). An interaction between a cytoplasm localized transcription activator NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEINS1 (NPR1) and SA receptors, NPR3 and 4, leads to NPR1 being converted to a monomeric form. The monomeric NPR1 is then transported to the nucleus where it interacts with TGA-class

transcription factors, activating defense related genes (Zhang et al. 1999; Mou et al. 2003, Fu et al. 2012; Mur et al. 2013). In healthy cells, NPR1 levels are regulated by proteasome based degradation to maintain minimal expression of defense genes (Spoel et al. 2009; Fu et al. 2012).

In cases of attack by necrotrophs, JA and ethylene regulate the plant HR response (Berrocal-Lobo et al. 2002; Ton et al. 2002; Mur et al. 2013). In this pathway, JAR1 conjugatase binds with JA to form (+)-7-iso-jasmonoyl-L-Ile (JA-Ile). JA-Ile will then interact with JASMONATE ZIMDOMAIN (JAZ) proteins targeting complex Skp-Cullin-F-box (SCF^{COI}) through CORONATIVE INSENSTIVE1 (COI1) protein (Chini et al. 2007). This will lead to the ubiquitination and proteasome dependent breakdown of JAZ repressors, which are negative regulators of transcriptional factors such as MYC 2, MYC 3, MYC 4 (Chini et al. 2007; Fernández-Calvo et al. 2011; Mur et al. 2013). COI1 histone deacetylases (HDA6 and HDA19) are both involved in JA signaling as transcription repressors (Devoto et al. 2002; Dombrecht et al. 2007). HDA19 suppresses transcription of defense genes that ETHYLENE RESPONSE FACTOR1 (ERF1) upregulates (Zhou et al. 2005).

In the ethylene pathway, ethylene receptors ETR1, ERS1, ETR2, ERS2 and EIN4 bind to ethylene and are relieved of their inhibitory role (Hua and Meyerowitz 1998). Their negative regulatory role in the pathway is carried out in conjugation with CTR1, a MAP3K (potentially through a MAP-Kinase cascade), which is also inhibited when receptors bind to ethylene. After the deactivation of the ethylene-CTR1 complex, Ethylne-insensitive 2 (EIN2), a positive regulator and key component in ethylene signaling, is dephosphorylated and transported to the nucleus. The EIN2 and EIN3 family

of transcription factors regulate the expression of transcription factors such as ORA59 and ERF1. Transcription of target genes essential for ethylene induced responses including PCD are then activated (Wang et al. 2002; Stepanova and Alonso 2009; Mur et al. 2013). When ethylene is not present, EIN3 is targeted by EIN3-binding F-box 1 and 2 (EBF1and EBF2) for degradation by the proteasome (Guo and Ecker 2003; Potuschak et al. 2003; Gagne et al. 2004; Mur et al. 2013). The JA-ethylene pathways interact primarily as JAZ repressors from the JA pathway interact with EIN3 (Zhu et al. 2011; Mur et al. 2013). The JA and ethylene pathways also integrate through ORA59 (Pré et al. 2008). ORA59 is essential for the expression of JA and ethylene induced defense genes such as PLANT DEFENSIN1.2 (PDF1.2). Due to the absolute importance of the plant hormones in plant PCD, genes involved in their biosynthesis and signal transduction are essential for plant PCD. Ethylene is not only involved in PCD during HR; it is also involved in plant developmental processes that employ PCD such as senescence (Reviewed in Graham et al. 2012), maize endosperm development (Young et al. 1997), and perforation formation in the lace plant (Dauphinee et al. 2012).

1.4 Execution of PCD in Mammals and Plants

1.4.1 Execution of PCD in Mammals

In mammals, execution of PCD revolves around cysteine aspartic proteases (caspases). Caspases are synthesized as inactive proenzyme forms called procaspases and they have to be cleaved to mature into active proteases (Yang et al. 1998). Once activated, they regulate the execution of processes that lead to the visible morphologies of apoptosis such as DNA fragmentation, formation of apoptotic bodies, degradation of

cellular proteins, chromatin condensation, and phagocytosis of recyclable cell components into neighboring cells (Reviewed in Elmore 2007). Biochemical changes such as crosslinking of proteins and protein cleavage also occur (Hengartner 2000). Caspases mediate the processing of specific molecules involved in the execution of cell death such as Caspase-activated DNase (CAD), a DNA helicase and Acinus; the protein responsible for chromatin condensation during apoptosis (Fisher et al. 2003; re-viewed in Woltering 2010). The aforementioned changes during apoptosis are an end result of a successful link between the initiation, signaling, regulation and execution of apoptosis; leading to cell demise. Signaling molecules that initiate phagocytosis are then expressed and engulfment genes activated to make sure cell debris is cleared away (Elmore 2007). Generally, caspase activation commits cells to undergo apoptosis. In humans, there are 14 caspases and those involved in apoptosis can be divided into two types: 1. initiator caspases (caspases 2, 8, 9 and 10) and 2. executioner caspases (caspases 3, 6 and 7) (MacKenzie and Clark 2012; Reviewed in McIlwain et al. 2013). The rest of the caspases are classified as inflammatory caspases.

1.4.1.1 Initiator Caspases

Initiator caspases are usually comprised of a small subunit, a large subunit (Figure 1.4) and a prodomain with either a caspase recruitment domain (CARD) or a death effector domain (DED; Taylor et al. 2008; McIlwain et al. 2013). Adaptor molecules such as FADD recruit caspases (especially 8 and 10) by interacting with their DED, forming a DISC. Initiator caspases are usually activated through dimerization instead of cleavage. They autocatalyze through a process termed induced proximity model; in which interaction with the upstream signal causes the intrinsic enzymatic activity possessing

procaspases to cluster together in close-proximity, form dimers and activate (Muzio 1998; Salvesen and Dixit 1999; Boatright et al. 2003; Chang et al. 2003; McIlwain et al. 2013). An active mature caspase is made up of dimers made from heterodimers consisting of two copies of both the small and large subunits (MacKenzie and Clark 2012). The active mature initiator caspase can either directly cleave executioner caspases to initiate the execution of apoptosis, or induce apoptosis by cleaving Bid: depending whether the apoptosis pathway is intrinsic or extrinsic (McIlwain et al. 2013).

The role and classification of caspase 2 is more complex. It can serve as an initiator in both intrinsic and extrinsic pathways. It can also act as an effector caspase. It is the only initiator caspase that requires proteolytic cleavage to be activated (Li et al. 1997; Baliga et al. 2004; MacKenzie and Clark 2012). Therefore its classification as an initiator caspase is questionable.

1.4.1.2 Executioner Caspases

Executioner caspases 3, 6 and 7 are activated from their inactive forms by initiator caspases. Once activated, they can also cleave and activate each other giving rise to a fast and aggressive activation loop. Executioner caspases are activated by cleavage of the intersubunit linker between the small and large subunits; allowing the subunits to orientate such that active sites are aligned together producing a mature active caspase **(**Ried and Shi 2004; McIlwain et al. 2013). The executor caspases also cleave some key proteins involved in execution of PCD: such as inhibitor of caspase activated DNase (ICAD), which will then release a caspase activated DNase (CAD). CAD is responsible for the DNA fragmentation during apoptosis (Enari et al. 1998; MacKenzie and Clark

2012). Other examples of caspase substrates include acinus and helicard (responsible for chromatin condensation), gelsolin, ROCK-1 and PAK2 (responsible for membrane blebbing; Reviewed in Fischer et al. 2003). Changes in cell shape during apoptosis may be due to cleavage of structural proteins such as cytokeratin-18 and vimentin, or Gas2 and plectin. Cell shrinkage is likely a result of cleaving focal adhesion kinase, Cas or paxillin; which adhere membrane proteins and actin filaments to the extracellular matrix (Gerner et al. 2002; Fischer et al. 2003). Nuclear matrix proteins are also degraded during apoptosis (Gerner et al. 2002; Fischer et al. 2003). The caspases can also switch off anti-apoptotic mechanisms; e.g. anti-apoptotic Bcl-2 members Bcl-2, Bcl-xL and Bid are cleaved and turned into proapoptotic proteins accelerating the apoptosis process (Reviewed in Fischer et al. 2003).

1.4.2 Execution of PCD in Plants

Some of the morphological and biochemical characteristics of apoptosis that are directly (or indirectly) a result of caspases in animal cells, such as DNA laddering, chromatin condensation, shrinkage of the cytoplasm, Cyt c release from mitochondria, caspase-like activity and activation of death proteases are also observed during plant PCD. Despite these similarities, plant genomes lack true caspases; instead they possess caspase-like enzymes (Piszczek and Gutman 2007; Gadjev et al. 2008). Proteases with similar sequences to caspases exist within plant genomes and they have shown increased expression during plant PCD, but most of these proteases do not possess caspase-like activity and do not cleave caspase substrates (Aravind et al. 1999; Uren et al. 2000; Vercammen et al. 2004; Watanabe and Lam 2005; Piszczek and Gutman 2007). This could mean that during plant PCD caspase substrates are not cleaved after the normal Asp

residue that the caspases prefer, or that other proteases, which do not share a significant sequence similarity with caspases are capable of hydrolyzing caspase substrates at the same site. Evidence supports the latter since other enzymes involved in plant PCD, with limited sequence similarity to animal caspases, have been shown to cleave synthetic caspase substrates at specific caspase recognition motifs (e.g. YVAD, VEID and TATD) (De Jong et al. 2000; Mlejnek and Prochazka 2002; He and Kermode 2003; Belenghi et al. 2004; Bozhkov et al. 2004; Chichkova et al. 2004; Danon et al. 2004; Piszczek and Gutman 2007; Bonneau et al. 2008; Vartapetian et al. 2011).

Specificity to cleave substrates at the caspase preferred site has been reported during plant PCD and caspase inhibitors such as p35 and Inhibitor of apoptosis (IAP) suppress plant PCD (Lincoln et al. 2002; Woltering et al. 2002; del Pozo and Lam 2003; Vartapetian et al. 2011); suggesting a requirement for caspase-like behavior during plant PCD. Caspase-1, -3, -4, -6 and -8-like activities have been reported during plant PCD (Cai et al. 2014; Tran et al. 2014). Plant caspase-like proteases identified so far fall into two broad groups: 1.subtilisin family serine endopeptidases (Saspases and Phytaspases) and 2. legumain family cysteine endopeptidases (metacaspases and vacuolar processing enzymes; Coffeen and Wolpert 2004; Piszczek and Gutman 2007; Vartapetian et al. 2011).

1.4.2.1 Saspases

Unlike caspases, which are cysteine dependent, saspases are serine dependent proteases. Despite this, they cleave synthetic caspase substrates, are Asp specific and also synthesized as inactive enzyme precursors. Saspases belong to a group of 56 subtilisin-like proteases in Arabidopsis, characterized by a catalytic triad consisting of amino acids:

aspartate, histidine and serine (Dodson and Wlodawer 1998; Rautengarten et al. 2005; Tripathi and Sowdhamini 2006; Vartapetian et al. 2011). Saspase precursors (preprosaspases), like all subtilisin-like proteases, comprise a signal peptide, a pro-domain and a peptidase domain. This domain architecture of saspase precursors is mainly inferred from other plant subtilisin-like proteases: since to date only saspase sequences known are partial sequences of SAS-1 and SAS-2 from *Avena sativa* (Coffeen and Wolpert 2004). A protease-associated domain is located within the peptidase domain (Figure 1.4; Tripathi and Sowdhamini 2006; Vartapetian et al. 2011).

Maturation of a preprosaspase occurs when the peptide and pro-domain are removed (Figure 1.5A; Vartapetian et al. 2011). The structure of saspases is different from that of caspases, which are generally made of an amino-terminal pro-region, large subunit and a small subunit (Figure 1.4). Additionally, mature saspases are single polypeptide chains of approximately 80 kDa, while mature caspases are made of a dimer of heterodimers, each subunit being roughly 20 and 12 kDa (Reviewed in Tripathi and Sowdhamini 2006). In *A. sativa* (oat), the two saspases are thought to be responsible for caspase-like activity involved in victorin induced PCD (Coffeen and Wolpert 2004). They displayed activity towards caspase substrate recognition sites VAD, VNLD, VEHD and VKMD (Coffeen and Wolpert 2004), but little to no peptidase activity to other caspase substrates with recognition sites YVAD, VDVAD, DEVD, LEVD, WEHD and VEID, which are preferred recognition sites for caspases 1, 2, 3, 4, 5 and 6 respectively (Coffeen and Wolpert 2004; Vartapetian et al. 2011). They also did not cleave general protease substrates such as casein, subtilisin A, chymotrypsin and cathepsin B (Coffeen and Wolpert 2004). Saspases seem to be indirectly involved in large subunit ribulose-

1,5-bisphosphate carboxylase/oxygenase (Rubisco) cleavage through a protease cascade (Coffeen and Wolpert 2004; Vartapetian et al. 2011).

Despite being able to cleave some caspase substrates and being inhibited by caspase inhibitors, classification of saspases as caspase-like enzymes is still questionable since they are serine instead of cysteine proteases. Saspase activity during plant PCD is also detected in the extracellular fluid instead of within cytoplasm, as displayed by caspases (Figure 1.5; Vartapetian et al. 2011). Additionally, there are serine proteases such as granzyme B and the 26s proteasome in animals that can also cleave caspase substrates (Thornberry et al. 1997; Kisselev et al. 2003; Bonneau et al. 2008), but are not classified as caspases.

1.4.2.2 Phytaspases

Phytaspases are also serine dependent subtilisin-like proteases with Asp specificity. They are synthesized as preproenzymes consisting of an N-terminal signal peptide, prodomain and protease domain. The N-terminal signal peptide and prodomain are removed to form a mature enzyme, which is approximately 80 kDa (Figure 1.4; Reviewed in Vartapetian et al. 2011). Phytaspases were identified in rice and tobacco while seeking plant proteases capable of cleaving the protein VirD2 from *Agrobacterium tumefaciens,* which causes the crown gall disease in plants (Chichkova et al. 2010; Vartapetian et al. 2011). VirD2 is known to orchestrate insertion of bacterial DNA within the infected plant's nuclear DNA. It is usually cleaved within the recognition motif TATD, a recognition site for caspase 3, which cleaves after the Asp.

Chichkova et al. (2004) discovered that tobacco plants containing the resistance gene (N gene) were able to cleave the VirD2 *in vivo* an hour after PCD has been induced

in response to tobacco mosaic virus (TMV; reviewed in Vartapetian et al. 2011). This suggested the presence of a plant protease possessing caspase-3 like specificity and activity during plant PCD in response to pathogen attack. The activity was not observed in healthy leaves; where PCD was not occurring or in tobacco plants lacking the N gene (Chichkova et al. 2004). They isolated the plant protease from both rice and tobacco and discovered that it was able to cleave synthetic caspase substrates such as YVAD-, IETD-, VDAD-, LEHD-, VAD- and VEID-AFC, but it did not cleave DEVD-AFC (Chichkova et al. 2010). Like procaspases, these phytaspase precursors displayed an autocatalytic processing ability (Chichkova et al. 2010; Vartapetian et al. 2011).

Overexpression of the phytaspases amplified PCD characteristics such as ROS accumulation, Cyt c release, and diminished cell viability during PCD in response to biotic and abiotic stresses. PCD was also suppressed when phytaspases were downregulated in TMV infected plants, and this made them more vulnerable to spread of the virus (Chichkova et al. 2010; Vartapetian et al. 2011). This evidence suggests the importance of phytaspases in HR and PCD caused by a wide range of stimuli. Even though they share some characteristics with caspases, active phytaspases are localized in the apoplast until PCD is induced, instead of being stored as inactive zymogens within the cytoplasm like caspases (Figure 1.5B; Chichkova et al. 2010; Fuentes-Prior and Salvesen 2004; Vartapetian et al. 2011). Vartapetian et al. (2011) suggested that they might be playing a protective role within the apoplast against effectors secreted by pathogens. Similarities in terms of specificity and roles in PCD, as well as differences in structure are discussed in more detail elsewhere (Chichkova et al. 2012). Like saspases, phytaspases are similar to animal and yeast subfamily S8B pro-protein convertases,

which are also subtilisin-like proteases (Steiner 1998; Vartapetian et al. 2011). More research needs to be carried out to identify natural substrates of phytaspases during plant PCD, to elucidate their specific role and unravel the protease cascade they employ during PCD.

1.4.2.3 Metacaspases

Metacaspases are cysteine dependent proteases, like caspases. Even though their sequences are different, the secondary structure and catalytic dyad (His-Cys) of metacaspases are similar to that of caspases (Vercammen et al. 2007). Only a few have a His-Ser catalytic dyad instead (Szallies et al. 2002). Metacaspases are only found in eukaryotes lacking true caspases, such as fungi and plants. They are absent in eukaryotes containing caspases, such as animals (Carmona-Gutierrez et al. 2010). Metacaspases are also synthesized as inactive precursors or proenzymes (Piszczek and Gutman 2007). There are two types of metacaspases: 1. type I metacaspases and 2. type II metacaspases. Type I metacaspase precursors possess an N-terminal pro-domain resembling that of initiator procaspases. Type II metacaspase precursors lack the pro-domain; and are similar to executioner procaspases, which also lack an extended N-terminal domain (Uren et al. 2000; Lord and Gunawardena 2012; Choi and Berges 2013). Additionally, both types of metacaspase precursors contain a large and a small subunit joined together by an interdomain linker; reminiscent of caspases (Figure 1.4). The interdomain linker in type II prometacaspases is longer (~130 amino acids) compared to the same in type I prometacaspases and in procaspases (~30 amino acids). It is not yet fully understood how prometacaspases are activated; most of them are able to autocatalyze and be active (Vercammen et al. 2004; Bozhkov et al. 2005b; González et al. 2007; Tsiatsiani et al.

2011; Watanabe and Lam 2011), while some do not need autolytic processing to be active (Lee et al. 2007; Moss et al. 2007; Ojha et al. 2010; Tsiatsiani et al. 2011).

Metacaspases are mainly localized in the cytoplasm (Figure 1.5C) but they can translocate to other organelles (such as the nuclei) during PCD (Woltering 2004; Bozhkov et al. 2005b). Most metacaspase precursors prefer a pH of 7-8.5 for activation; and some require elevated concentration levels of Ca^{2+} (Figure 1.5C; Vercammen et al. 2004; Bozhkov et al. 2005b; Watanabe and Lam 2005; Lee et al. 2007; Moss et al. 2007; He et al. 2008; Tsiatsiani et al. 2011). Under elevated levels of Ca^{2+}, a highly conserved site AKDK(225) is cleaved to activate the prometacaspase (Tsiatsiani et al. 2011; Watanabe and Lam 2011). When overproduced in *Escherichia coli*, type II metacaspase precursors were able to autoprocess, while type I prometacaspases (from Arabidopsis) did not (Vercammen et al. 2004; Watanabe and Lam 2005; Vercammen et al. 2006, 2007). Similar to mammalian initiator procaspases, type I prometacaspases may need to be mobilized to activation platforms, which are conducive for dimerization and activation (Fuentes-Prior and Salvesen 2004; Vercammen et al. 2007). They may require suitable conditions like optimal pH and a high Ca^{2+} concentration to induce autocatalysis.

Metacaspases prefer cleaving their substrates after the basic residues arginine (Arg) or lysine (Lys) residues instead of the acidic caspase preferred Asp residues (Vercammen et al. 2004; Watanabe and Lam 2005; Carmona-Gutierrez et al. 2010). Therefore, whether they cleave many similar PCD related substrates is unknown. Only one natural substrate (Tudor staphylococcal nuclease; TSN) common for metacaspases and caspases (caspase 3) has been identified so far, however the cleavage sites are not the same (Sundström et al. 2009; Carmona-Gutierrez et al. 2010). Metacaspase inhibitor,

EGR-chloromethyl ketone, and a catalytic Cys mutation abolished metacaspase processing of TSN (Sundström et al. 2009). Human TSN is involved in gene expression regulation (splicing) and is involved in PCD (Sundström et al. 2009; Carmona-Gutierrez et al. 2010). The function of TSN in plants is unknown and it is unlikely that it is involved in splicing since in plants TSN is localized in the cytoplasm (Sundström et al. 2009; dit Frey et al. 2010). Since metacaspases cleave TSN at about five different sites and caspase 3 cleaves at a single site, it is likely that metacaspases degrade TSN while caspase 3 activates it. Tsiatsiani et al. (2011) proposed a model by which plant TSN could be regulating the expression of protease inhibitors to protect cells from PCD. According to this untested model, when metacaspases degrade TSN, protease inhibitor levels will be decreased, thus allowing PCD to occur.

Metacaspases have been implicated in different plant PCD processes such as oxidative stress induced PCD (He et al. 2008), HR (Hoeberichts et al. 2003), and developmental PCD during embryonic pattern formation (Bozhkov et al. 2004; Suarez et al. 2004; Bozhkov et al. 2005a). A serine protease inhibitor that inhibits caspase 1, 8 and 10 by being cleaved by these caspases at its reactive center loop, and binding to them, also inhibits Arabidopsis metacaspase 9 in a similar way. Despite all this evidence, it is not conclusive that metacaspases perform caspase-like duties in plants. More research is needed to determine if there are any natural caspase substrates that metacaspases process in a similar manner, and whether they process any natural substrates directly related to plant PCD.

1.4.2.4 Vacuolar Processing Enzymes

Four vacuolar processing enzymes (VPEs) were identified in Arabidopsis and they were classified into seed type and vegetative type VPEs (Kinoshita et al. 1995a, 1995b; Nakaune et al. 2005; Yamada et al. 2005). Similar to caspases, VPEs are cysteine proteases; they have a His–Cys catalytic dyad (Figure 1.4), but they cleave their substrates specifically after asparaginyl (Asn) residues instead of the caspase preferred Asp residues (Crawford and Wells 2011; Tsiatsiani et al. 2011; Misas-Villamil et al. 2013).

Despite being Asn specific, VPEs have a capacity to process substrates and inhibitors with Asp residues preceding the cleavable peptide bond (Hatsugai et al. 2004; Rojo et al. 2004; Misas-Villamil et al. 2013). Hatsugai et al. (2004) also demonstrated that VPEs process natural substrates after Asp residues, but at low rates. Their ability to process these substrates was attributed to the cellular localization of VPEs within the acidic vacuole, where the pH (~5.5) is capable of extinguishing the negative charge of the Asp residue through partial protonation of its side chain (Kato et al. 2005; Misas-Villamil et al. 2013). Synthetic caspase inhibitors (mostly caspase-1 inhibitors) that specifically inhibit VPEs include Ac-YVAD-CHO, YVAD-, and YVKD-CMK (Hatsugai et al. 2004; Rojo et al. 2004; Misas-Villamil et al. 2013). VPEs are only able to cleave after Asp residues when they are part of YVAD, a caspase 1 substrate sequence. The same phenomenon is characteristic of caspase-1 (Stennicke and Salvesen 1998; Earnshaw et al. 1999).

In addition, VPEs also share three other properties with caspase 1. First, His237 and Cys285 within the catalytic dyad of human caspase-1 are comparable to His174 and

Cys216 in tobacco VPE (NtVPE-1a; Figure 1.4; Cohen 1997; Hiraiwa et al. 1999; Nicholson 1999; Hara-Nishimura et al. 2005). Secondly, the pentapeptides for active sites, QACRG and E(A/G)CES, of caspase-1 and VPEs respectively, are similar (Sanmartin et al. 2005). Finally, three amino acids (Arg179, Arg341 and Ser347), which form the caspase-1 substrate-binding pocket, are also present in all VPEs identified so far (Wilson et al. 1994; Nicholson 1999; Hara-Nishimura et al. 2005). VPEs are synthesized as inactive proprotein precursors (ppVPE) containing a signal peptide (SP), N-terminal pro-peptide (NTPP), active domain and a C-terminal inhibitory pro-peptide (CTPP; Hara-Nishimura et al. 2005). During translation in the endoplasmic reticulum, the SP is removed; giving rise to another inactive form of VPE, proVPE (Figure 1.5D; Kuroyanagi et al. 2002). Once transported to the vacuole, the acidic pH will initiate self-autocatalysis of proVPE resulting in the removal of the auto-inhibitory CTPP; the active intermediate isoform (iVPE) is produced. NTPP will then be removed from iVPE producing a mature VPE, which is also active (Kuroyanagi et al. 2002; Reviewed in Misas-Villamil et al. 2013). Similarly to VPEs, caspase-1 is also synthesized as an inactive proenzyme and undergoes self-catalysis to form an active protease through a process involving propeptide removal (Cohen 1997; Raff 1998; Hara-Nishimura et al. 2005).

Hatsugai et al. (2004) determined that VPEs are responsible for the caspase-1 activity observed during HR in *Nicotiana benthamiana* leaves infected with TMV. As a response to attack by TMV, *N. benthamiana* leaves usually form lesions through PCD to prevent spread of the bacteria to surrounding healthy cells (Hatsugai et al. 2004). PCD characteristics observed during this lesion formation include cell shrinkage, tonoplast rupture and DNA fragmentation (Hatsugai et al. 2004; Hara-Nishimura et al. 2005).

Lesion formation and PCD characteristics were absent in VPE silenced plants infected with TMV. Similarly, no lesions were formed in TMV infected plants treated with a caspase-1 inhibitor. Using the biotin-labeled caspase-1 inhibitor, Hatsugai et al. (2004) identified active forms of VPE during this HR response. The caspase-1/VPE activity peaked and disappeared before obvious signs of lesion formation, which coincides with requirement of this activity during early stages of HR cell death (Hatsugai et al. 2004; Hara-Nishimura et al. 2005). Hatsugai et al. (2004) suggested that VPEs are localized in the tonoplast and may be responsible for its degradation and rupture; releasing hydrolytic enzymes that degrade contents of the cytoplasm and nucleus. This is supported by findings showing that many hydrolytic enzymes are up-regulated during plant PCD (Fukuda 2004). The vacuole is an essential organelle in plant PCD, since its collapse determines the beginning of rapid cellular degradation. If VPEs play the role of mediating tonoplast rupture then they play a key role in vacuolar-mediated plant PCD. VPEs play a role in other forms of plant PCD involving the vacuole: such as leaf senescence, PCD during lateral root formation, seed development and embryogenesis (Kinoshita et al. 1999; Hara-Nishimura and Maeshima 2000; Kuriyama and Fukuda 2002; Lam 2004; van Doorn and Woltering 2005).

This evidence alone does not prove beyond reasonable doubt that VPEs are the ones that directly compromise tonoplast membrane integrity, but they may activate some other enzymes within the vacuole or in the tonoplast that degrade the membrane. Nevertheless, their role in plant PCD is still pivotal.

1.5 Conclusion and Future work

It is evident that there are some similarities between PCD in plants and apoptosis in animals (Table 1.1). Similarities in signaling molecules, regulation, as well as some components involved in execution highlight commonalities between the two PCD systems. The use of animal transgenes in plants, cleavage of animal substrates by plant proteases, use of animal based inhibitors on plants and vice versa also suggests the existence of a common PCD machinery that plants and animals used to share. This common PCD machinery was likely neither apoptosis nor plant PCD as we know them today, but some traces of this ancient PCD mechanism are still conserved. Considering animal PCD is more understood at the moment, experiments in plants, based on the animal PCD, system such as finding plant orthologs and functional homologs of animal PCD genes have helped elucidate many components within plant PCD. More research using this approach needs to be carried out. As in Shabala et al. (2007), the research also needs to go further than just studying the effect of animal genes on plant PCD; we also need to identify what they do specifically and identify plant proteins that they interact with.

Whole plant genome screens for similar genes have also provided insights into which genes are conserved. With sequencing of more plant genomes we will be able determine the extent of conservation of PCD genes, and possibly elucidate more novel PCD genes that are exclusive to plants. More gene knockouts and mutations will also help elucidate functions of many plant genes in PCD. Protein interaction assays are also crucial in understanding the role of different proteins in plant PCD. For example, natural substrates for plant caspase-like enzymes during PCD are still largely unknown; therefore

Table 1.1 Comparison of the Components Involved in Signaling, Regulation and Execution of Mammalian Apoptosis and Plant PCD

	Mammalian	Plant
Signaling	**Inducers**	
	- *Reactive oxygen species*	- *Reactive oxygen species*
	- *Nitric oxide*	- *Nitric oxide*
	- *Stress (e.g., heat, UV light)*	- *Stress (e.g., heat, cold)*
	- *Developmental cues*	- *Developmental cues*
	- Death receptor ligands	- Pathogen attack
	Receptors	**Receptor-like kinases**
	- TNF subfamily of receptors	FLS2
	FasR	BAK1
	TNFR1	CRKs
	DR3	Syntaxins
	DR4	CDPKs
	DR5	RBOHD
	TRAILR1	SYP121
	TRAILR2	H+-ATPase
	Ectodysplasin-A receptor	
Regulation	**Hormones**	
	- Thyroid hormone	- Jasmonic acid
	- Prolactin	- Ethylene
	- Human growth hormone	- Salicylic acid
	- Steroids	- Gibberelin
	- Testosterone	- Cytokinins
	- Estrogen	- Abscisic acid
	- Progesterone	
	- Dihydrotestosterone	
	- Dexamethasone	
	Non-hormonal	
	- *Ca^{2+} cascade*	- *Ca^{2+} cascade*
	- *MAPKs*	- *MAPKs*
	- *Bcl-2 family proteins*	- *Bcl-2? Bax? Lrg?*
	- *Cytochrome c*	- *Cytochrome c*
	- *DAD1*	- *DAD1*
	- *Bax inhibitor*	- *Bax inhibitor*
	- *Calmodulin*	- *Calmodulin*
	- Apaf-1	- BAP1 and BAP2 genes
	- Inhibitors of apoptosis (IAPs)	
Execution	**Proteases**	
	- *Caspases*	- *Caspase-like enzymes*
	- *Proteasome*	- *Proteasome*
	- Cathepsins	
	Nucleases	
	- DAD1	- ZEN1
	- CAD	- BFN1
	- Endonuclease G	- Ca^{2+}/Mg^{2+} nuclease
	- DNase II	- Chromatin loop nuclease

Note: Italic font highlight similarities and inconclusive evidence is indicated by a question mark.

more research is needed to identify specific death proteases that these enzymes activate. Plants and animals went through different evolutionary pathways, and this is also evident in terms of PCD mechanisms. Plants have unique components that are important in PCD, such as the phytohormones discussed above, cell wall and organelles like chloroplasts, which are absent in animals. These introduce obvious differences between plant and animal PCD and it would be interesting to study the evolutionary significance of these unique organelles in plant PCD. During apoptosis in animals, mitochondria play a central role. Even though the entire plant PCD system is far from being fully elucidated, evidence already suggests that there are many variations of plant PCD in terms of which organelles play a central role. The vacuole plays a central role in most plant PCD systems; but some systems are more chloroplast (Kim et al. 2012) and mitochondrial dependent (Li and Xing 2011). Vacuolar and chloroplast dependent forms of plant PCD might need a unique approach since they deviate more from animal PCD. Overall, more needs to be uncovered within the evidently complex molecular mechanism of plant PCD, to match the morphological studies of plant PCD, which are relatively more documented.

1.6 Acknowledgments

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1.7 The Lace Plant

The lace plant (*Aponogeton madagascariesis;* Figure 1.6A) is an aquatic plant species belonging to the Aponogetonaceae family of monocots. Out of the forty species within Aponogetonaceae, it is the only species that forms perforations in its leaves. These perforations are part of normal leaf development and form through developmentally regulated PCD (Gunawardena et al. 2004). The formation of perforations through developmentally regulated PCD is not only rare within Aponogetonaceae family. So far in all the known plant species, it occurs in only one other plant family known as Araceae (Gunawardena and Dengler et al. 2006). However, unlike in Araceae species in which perforations form at unpredictable and less accessible areas, lace plant perforations are spatially and temporally predictable. They form in between longitudinal and transverse veins, known as areoles, at a predictable stage of leaf development (window stage). PCD begins in the center of an areole of young leaves and develops towards the veins and stops 4-5 cell layers from the vascular tissue. Lace plant leaves are also thin and excellent for microscopy. In addition, a sterile propagation method of the lace plant has been developed (Figure 1.6B; Gunawardena and Dengler 2006), which provides microbe free tissue for experimentation. During formation of perforations, cells that are destined to undergo PCD to give rise to perforations (PCD cells) are easily distinguishable from those not destined to die at this stage (NPCD cells). At the early stages of perforation formation, the PCD cells start to lose their pigmentation and become somewhat transparent while pigmentation is maintained within NPCD cells (Figure 1.6C-E; Lord et al. 2011). The suitability of the lace plant as a model organism to study developmentally regulated PCD in plants has allowed for numerous studies to be performed, and most of

these studies detail the morphological changes that occur during PCD (Dauphinee and Gunawardena 2015).

Based on the morphological studies, formation of perforations was divided into five stages (Gunwardena et al. 2004). Some of the morphological changes that occur in the PCD cells during perforation formation include shrinkage of chloroplasts and nucleus, increased visibility of transvacuolar strands, ring formation of chloroplasts around the nucleus, enlargement of the vacuole, formation of organelle clusters within the vacuole, rupture of the vacuolar membrane, termination of mitochondrial streaming, collapse of the plasma membrane and disappearance of the cell wall (Wright et al. 2009; Wertman et al. 2012, Lord et al. 2013). Indirect evidence has suggested the involvement of ethylene and caspase-like enzymes in lace plant PCD (Gunawardena et al. 2006; Lord et al. 2013). Despite morphological changes that occur during PCD being well documented, little is known about the molecular mechanisms that regulate lace plant PCD.

1.8 Objectives

The broad objective of this dissertation was to provide insights on the molecular regulation of PCD in the lace plant. This main objective was subdivided as follows:

1.8.1 The Role of Ethylene and Ethylene Receptors as Regulators of PCD

Based on indirect evidence suggesting the involvement of ethylene in lace plant PCD, the role of ethylene receptors was investigated. Ethylene receptors percieve the ethylene signal and they are negative regulators of ethylene-induced responses. The aim of this research was to isolate lace plant ethylene receptors and investigate their transcript expression pattern at different stages of leaf deveolpment. The presence of cells types,

which responded differently to ethylene-induced PCD within the lace plant system also provided an opportunity to unravel underlying mechanisms that resulted in different responses. Therefore, PCD and NPCD cells were separated and their ethylene receptor transcript levels were detemined.

1.8.2 Vacuolar Processing Enzymes and Lace Plant PCD

Following indirect evidence suggesting the involvement of caspase-1 like activity in lace plant PCD, the aim of this research was to isolate lace plant VPEs and study their transcript expression pattern and activity during PCD. VPEs are plant caspase-like enzymes and are known to possess caspase-1 like activity. Plant caspase-like enzymes have been described as executors of PCD. Therefore, the transcript expression patterns of lace plant VPEs were studied in leaves at different developmental as stages, as well as between PCD and NPCD cells.

1.8.3 Effect of Ethylene on VPE Transcription Rates During Lace Plant PCD

Following the investigation of the role of ethylene, ethylene receptors and VPEs, the effect of ethylene on VPE transcript expression levels during lace plant PCD was investigated. Ethylene is known to be within the regulatory phase of the plant PCD cascade, while VPEs are within the execution phase. The results were summurized into a proposed PCD regulatory mechanism in the lace plant involving ethylene, ethylene receptors and VPEs.

Figure 1.1 Apoptosis Pathways in Mammalian Cells

The extrinsic pathway involves a death ligand such as Fas ligand (FasL), which binds to a membrane-bound receptor Fas. Adaptor molecules such as Fas-associated protein with death domain (FADD) are then recruited to the receptor, upon which procaspase 8 (Procas 8) is also recruited and activated. The active caspase 8 (Cas 8) then activates precursors (Procas 3 and 7) of executioner caspases, caspase 3 (Cas 3) and caspase 7 (Cas 7), which cleave death substrates. In some instances, the extrinsic pathway employs the extrinsic pathway to amplify the death signal. After activation, caspase 8 will cleave Bid into tBid; which interacts with other Bcl–2 type proteins such as Bax to compromise the integrity of the mitochondrial inner membrane. This results in Cyt c escaping from the mitochondria into the cytosol where it forms an apoptosome with Apaf-1, and a caspase 9 (Cas 9) precursor in the presence of adenosine triphosphate. The caspase 9 precursor is activated into an active caspase 9 (Cas 9) that activates the executioner caspases, which cleave death substrates. Image by Gaolathe Rantong; information gathered from Elmore (2007), Kantari and Walczak (2011), McIlwain et al. (2013), and Mariño et al. (2014).

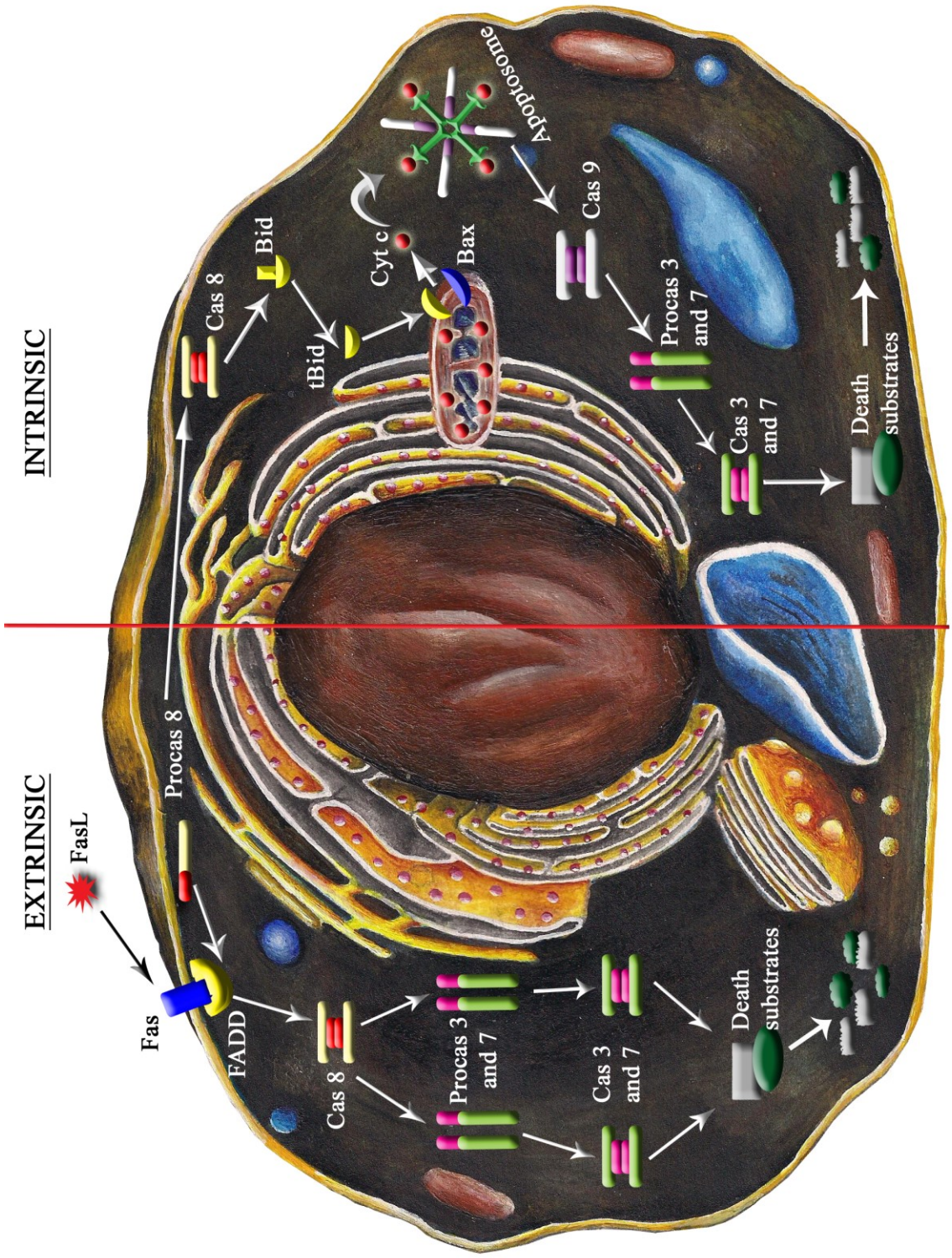

Figure 1.1 Apoptosis Pathways in Mammalian Cells

Figure 1.2 General Model for Plant PCD

General model for plant PCD induced by different stimuli such as UV light, hydrogen peroxide (H_2O_2), pathogen attack, developmental cues and stress conditions such as heat and drought. This model is based around ROS and hormonally regulated PCD. Over exposure to UV light triggers ROS production by damaging DNA and the nucleus, activating a MAPK cascade, which will induce ROS production. H_2O_2, pathogen attack, different stress conditions and developmental signals result in an increased ROS production. H_2O_2 triggers a loop of intracellular ROS production and cell recognition of microbe associated molecular patterns (MAMPS) through pattern recognition receptors (PRRs) can induce increased ROS production by enhanced activity of NADPH oxidases, amine oxidases and cell wall bound peroxidases. The ROS produced will be recognized through unidentified ROS receptors. It is unknown whether these receptors are either two-component receptors or receptor-like protein kinases. Perception of ROS through the sensors will activate a signaling cascade (a MAPK, Ca^{2+}, calmodulin, and possibly other cascades). ROS can also induce hormonal regulation through salicylic, jasmonic acid or ethylene. There is a complex cross talk between these hormones during PCD regulation. Also, PCD signals that employ hormonal regulation can trigger increased ROS production. The hormonal regulation and or the MAPK, Ca^{2+} and calmodulin cascades will activate transcription factors that regulate PCD genes. Transcription factors regulating anti-PCD genes may be suppressed. Genes that play a central role in plant PCD are caspase-like enzymes phytaspases, saspases, metacaspases, and VPEs. They are all produced as inactive zymogens and are self-autocatalytically processed into active forms; except phytaspases, who are recruited from the apoplast active already. The caspase-like enzymes will cleave and activate other enzymes involved in cell death such as nucleases, other proteases and cell wall modifiers. There are likely many other components involved in the plant PCD model that are still unknown. Evidence also suggests that there are alternative pathways within plant PCD, and more interactions within the components of plant PCD cascade. Therefore this model is a general overview and lacks many components that are still unclear. Figure created by Gaolathe Rantong; information gathered from Mittler (2002), Mittler et al. (2004), Piszczek and Gutman (2007), and Mittler et al. (2011).

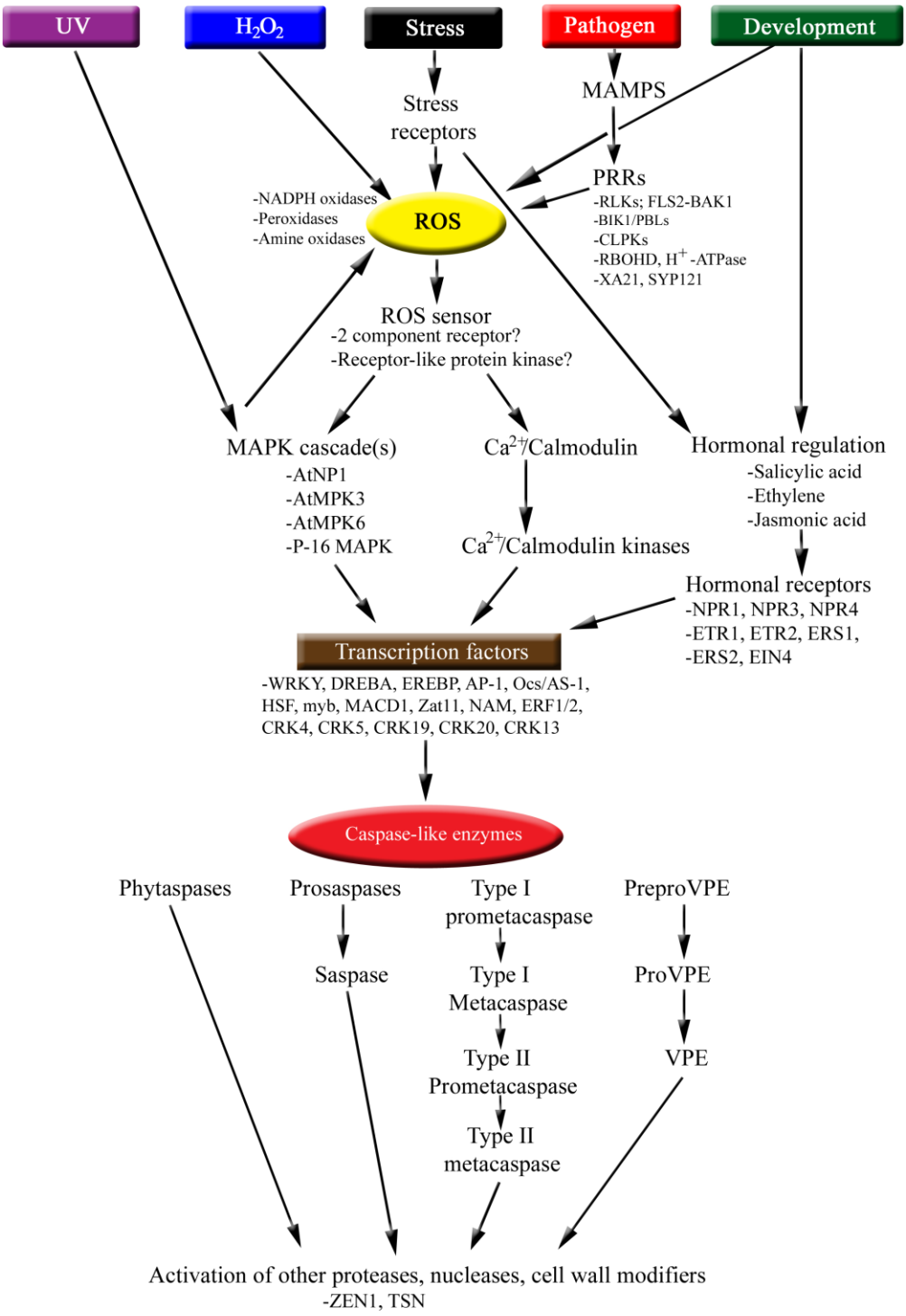

Figure 1.2 General Model for Plant PCD

Figure 1.3 A Model for Reactive Oxygen Species (ROS), Salicylic Acid (SA), and Nitric Oxide (NO) Mediated Plant Programmed Cell Death (PCD) in Response to Pathogen Attack

Upon detection of pathogen attack, activity of NADPH oxidases, amine oxidases, and cell wall bound peroxidases are enhanced, producing large amounts of ROS. Along with SA and NO, ROS will then diffuse into the cells through aquaporins. Elevated levels of ROS will induce an increase in Ca^{2+} levels. The Ca^{2+} will enter into mitochondria, and they will release Cyt c and produce more ROS. SA and NO inhibit ascorbate peroxidase and catalase, which are involved in scavenging cellular ROS. Cyt c will induce the transcription of defense genes, including PCD genes such as VPEs. Elevated SA levels in the cells also induce transcription of defense genes. Amid the high levels of SA, SA receptor NPR3 will bind and interact NPR1, possibly converting it to its monomeric form. Monomeric NPR1 is thought to then interact with transcription factors in the nucleus to facilitate transcription of defense and PCD genes, such as vacuolar processing enzymes (VPEs). The role of NPR1 in this model is still under investigation; some evidence suggests that it is an SA receptor. VPEs are produced as inactive pro-enzymes (proVPEs), which relocate to the vacuole, where they get cleaved into active intermediate (iVPE) and mature (mVPE) forms. In the vacuole, the active VPEs will cleave and activate their targets (which may include hydrolases). VPEs are also thought to weaken the tonoplast eventually leading to its rupture. Tonoplast rupture will result in the release of hydrolyses and other vacuolar contents into the cytoplasm where they will cause rapid cell death. The nucleus and other cellular components will be degraded and pathogens within the cell affected. This model highlights the importance of the vacuole and vacuolar processing enzymes in plant PCD. Image by Gaolathe Rantong; information gathered from Mou et al. (2003), Attaran and He (2012), Fu et al. (2012) and Mur et al. (2013).

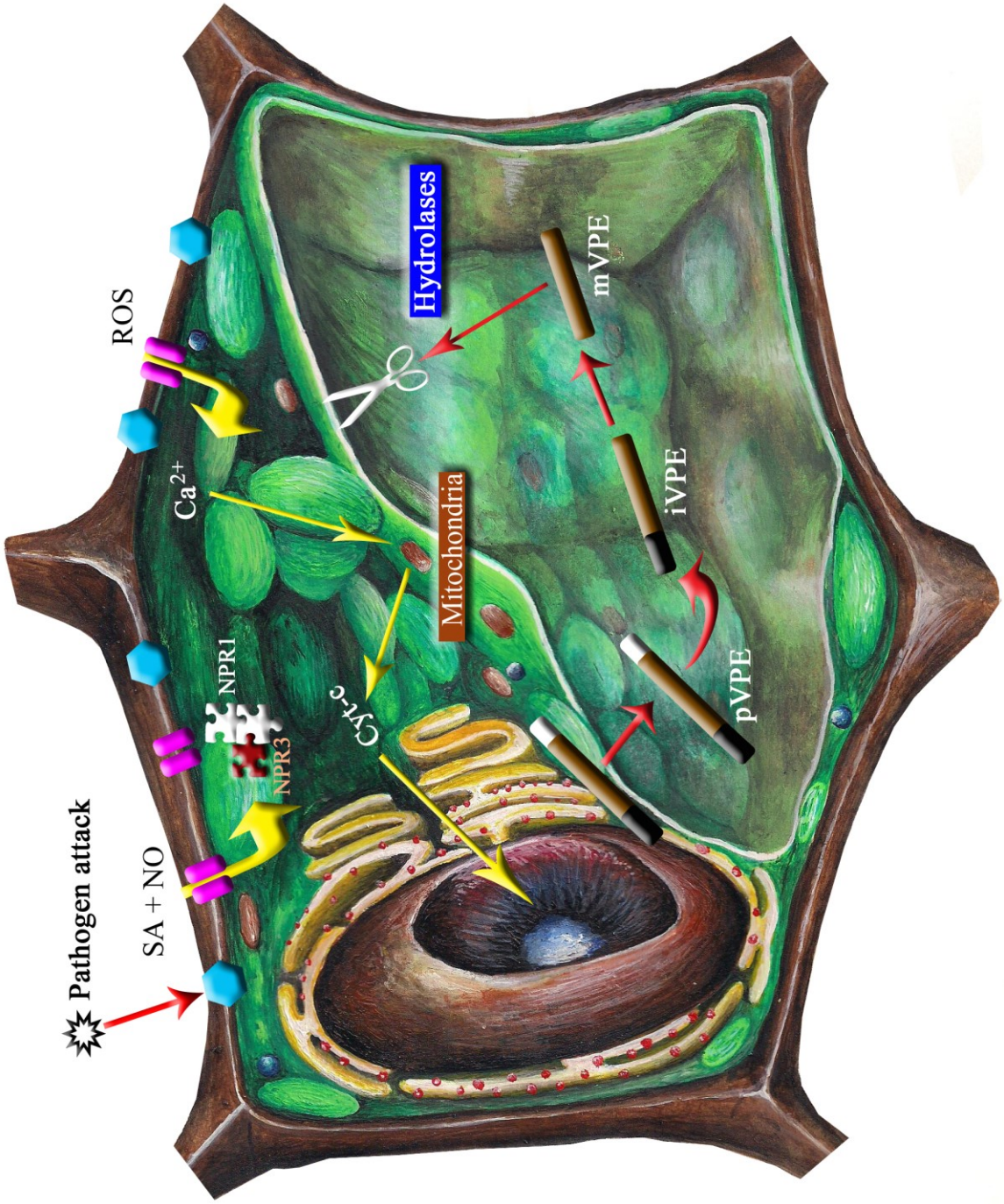

Figure 1.3 A Model for Reactive Oxygen Species (ROS), Salicylic Acid (SA), and Nitric Oxide (NO) Mediated Plant Programmed Cell Death (PCD) in Response to Pathogen Attack

Figure 1.4 Schematic Representations of a Human Caspase and Plant Caspase-Like Enzymes

Caspase-1 is shown to illustrate the different domains found in an inactive mammalian caspase precursor. These domains usually include the N-terminal prodomain, a large (L) subunit and a small (S) subunit, joined together by an interdomain linker. Essential amino acids within the catalytic dyad, His237 and Cys285 are shown. Arg179, Arg341, and Ser347 are three amino acids found within the human caspase-1 substrate-binding pocket. A plant vacuolar processing enzyme proprotein precursor (N. tabacum ppVPE-1a) consists of a signal peptide (SP), an N- terminal propeptide (NTPP), a protease domain, and a C-terminal propeptide (CTPP). Its essential amino acids within both catalytic dyad (His174 and Cys216) and substrate binding pocket (Arg109, Arg386, and Ser392) are comparable to those found in the human caspase-1. Gln283 in hcaspase-1 is also comparable with Gln214 in NtVPE-1a. A general type I metacaspase is synthesized as an inactive zymogen, prometacaspase, consisting of an N-terminal prodomain, large subunit, small subunit, and interdomain linker structurally resembling those of mammalian procaspases. Essential amino acids within the catalytic dyad (His and Cys) are also conserved between caspases and plant metacaspases. Unlike type I, type II metacaspase precursors lack an N-terminal prodomain and their interdomain linker is longer than that of caspases. Nevertheless, they also still consist of a large and small subunit. The His and Cys within the catalytic dyad are also conserved in type II metacaspases. Sapspases and phytaspases are also synthesized as zymogens, preprosaspase, and phytaspase precursor, respectively, consisting of a signal peptide, propeptide, and protease domain. The His and Cys residues conserved among caspases and other caspase-like enymes are lacking in saspases and phytaspases. Figure created by Gaolathe Rantong; information gathered from Hara-Nishimura et al. (2005), Piszczek and Gutman (2007).

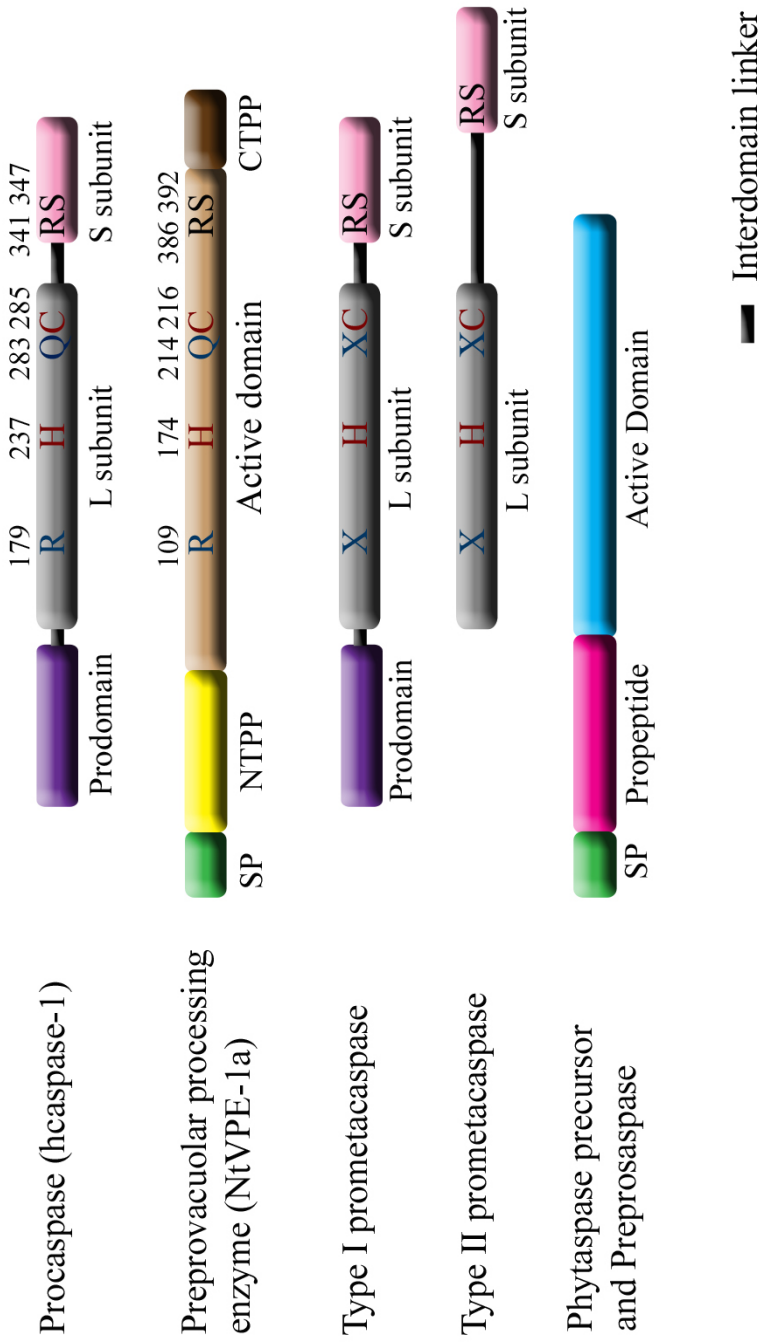

Figure 1.4 Schematic Representations of a Human Caspase and Plant Caspase-Like Enzymes

Figure 1.5 Processing, Activity, and Subcellular Localization of Plant Caspase-Like Enzymes

(A) Saspases are synthesized as zymogens and, after translation, the resultant prosaspase consists of a propeptide and protease domain. The propeptide is cleaved to produce an active mature saspase. Active mature saspases are almost instantly detected in the apoplast upon programmed cell death (PCD) induction, but it is not known whether these are from prosaspases synthesized and quickly secreted to the apoplast upon induction of PCD (1), or from prosaspases stored in the apoplast awaiting activation by a PCD signal (2). Either way, saspases carry out their caspase-like activities in the apoplast. (B) During translation, preprophytaspases lose their signal peptide and the resultant inactive protein, prophytaspase, consists of a propeptide and protease domain. During non-PCD conditions, prophytaspases are constitutively produced and cleaved to produce mature phytaspases. The mature active phytaspase (lacking the propeptide) is then relocated to the apoplast. They maintain their activity in the apoplast and are thought to be involved in pathogen-related defense mechanisms in the extra cellular fluid. Upon PCD induction, the phytaspases are recalled to the cytoplasm, where they are thought to perform their caspase-like activities. (C) Prometacaspases (prometacasp) are inactive and made of a large and small subunit. In addition, type I prometacaspases also consist of an N-terminal prodomain. This prodomain is cleaved and removed upon activation. It is not fully understood how type II metacaspases are activated; most of them are able to autocatalyze, while others do not require autolytic processing to be active. Both type I and type II prometacaspases are localized in the cytosol and require optimal conditions like elevated calcium ion levels and a pH between 7 and 8.5 to be activated. The active metacaspases display their proteolytic activity in the cytosol. (D) Signal peptides within preproVPEs are co-translationally removed; the resultant proVPE (pVPE) is inactive and consists of an N-terminal propeptide, protease domain and a C-terminal propeptide. The proVPE is transported to the vacuole, where the acidic vacuolar pH of ~5.5 induces the self-autocatalytic removal of the inhibitory C-terminal propeptide resulting in an active intermediate VPE (iVPE). The N-terminal propeptide is then also removed to produce a fully mature VPE (mVPE), which is active within the vacuole. Image by Gaolathe Rantong; information gathered from Woltering (2004), Bozhkov et al. (2005b), Vartapetian et al. (2011), and Misas-Villamil et al. (2013).

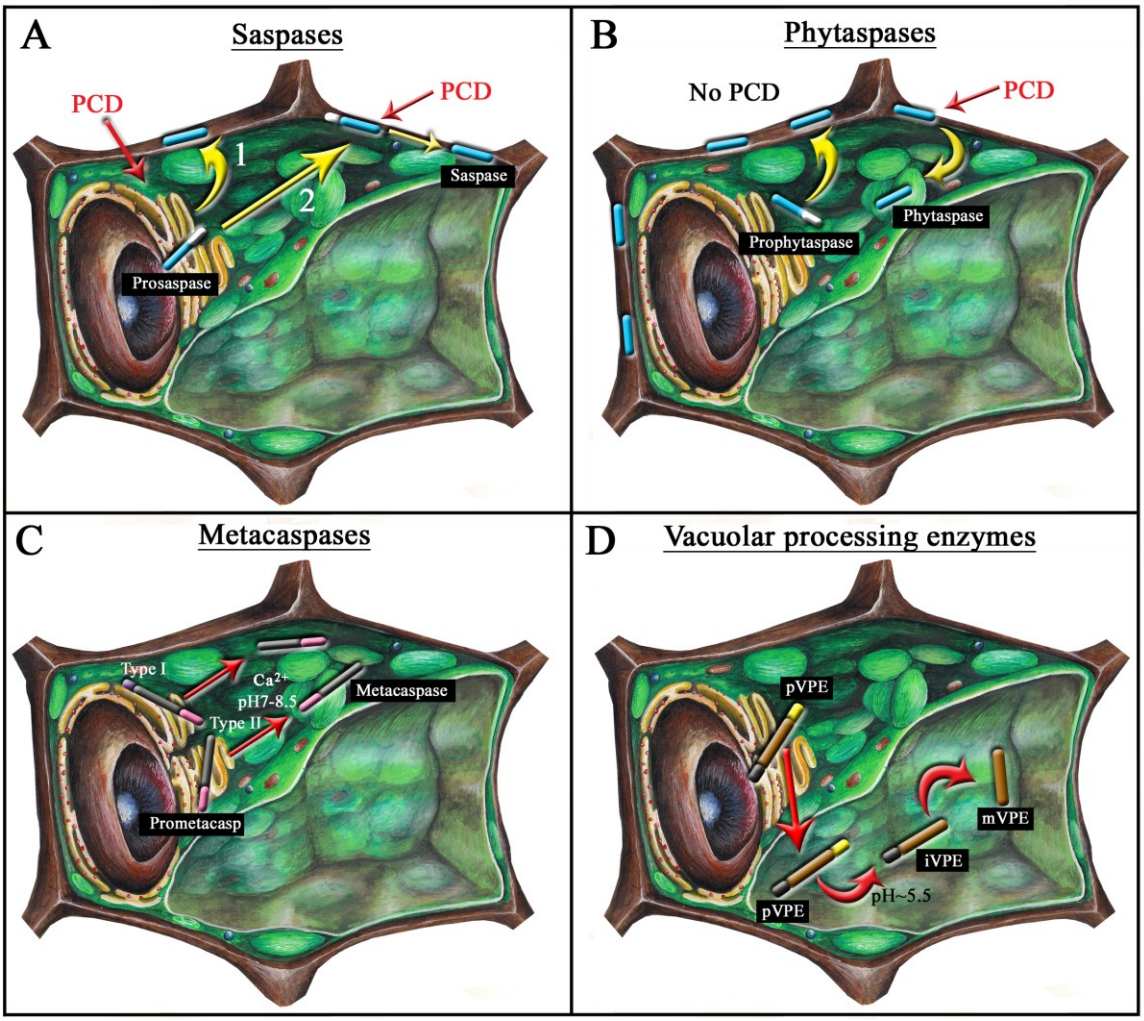

Figure 1.5 Processing, Activity, and Subcellular Localization of Plant Caspase-Like Enzymes

Figure 1.6 The Lace Plant (*Aponogeton madagascariensis*)

Lace plant propagation and leaf development through PCD. (A) Lace plant perforations forms perforation in its leaves through PCD. It can be grown in axenic conditions in Magenta GA7 boxes (B) for experimental purposes. Lace plant perforations form at highly predictable areas between longitudinal and transverse veins (C and D). The dying cells (PCD cells) are easily distinguishable from the non-PCD (NPCD) cells. They lose pigmentation, become somewhat transparent and eventually disintergrate: leading to a preforation. (E) A fully formed perforation in a mature leaf. Bars = 1 cm in A-B, C = 40 μm, D = 100 μm and E = 150 μm.

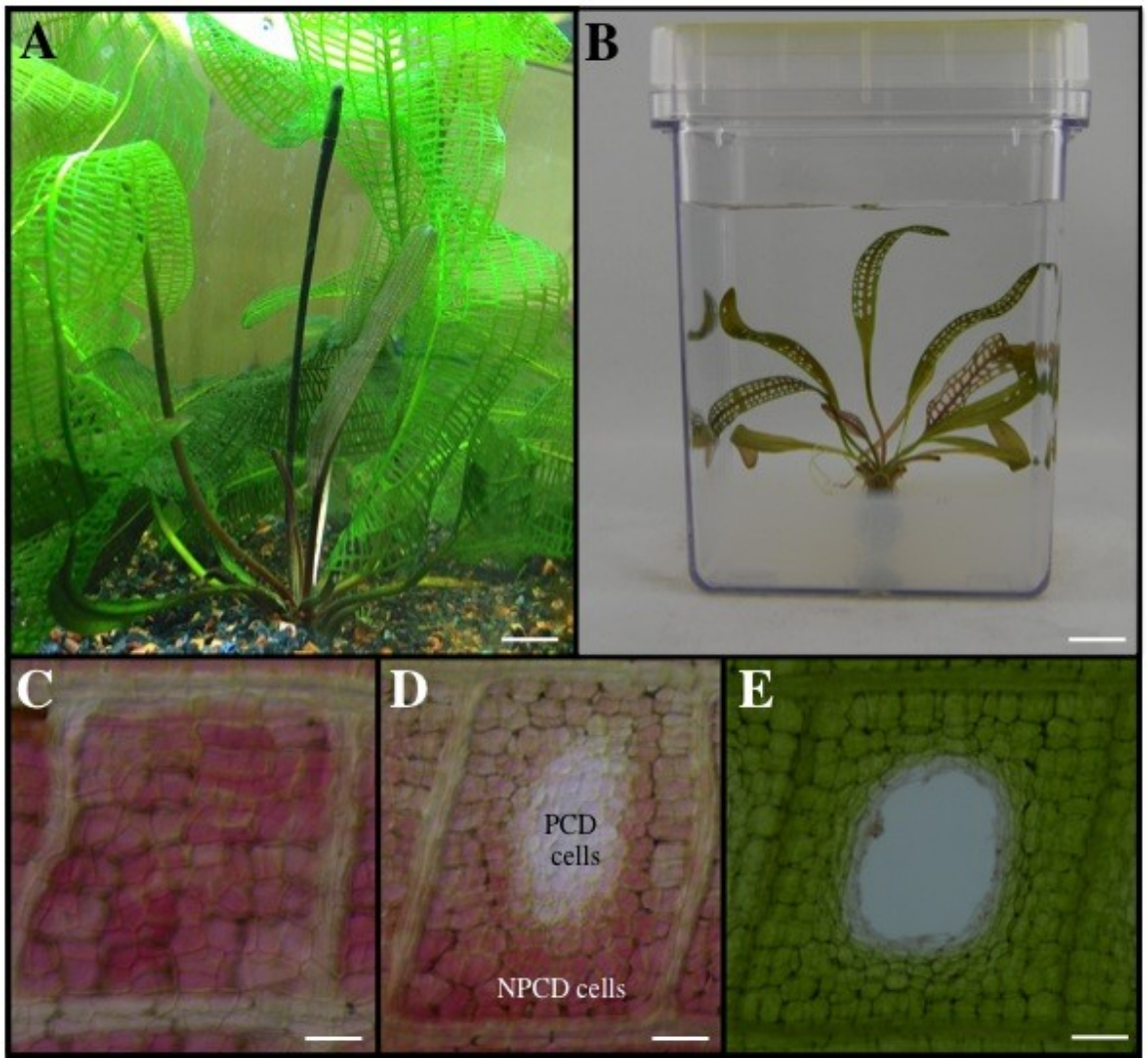

Figure 1.6 The Lace Plant (*Aponogeton madagascariensis*)

Chapter 2 Lace Plant Ethylene Receptors, AmERS1a and AmERS1c, Regulate Ethylene-Induced Programmed Cell Death During Leaf Morphogenesis

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Author's contributions

GR designed and carried out all experiments. GR wrote the first draft of the manuscript. RE assisted with phylogenetic analysis. AHLANG, conceived the study, participated in its design and coordination, and helped in drafting and revising the manuscript as well as supervised all experimental work.

2.1 Abstract

The lace plant (*Aponogeton madagascariensis*), an aquatic monocot, forms perforations in its leaves as an element of normal leaf development. Perforation formation occurs through developmentally regulated programmed cell death (PCD). The molecular basis of PCD regulation in the lace plant is unknown, however ethylene has been shown to play a significant role. In this study, we examined the role of ethylene receptors during perforation formation. We isolated three lace plant ethylene receptors AmERS1a, AmERS1b and AmERS1c. Using quantitative PCR, we examined their transcript levels at seven stages of leaf development. Through laser-capture microscopy, transcript levels were also determined in cells undergoing PCD and cells not undergoing PCD (NPCD cells). AmERS1a transcript levels were significantly lower in window stage leaves (in which perforation formation and PCD are occurring) as compared to all other leaf developmental stages. AmERS1a and AmERS1c (the most abundant among the three receptors) had the highest transcript levels in mature stage leaves, where PCD is not occurring. Their transcript levels decreased significantly during senescence-associated PCD. AmERS1c had significantly higher transcript levels in NPCD compared to PCD cells. Despite being significantly low in window stage leaves, AmERS1a transcripts were not differentially expressed between PCD and NPCD cells. The results suggested that ethylene receptors negatively regulate ethylene-controlled PCD in the lace plant. A combination of ethylene and receptor levels determines cell fate during perforation formation and leaf senescence. A new model for ethylene emission and receptor expression during lace plant perforation formation and senescence is proposed.

2.2 Introduction

2.2.1 Programmed Cell Death (PCD) and Plants

Programmed cell death (PCD) is a genetically controlled cell suicide that eliminates undesirable cells in most multicellular organisms (Greenberg 1996). PCD occurs throughout normal development in plants; starting from the fertilization of the ovule to death of the whole plant (van Doorn and Woltering 2005), and is involved in processes such as death of the embryonic suspensor (reviewed in Lombardi et al. 2007), leaf and flower senescence (reviewed by Lim et al. 2007; Rogers 2012), aerenchyma formation (Gunawardena et al. 2001; Lenochová et al. 2009), tracheary element differentiation (Groover and Jones 1999; Fukuda, 2000), dehiscence of anthers (Bonner and Dickinson 1989), root cap shedding (Wang et al. 1996), and perforation formation during leaf morphogenesis in *Monstera* and lace plant (Gunawardena et al. 2004; Gunawardena et al. 2005; Wright et al. 2009; Wertman et al. 2012)

In plants, several genetic components have been associated with PCD: these include receptor-like/Pelle kinases, pattern recognition receptors, stress receptors, reactive oxygen (ROS) sensors, MAPK cascade, hormonal regulators, transcription factors and caspase-like enzymes (reviewed in Rantong and Gunawardena 2015). Hormones involved in plant PCD include, but are not limited to salicylic acid (Cao et al. 1994; Mur et al. 2013), jasmonic acid (Mur et al. 2013), and ethylene (Zhao and Schaller 2004; Dauphinee et al. 2012).

2.2.2 Ethylene and Plant PCD

The plant hormone ethylene has been implicated as an important regulator of PCD in plants (Zhao and Schaller 2004). Examples of plant PCD that are thought to involve ethylene include, but are not limited to: the hypersensitive response, organ senescence, aerenchyma formation, leaf and petal abscission, endosperm cell death (Young et al. 1997; reviewed in Bleecker and Kende 2000; Trobacher 2009; Rogers 2012) and perforation formation in the lace plant (Dauphinee et al. 2012). Ethylene has been shown to promote the onset of senescence (Zacarias and Reid 1990; Jing et al. 2005) and ethylene-insensitive mutants often display delayed senescence (Grbic´ and Bleecker 1995; Oh et al. 1997; Jing et al. 2005). Also, tomato plants that had suppressed ethylene production showed delayed leaf senescence (John et al. 1995; Jing et al. 2005). Ethylene biosynthesis and action inhibitors have been shown to stop aerenchyma formation in maize roots subjected to low oxygen conditions (reviewed in Drew et al. 2000). Also, low concentrations of ethylene induced PCD in cells pre-determined to die during aerenchyma formation (Drew et al. 2000). These examples demonstrate the importance of ethylene in PCD and the significance of both ethylene and PCD during plant development.

2.2.3 Ethylene Biosynthesis and Signalling

Within plant cells, ethylene biosynthesis begins with the conversion of methionine to S-adenosyl-methionine (S-AdoMet) by AdoMet synthetase. Through an ATP dependent cellular process, about 80% of methionine is converted to S-AdoMet (Ravanel et al. 1998; Wang et al. 2002). The S-AdoMet is then used to make 1-

aminocyclopropane-1-carboxylic acid (ACC) through ACC synthase. The conversion of S-AdoMet to ACC is considered the first committed and rate-limiting step in ethylene biosynthesis (reviewed in Yang and Hoffman 1984; Kende 1993; Wang et al. 2002). ACC is then oxidized by ACC oxidase, producing ethylene, CO_2 and cyanide, of which the latter is degraded.

In order to trigger ethylene-induced responses, ethylene is perceived through a signal transduction pathway. Within the pathway (in *Arabidopsis*), it is recognised by a family of membrane-bound receptors found on the endoplasmic reticulum (ER): ETR1, ETR2, ERS1, ERS2 and EIN4 (reviewed in Chang and Stadler 2001; Wang et al. 2002). The ethylene receptors act constitutively to negatively regulate the ethylene signal transduction pathway and suppress ethylene responses; hence, decreasing the number of ethylene receptors increases the cell's sensitivity to ethylene (reviewed in Trobacher 2009). Downstream of ethylene receptors is a Raf family serine/threonine kinase, CTR1, which is also a negative regulator of the ethylene signal transduction pathway.

When ethylene is limited, ethylene receptors and CTR1 form an ER bound CTR1-receptor complex that releases an inhibitory signal to downstream components and suppresses ethylene responses (Hall et al. 2007). In the abundance of ethylene, the ethylene binds to the receptors, interrupting the CTR1-receptor complex's inhibitory effect on the next component (EIN2) in the pathway (reviewed in Chang and Stadler 2001). Interrupting the inhibitory effect of the complex activates EIN2, which is a positive regulator of the pathway (Li and Guo 2007), and it transfers the signal to downstream components (Alonso et al. 1999; Wang et al. 2002). Upon receiving the

signal, a transcription factor downstream of EIN2 (EIN3) then stimulates expression of ethylene-responsive DNA-binding factors (EDFs) and other transcription factors (such as ethylene-response factors; ERFs; Li and Guo 2007). This leads to initialization of specific ethylene-induced responses.

2.2.4 Ethylene Receptors

Ethylene receptors resemble bacterial two-component histidine kinases. They both are typically made of two proteins: a sensor histidine kinase and a response regulator (Wurgler-Murphy and Saito 1997; Pirrung 1999; Wang et al. 2002). Only three receptors (ETR1, ETR2 and EIN4) among the *Arabidopsis* ethylene receptor family possess a receiver domain. However, the receptors that lack a receiver domain (ESR1 and ERS2) are thought to form heterodimers with those that do (Hua et al. 1998; Wang et al. 2002). Based on structural similarities, the ethylene receptor family in Arabidopsis can be categorized into two subfamilies: subfamily I (ETR1-like) and subfamily II (ETR2-like). Subfamily I is made up of ETR1 and ERS1, which consist of three membrane-spanning regions (the ethylene binding regions) on their N-terminal regions (Schaller and Bleecker 1995; Hall et al. 2000; Wang et al. 2002), and a highly conserved C-terminal histidine kinase domain. Subfamily II consists of ETR2, ERS2 and EIN4; they possess four N-terminal hydrophobic extensions and a histidine kinase domain that lacks one or more elements necessary for catalytic activity (Wang et al. 2002). Due to the structural differences between the subfamilies, the receptors may serve different functions. However, dominant mutations in any single member of the receptor family have been demonstrated to cause insensitivity to ethylene in plants (Chang et al. 1993; Hua et al. 1998; Sakai et al. 1998; O'Malley et al. 2005). In addition, loss of function mutations in

any two (or more) of the *Arabidopsis* receptors causes a constitutive ethylene response phenotype (Hua and Meyerowitz 1998; Wang et al. 2003; O'Malley et al. 2005). Even though less is known about the specific roles of each ethylene receptor family member, it is evident that they all are involved in signal transduction and inhibition of ethylene-induced responses. It has also been shown that in general, at least one member from subfamily I (either ETR1 or ERS1) is required for most ethylene responses (Wang et al. 2003). Like *A. thaliana*, *Oryza sativa* (rice) ethylene receptor gene family consists of five members. These are ERS1, ERS2, ETR2, ETR3 and ETR4 (reviewed in Wuriyanghan et al. 2009). *Zea mays* (maize) ethylene receptor gene family is made-up of ERS1a, ERS1b, ETR2a and ETR2b (Gallie and Young 2004). In the lace plant, ethylene receptors have not been isolated but it has been shown that ethylene plays a significant role during perforation formation through PCD (Dauphinee et al. 2012).

2.2.5 The Lace Plant

The lace plant is a submerged aquatic monocot belonging to the family Aponogetonaceae and employs PCD during leaf morphogenesis (Figure 2.1A). The plant forms perforations in its leaves through PCD and can be grown in magenta boxes in axenic conditions for experimental purposes (Gunawardena et al. 2006; Figure 2.1B). The formation of perforations in lace plant leaves has been previously characterised and divided into five developmental stages (Gunawardena et al. 2004). In "window" stage leaves, cells at the center of a perforation site (PCD cells; Figure 2.1C) begin to undergo PCD. These cells lose their pigmentation and appear somewhat transparent compared to their non-dying (NPCD) counterparts, which turn pink due to high amounts of

anthocyanin. The NPCD cells do not undergo PCD during perforation formation and occupy 4-5 cells layers away from vascular tissue (Figure 2.1C and D).

The process of perforation formation and the morphological aspects of PCD in lace plant have been well studied (Gunawardena et al. 2004, 2005, 2006, 2007, Gunawardena, 2008, Wright et al. 2009, Elliott et al. 2010, Lord et al. 2011, Wertman et al. 2012). Despite the lace plant being an excellent model for the study of PCD, little to no molecular work has been carried out on the species and the developmental signalling pathways involved during perforation formation remain unclear. However, lace plant leaves undergoing PCD during perforation formation and senescence emit a significantly high amount of ethylene, while inhibition of ethylene biosynthesis aminoethoxyvinylglycine (AVG) inhibits perforation formation in lace plant leaves (Dauphinee et al. 2012). An ethylene receptor inhibitors silver nitrate ($AgNO_3$) (Gunawardena et al. 2006), was also shown to result in significant reductions in the number of perforations within leaves. These inhibitor experiments provided indirect evidence for the involvement of ethylene and ethylene receptors in perforation formation. Insight into what signals trigger, and or regulate perforation formation will provide a better understanding of PCD regulation during normal development in plants.

The objective of the following study was to provide more evidence for the involvement of ethylene during lace plant PCD and investigate the role of ethylene receptors in regulation of lace plant PCD. Lace plant ethylene receptors were isolated and their transcript expression patterns were studied in different stages of leaf development and between PCD versus NPCD cells. Based on the results, a model for regulation of

PCD during perforation formation and senescence is proposed. This study is the first molecular study of perforation formation via PCD in the lace plant.

2.3 Materials and Methods

2.3.1 Plant Materials

Lace plants were propagated under axenic conditions in Magenta GA7 boxes as described by Gunawardena et al. (2006). Plants were grown at 24 ºC under daylight simulating fluorescent bulbs (Philips, Daylight Deluxe, F40T12/DX, Markham, Ontario, Canada) providing 12 h light/ 12 h dark cycles at approximately 125 μmol m^{-2} s^{-1}. Leaves at seven different stages of development were selected and harvested from these plants to be used for RNA extraction. For each RNA sample, tissue was collected from at least 3 leaves obtained from different plants. Analysis was based on data from 28 independent RNA samples (4 RNA samples per leaf developmental stage).

2.3.2 RNA Extraction and cDNA Synthesis

The TRI-reagent (Sigma, Oakville, Ontario, Canada) was used for RNA extraction with some modifications to the standard method. Twice the recommended volume of TRI-reagent was used and the RNA pellet was not air-dried. Leaf tissue (without midrib) of approximately 200 mg was used in RNA extraction. The midrib was removed because it contains phenolic compounds, which interfere with RNA extraction. RNA quality for each sample was determined through gel electrophoresis and spectrometry (at 260 nm). RNA was treated with DNase 1 (Fermentas, Burlington, Ontario, Canada) prior to cDNA synthesis, to degrade genomic DNA. cDNA was synthesised using M-MuLV reverse transcriptase (New England Biolabs, Pickering,

Ontario, Canada). Two µg of RNA, 1 µl of 10 µM dT primer and 1 µl of 10 mM dNTP mix were added to a nuclease free tube. The mixture was then incubated at 65 ºC for 5 min in a water bath, quickly chilled on ice and briefly spun to collect the contents. Four µl of 5X First Strand Buffer (Invitrogen, Burlington, Ontario, Canada), 1 µl of RNase inhibitor (40 U/µl) (New England Biolabs, Pickering, Ontario, Canada) and 2 µl of 0.1 M DTT (Invitrogen, Burlington, Ontario, Canada) were then added to each sample. The mixtures were incubated at 37 ºC for 2 min in a water bath. Two microliters of the M-MuLV reverse transcriptase (200 U/ µl) was then added and the contents mixed by pipetting. Samples were incubated at 37 ºC for 1 h; the reaction was then heat inactivated by incubating the samples at 70 ºC for 15 min. Each sample was diluted with nuclease free water to a total volume of 50 µl.

2.3.3 Laser Capture Microscopy

In early window and window stage leaves, NPCD cells are pink due to anthocyanin while PCD cells have lost their anthocyanin (Figure 2.1C). Therefore, the cell types are easily distinguishable due to their color differences. The cells were separated using a Zeiss PALM Laser Capture Microdissection and Imaging System. A total of 8 different samples (4 samples per cell type) were used for RNA extraction, and each sample was collected from at least 3 different leaves obtained from different plants. RNA was extracted from the cells using a ReliaPrep RNA Cell Miniprep kit (Promega, Nepean, Ontario, Canada), following manufacture's instructions. DNase 1 was used to degrade trace amounts DNA, and cDNA was synthesized using Protoscript M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs, Pickering, Ontario, Canada) according to manufacture's instructions.

2.3.4 Isolation of Lace Plant Ethylene Receptors

For isolation of lace plant ethylene receptors, cDNA from preperforation, window and mature stage leaves was used. Initial fragments of the ethylene receptors were amplified using forward and reverse degenerate primers; 5′-TGGGTKCTTGTTCAGTT YGGTGC-3′ and 5′-CATTCTCACATGCYTTCCWGTYTC-3′, respectively. These degenerate primers were designed from an alignment of the following sequences; *Arabidopsis thaliana* ecotype Columbia (Col) (NM_105305), *Lycopersicon esculentum* (AF043084), *Oryza sativa* (AB107219), *Pelargonium x hortorum* (AF141928), *Vitis vinifera* (AF243474), *Populus trichocarpa* (XM_002302696) and *Physcomitrella patens ssp. patens* (XM_001751468). The PCR reaction mixture prepared for amplification consisted of 11.15 µl of nuclease free water, 2 µl 10X Thermobuffer (New England Biolabs, Pickering, Ontario, Canada**),** 1 µl of 10 mM dNTP mix (New England Biolabs, Pickering, Ontario, Canada**),** 1 µl of 10 mM forward primer, 1 µl of 10 mM of reverse primer and 0.35 µl of Taq DNA polymerase (5 U/ µl) (New England Biolabs, Pickering, Ontario, Canada). As a template, 3.5 µl of cDNA was used. PCR conditions used were 94ºC for 5 min, 40 cycles of 94ºC for 30 seconds, 45ºC for 30 seconds and 72ºC for 1 min. Following the 40 cycles, a final primer extension was carried out at 72ºC for 10 min. PCR products were separated on 1.5% agarose gels stained with ethidium bromide (Sigma Aldrich, Oakville, Ontario, Canada) and visualized using DNR F- ChemiBIs 3.2M Pro (Bio- imaging Systems, Montreal, Quebec, Canada). Amplified products were cloned using the pGEM-T Easy Vector System (Promega, Nepean, Ontario, Canada) following the manufacturer's instructions. A GenElute plasmid miniprep kit (Sigma, Oakville, Ontario, Canada) was used for plasmid purification. Clones were sent to

Macrogen Corp (Rockville, Maryland, USA) for sequencing. The rest of the 3′ end (including 3′ UTR) for each of the ethylene receptors was isolated through 3′-RACE; using an anchored primer (AP; 5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTT TTTT-3′) and an abridged universal amplification primer (AUAP; 5′-GTACTAGTCG-ACGCGTGGCC-3′). An actin gene fragment was also isolated using the degenerate primers 5′-AATGGHACTGGAATGGTCAAGG-3′ and 5′-CAYTTCATGATGGARTT GTA-3′. BioEdit Sequence Alignment Editor (Carlsbad, Ottawa, Ontario, Canada) was used to analyse sequences. Sequences were compared with National Center for Biotechnology Information (NCBI) nonredundant protein (blastx) database sequences for sequence identity analysis.

2.3.5 Phylogenetic Analysis

A total of 10 ethylene receptor amino acid sequences from maize, rice and and *Arabidopsis* were obtained from the NCBI protein database. The GenBank accession numbers of these ethylene receptors are AAR25566 (ZmERS1a), NP_001137032 (ZmERS1b), NP_001104852 (ZmETR2a), XP_008667201 (ZmETR2b), AAB72193 (OsERS1), AAL66363 (OsERS2), CAD39679 (OsETR2), AAL29303 (OsETR3), AAQ07254 (OsETR4) and NP_187108.1 (AtEIN4). These amino acid sequences were aligned to the three lace plant amino acid sequences obtained here using MEGA version 6.06 (Tamura, Dudley, Nei and Kumar, 2007). Prior to phylogenetic tree construction, the large gap at the 5′ end of lace plant sequences (see Figure 2.2), and corresponding amino acids in the reference sequences, were deleted. A single tree was constructed, with the A. thaliana sequence designated as an outgroup, using the Neighbor-Joining method in

MEGA version 6.06. Branch strength within the resulting tree was calculated using 1000 replicates in a nonparametric bootstrap test.

2.3.6 Quantitative PCR

AmERS1a primers used in QPCR are: 5′-TGATCAGGTAGCAGTTGCTC-3′and 5′-AGCCTC TCTTCGAGCTGAGTCC-3′. AmERS1c primers used are 5′-AGATCAGGTTGCCGTTGCCC -3′ and 5′-CTAGCTGCATCCAAGGCAAC-3′. 5′-TGATCAGGTAGCTGTTGCAC-3′ and 5′-TGCCTCTCGTCGTGCAGAGTCT-3′ were used for AmERS1b QPCR. For actin QPCR, 5′-TACGACAGGTATCGTGCTTG-3′ and 5′-CAAGCACGATACCTGTCGTA -3′ were used. Prior to QPCR, each primer pair was verified to produce a single amplicon through PCR. The fragments amplified by each of the primer pairs were cloned, sequenced and verified. For QPCR, DNA standards and cDNA samples were amplified using a QuantiTect SYBR Green PCR Kit (Qiagen, Mississauga, Ontario, Canada) following the manufacturer's instructions. For negative controls, the reverse transcriptase was omitted in the cDNA synthesis reactions and these samples were also subjected to QPCR. Thermal cycling and fluorescence detection were performed using a Rotor-Gene 3000 system (Corbett Research, Sydney, Australia). The QPCR was performed in 20 μl reaction volume and PCR conditions were initial holding at 95ºC for 15 min, 40 cycles of denaturing at 95ºC for 20 s, annealing temperature (59ºC for AmERS1a, 60ºC for AmERS1b, AmERS1c and actin) for 30 s and elongation at 72ºC for 30 s. Melting temperature of the PCR product was monitored after completion of PCR and was used as an indicator that a single specific product was amplified and is responsible for the total fluorescence. The fluorescence was measured at the end of each

cycle and standard curves were used to determine mRNA copy numbers of actin and each of the ethylene receptors, as explained in Bustin et al. (2005). Relative steady-state levels of ethylene receptor transcripts were determined by dividing their absolute copy numbers by the copy number of actin transcripts in each sample.

2.3.7 Statistical Analysis

The Quantitative PCR data was analysed via GraphPad Prism version 5.00 (San Diego, California, USA). The relative abundance of transcripts encoded by each gene is presented as mean ± S.E.M. A one-way ANOVA was used to determine whether there was a significant difference in relative abundance of transcripts among leaf developmental stages. A Tukey's HSD test was used to conduct post hoc comparisons. For relative transcript levels between PCD and NPCD cells, an unpaired t-test was used. Data was determined to be statistically significant if $P < 0.05$.

2.4 Results

2.4.1 Lace Plant Ethylene Receptors

Three lace plant ethylene receptors were isolated, namely AmERS1a, AmERS1b and AmERS1c. AmERS1a fragment is 1890 bp (including the 3´ untranslated region; KR349966), and translated into a 572 amino acid protein fragment (Figure 2.2). AmERS1b fragment was 1867 bp (including the 3´ untranslated region; KR349967), translating into a 549 amino acid fragment (Figure 2.2). AmERS1c was 1604 bp (KR349968) and translated into a protein fragment of 549 amino acids. The ethylene receptors shared conserved domains, sites and motifs, such as, the GAF domain, histidine

kinase domain, dimer interface domain, HATPase_c, Mg^{2+} binding site, G-X-G motif, phosphorylation site, and adenosine triphosphate (ATP) binding site. These are also conserved in *Z. mays* subfamily I ethylene receptors (Figure 2.2). Amongst themselves, lace plant ethylene receptors share high levels of amino acid sequence identity. AmERS1a amino acid fragment shares 90.35 and 75.84 percentage identities with AmERS1b and AmERS1c respectively. AmERS1b and AmERS1c share 77.53 % identity. The percentage identity between the lace plant and *Z. mays* subfamily I ethylene receptors ranged between 71.5 and 74.5 %.

2.4.2 Structural Features of Lace Plant Ethylene Receptors

Lace plant ethylene receptors shared the same structural characteristics with each other (Figure 2.3). Compared with rice and maize ethylene receptors, they shared more characteristics with subfamily I (ZmERS1a, ZmERS1b, OsERS1 and OsERS2) than subfamily II receptors (ZmETR2, OsETR2, OsETR3 and OsETR4). They posses the conserved essential residues (H, N, G1, F and G2) within the histidine kinase domain, characteristic of subfamily I receptors, and required for histidine kinase activity. Subfamily II maize and rice receptors lack some or all of the essential residues within the histidine kinase activity. Within all these lace plant ethylene receptors, there is part of the ethylene binding domain, the GAF domain, and a functional histidine kinase domain. They lack a C-terminal receiver domain, which is a response regulator and is present in maize and rice subfamily II ethylene receptors.

A phylogenetic analysis consisting of maize, rice and lace plant ethylene receptors showed that the three lace plant ethylene receptors (AmERS1a, AmERS1b and

AmERS1c) are more closely related to each other than they are to other monocot receptors (Figure 2.4). AmERS1a and AmERS1b are more closely related to each other than they are to AmERS1c. The three lace plant ethylene receptors are more closely related to subfamily I than subfamily II maize and rice ethylene receptors. Within subfamily I, they are also more closely related to the ERS1 receptors (ZmERS1a, ZmERS1b, OsERS1) than OsERS2.

2.4.3 AmERS1a, AmERS1b and AmERS1c Expression Levels in Different Stages of Lace Plant Leaf Development

To provide insights into the role of ethylene receptors in lace plant leaf development and PCD, quantitative PCR was performed to determine transcript levels of each of the receptors during seven stages of lace plant leaf development (Figure 2.5). Stage 1 (early preperforation; EPP), the leaves are young, tightly furled and have just emerged from the corm. There are no visible signs of PCD or perforation formation at this stage. Stage 2 (preperforation; PP), the leaves are still furled, vasculature is well pronounced, but there are still no signs of PCD or perforation formation. During stage 3 (early window; EW), about half of the leaf is unfurled and perforation sites are visible. Cells that do not undergo PCD (NPCD cells) during perforation formation appear pink (due to the pigment anthocyanin) while PCD cells that are destined to die during perforation formation have already lost anthocyanin. In stage 4 (window; W), the entire leaf is unfurled; perforation sites start to become somewhat transparent (PCD cells appear to lose some of their chlorophyll; Figure 2.1C). During stage 5 (late window; LW), actual holes start to form at the perforation sites, as some of the cells have died and disintegrate. Some cells at the perforation border are still undergoing PCD. Stage 6 is the mature stage

(M), where perforations are fully formed, there are no more signs of PCD and leaves are completely green again. At this stage, only NPCD cells remain, and they occupy 4-5 cell layers between the perforation and vascular tissue (Figure 2.1D). The last stage, stage 7 (senescence; S), the leaves are starting to yellow and there are some brown spots on the leaf blade.

Quantitative PCR results showed that AmERS1a transcript levels were similar from early preperforation to early window stage (Figure 2.6A). The AmERS1a transcript levels declined to significantly ($P < 0.05$) lower levels during the window stage, in which perforation formation and PCD were occurring. During the mature stage, where PCD and perforation formation are no longer occurring, AmERS1a transcripts increased to the highest levels. The levels, however, declined significantly ($P < 0.05$) during leaf senescence. AmERS1b was constitutively expressed throughout leaf development (Figure 2.6B). AsERS1c was constitutively expressed from early preperforation to late window stage (Figure 2.6C). However, similar to AmERS1a, the AmERS1c transcript levels increased significantly ($P < 0.05$) during the mature stage. AmERSlc transcript levels also declined significantly to the lowest levels during leaf senescence. Of the three lace plant ethylene receptors, AmERS1c appeared to have the highest transcript levels in leaves, followed by AmERS1b, and AmERS1a had the least transcript levels throughout leaf development. Actin, the reference gene, was constitutively expressed throughout leaf development (Figure 2.6D).

2.4.4 Expression Levels of AmERS1a, AmERS1b and AmERS1c in PCD and NPCD Cells

To further investigate the role of ethylene receptors in lace plant perforation formation and PCD, transcript levels between the dying (PCD) and non-dying (NPCD) cells were determined (Figure 2.7). The cells were separated and isolated from window stage leaves using a Zeiss PALM Laser Capture Microdissection and Imaging System. AmERS1a and AmERS1b transcript levels were not significantly different between PCD and NPCD cells (Figure 2.7A and B). AmERS1c had significantly higher ($P < 0.05$) transcript levels in NPCD cells than in PCD cells (almost 2 fold; Figure 2.7C). Even at the cellular level, AmERS1c had the highest transcript levels, then AmERS1b and lastly, AmERS1a. Actin was constitutively expressed between the two cell types (Figure 2.7D).

2.5 Discussion

Recent research shows ethylene is involved in regulation of PCD in lace plant during perforation formation and senescence, in a climacteric-like pattern (Dauphinee et al. 2012). Dauphinee et al. (2012) provided the first evidence for the involvement of ethylene in a climacteric-like pattern during normal leaf morphogenesis and development. They showed that ethylene production peaks during the window and senescence stages, both in-which PCD is occurring. Lace plant is a unique example of ethylene climacteric-like behaviour during leaf morphogenesis through PCD. To determine the role of ethylene perception in regulation of lace plant leaf development and PCD, through ethylene receptors, we isolated three lace plant receptors. These ethylene receptors, AmERS1a, AmERS1b and AmERS1c, showed high sequence similarity to other monocot ethylene receptors, from maize and rice. Rice has five ethylene receptors

and they have been divided into two subfamilies (Bleecker 1999; Yau et al. 2004). One family consists of ERS receptors (OsERS1 and OsERS2), and the other consists of ETR receptors (OsETR2, OsETR3 and OsETR4). Maize consists of four ethylene receptors and they also divided into the same two categories found in rice, but maize ERS category consists of ZmERS1a and ZmERS1b. It lacks an ERS2 receptor found in rice. The second category consists of ZmETR2a and ZmETR2b, it lacks the ETR3 and ETR4 receptors found in rice. The ERS monocot receptors all have the conserved residues within the histidine kinase domain and lack a receiver domain. The three lace plant ethylene receptors share these characteristics with the monocot ERS receptors. ETR receptors in maize and rice lack all or some of the essential residues within their histidine kinase domain an posses a receiver domain. Phylogenetic analysis, based on amino acid sequence similarity, also show that the lace plant ethylene receptors are more similar to ERS than ETR monocot ethylene receptors. All three isolated lace plant receptors also seem to be ERS1 isoforms. This is also supported by the phylogenetic analysis, which grouped them with ZmERS1a, ZmERS1b and OsERS1.

The three isolated lace plant ethylene receptors are subfamily I receptors, and it is most likely that the lace plant genome possesses subfamily II ethylene receptors as well. So far, all the plant species that have their ethylene receptors isolated have both subfamily I and II ethylene receptors. These include *Arabidopsis* (Bleecker et al. 1998), tomato (Klee and Tieman 2002), maize (Chen and Gallie 2010) and rice (Yau et al. 2004). Subfamily 1 ethylene receptors in *Arabidopsis* play a predominant role in regulation of ethylene responses (Wang et al. 2003; Shakeel et al. 2012). The ethylene receptors overlap in terms of functions during the control of ethylene responses.

However, Wang et al. (2006) showed that the lack of a subfamily I receptor in *Arabidopsis* results in a constitutive ethylene response, in which the inhibitory effect of ethylene receptors in ethylene induced responses is lacking. Hall and Bleecker (2003) also showed that *Arabidopsis* subfamily 1 (*ers1* and *etr1*) double loss of function mutants are severely developmentally defective, providing more evidence for the paramount importance of subfamily I receptors in development and regulation of ethylene induced responses.

Ethylene receptors also have non-overlapping roles; some are mostly involved in pathogen responses (Knoester 1998; Plett et al. 2009a), response to silver ions (McDaniel and Binder 2012), growth recovery after exposure to exogenous ethylene (Kim et al. 2011), trichome development (Plett et al. 2009a; Plett et al. 2009b), and nutational bending (Binder et al. 2006; Kim et al. 2011). In the lace plant, ethylene receptors seem to play a role in leaf development during perforation formation through PCD. An ethylene receptor inhibitor, silver nitrate ($AgNO_3$), reduced the number of perforations (Gunawardena et al. 2006). To determine the role of three lace plant ethylene receptors in leaf development and developmentally regulated PCD, we studied the transcript levels of each of the receptors throughout seven stages of lace plant leaf development. In general, AmERS1c had the highest transcript levels in leaf tissue. Its transcript levels were approximately 2000-fold the amount of AmERS1a and 3-fold the amount of AmERS1b. This suggests that AmERS1c may play a predominant role in ethylene perception during leaf development. AmERS1a also seems to be involved in lace plant PCD, despite its generally low transcript levels in leaves. Its transcript levels were significantly lower in window stage leaves, in which perforation formation and PCD occur. This is also when

ethylene levels peak (Dauphinee et al. 2012). The AmERS1a transcript levels then significantly increase during the mature stage, when the perforation is complete and ethylene levels are low. During senescence, when PCD is occurring and ethylene levels peak again, AmERS1a levels are reduced. AmERS1c levels are significantly high in mature stage leaves and significantly low during senescence. In window stage leaves, AmERS1c levels are lower in PCD cells than in NPCD cells. Even though AmERS1a levels are generally significantly lower in the window stage leaves, its transcript levels are not significantly different between PCD and NPCD cells. AmERS1b is constitutively expressed throughout leaf development and between the two types of cells and therefore unlikely to play a significant role in regulation of PCD during perforation formation. AmERS1a and AmERS1c seem to be the key players in regulation of ethylene perception and regulation of ethylene-dependent PCD during perforation formation in lace plant.

Ethylene receptors are negative regulators to the ethylene signal transduction pathway (Hall et al. 2007). In the absence of (or low) ethylene, the receptors form a complex with CTR1 and this complex has a negative effect on the rest of the ethylene signal transduction pathway, inhibiting ethylene-induced responses including PCD. When there is abundant ethylene, the ethylene binds to the receptor-CTR1 complex, deactivating the complex and extinguishing its inhibitory effect on the rest of the signal transduction pathway (Hall et al. 2007). The effect of ethylene on ethylene-induced responses is dependent on the amount of ethylene and ethylene receptors. When there is enough ethylene to bind to all or most of the receptors, the inhibitory action of receptor-CTR1 complex is lost, the pathway is activated and ethylene induced responses are expressed (Hall et al. 2007). Conversely, when there are high receptor levels available,

ethylene is not able to bind to all or a significant portion of the receptors, and the unbound receptor-CTR1 complexes remain active and inhibit the rest of the pathway, and thus, ethylene induced responses are not observed (Hall et al. 2007).

Ethylene levels are known to vary between species, different developmental stages, and different tissues within a plant (Ievinsh and Ozola 1998). Also, plants are known to increase sensitivity to ethylene by either reducing their ethylene receptor levels or producing more endogenous ethylene (Chang et al. 1993; Zhao and Schaller 2004; reviewed in Arora 2005). A proposed model of how ethylene receptor (AmERS1a and AmERS1c) transcript and endogenous ethylene levels regulate perforation formation and PCD in the lace plant is illustrated in Figure 2.8. In the lace plant, it has been shown in window stage leaves there are significantly higher ethylene levels, than in mature stage leaves (Dauphinee et al. 2012). Through ethylene biosynthesis inhibitor studies, it was shown that the high ethylene is necessary for perforation formation and PCD to occur (Dauphinee et al. 2012). In this high ethylene environment in window stage leaves, only PCD cells undergo PCD, and NPCD cells seem to be resistant to the high ethylene levels. This resistance can be attributed to the increase in AmERS1c transcript levels within NPCD cells that we observed in this study (Figure 2.7). We hypothesize that since the PCD cells seem to lower their AMERS1c (the most abundant receptor by far) levels, they become susceptible to ethylene and the ethylene-induced PCD occurs in these cells. After being exposed to the high ethylene levels during the window stage, NPCD cells seem to maintain their high AmERS1c and increase their AmERS1a transcript levels to withstand ethylene induced PCD. These high transcript levels are evident in mature stage leaves where developmental PCD is no longer occurring as perforation formation is

complete. Less ethylene is also produced in the mature leaves (Dauphinee et al. 2012). During senescence ethylene levels peak again (Dauphinee et al. 2012), and AmERS1a and AmERS1c transcript levels significantly decline, making the cells susceptible to ethylene and giving rise to the ethylene-regulated PCD in all the cells.

2.6 Conclusions and Future Work

The lace plant is an excellent model for studying cell biological aspects of PCD. It had been shown previously that the plant hormone ethylene plays an important role in regulation of lace plant PCD. Genetic regulation of developmentally regulated PCD in the lace plant has been unclear. This study provides some insight into how it may be genetically determined which cells are supposed to undergo PCD during perforation formation in the lace plant. The proposed model involving ethylene and ethylene receptors (Figure 2.8) explains why despite being within the same leaf tissue and environment, some cells die and others survive. Ethylene has been implicated as the trigger and regulator in other plant PCD systems, but in the lace plant the intrinsic signal that triggers increases in endogenous ethylene production and adjustment of ethylene receptors to determine cell fate is still unknown. Three lace plant ethylene receptors were isolated in this study, all of them are subfamily I receptors. It is unlikely that more of the subfamily I receptors exist, but it is almost certain that lace plant has undiscovered subfamily II receptors. Ethylene receptors are known to have overlapping roles in the ethylene signal transduction pathway (Hua and Meyerrowitz 1998). Sometimes, each receptor has some unique role in different ethylene induced responses (Plett et al. 2009a). Isolating the remaining ethylene receptor family members and studying their expression patterns would provide more insight into how each of the receptors is involved in

perforation formation in lace plant. Other genes within the ethylene biosynthesis and signal transduction pathways also need to be isolated and this will allow for more in-depth studies of the role of ethylene during perforation formation. Transcript level studies may also be supplemented with ethylene receptor mutants, to provide more insight into receptor function. Other genes that play a role in signalling, regulation and execution of lace plant PCD also need to be isolated and their roles investigated.

2.7 Acknowledgments

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Figure 2.1 The Lace Plant

(A) A typical lace plant from an aquarium. Leaves emerge from a corm (arrow). The corm also has several roots which function in anchoring the plant to growth medium. (B) Lace plant growing in a magenta box. This method of growing lace plant was developed to propagate lace plant in axenic conditions. Different developmental stage leaves, such as leaf number 1 (preperforation), 2 (late window) and 3 (mature), as shown in the magenta box grown plant, were harvested and used in experiments. The first few leaves produced by the lace plant do not form perforations (leaf number 4). (C) An areole from a "window" stage leaf, in which perforations are actively forming, depicting 4 to 5 cell layers of non-dying (NPCD) cells around the perforation site and dying (PCD) cells within the perforation site. (D) A perforation site, with NPCD located between the perforation and vascular tissue. Bars = 5 cm in A, 2.7 cm in B, 200 μm in C and 150 μm in D.

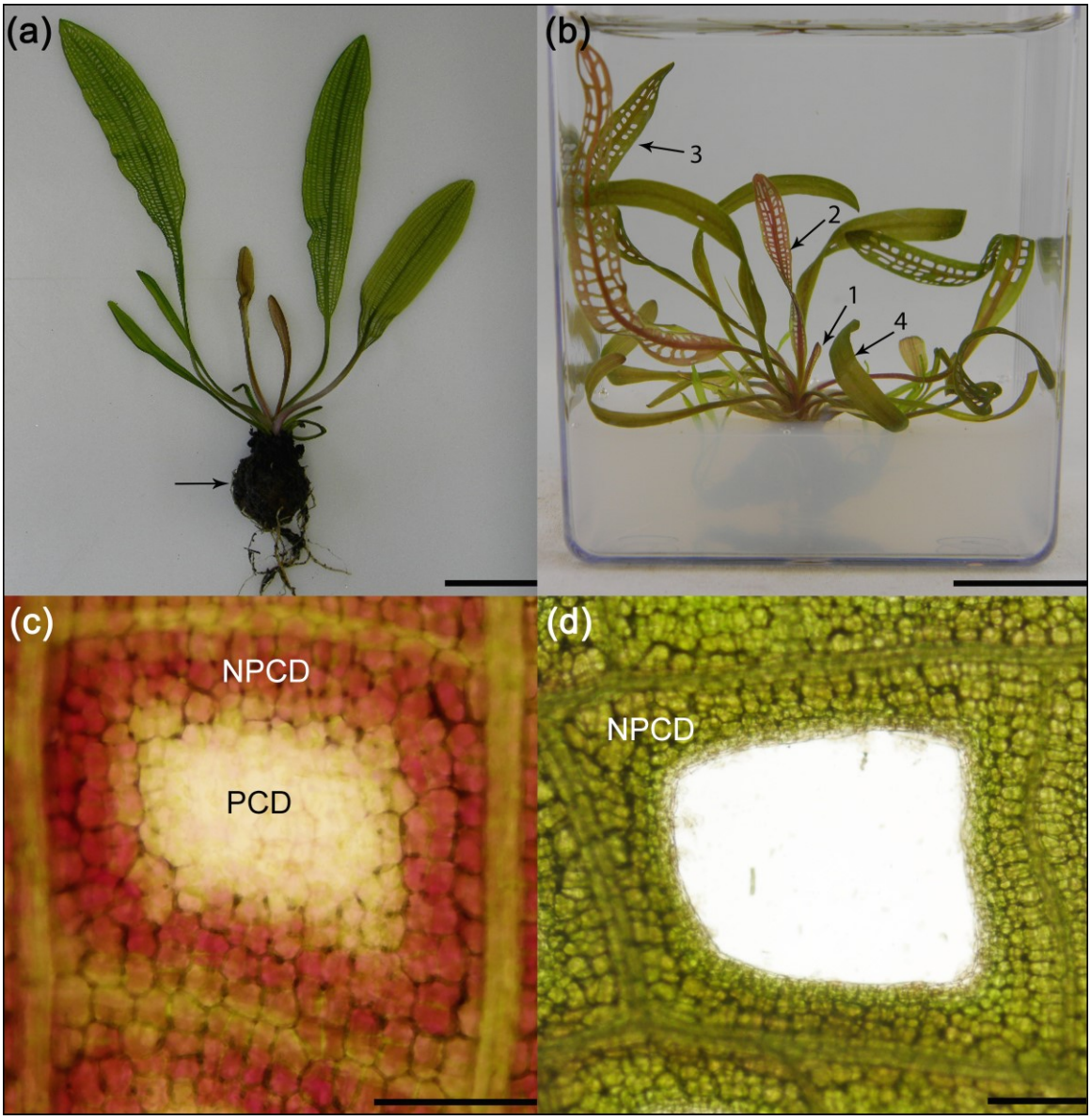

Figure 2.1 The Lace Plant

Figure 2.2 Amino Acid Sequences of the Lace Plant Ethylene Receptors and Their Alignment to *Z. Mays* ERS1a and ERS1b

Several important domains within the ethylene receptors are highlighted; GAF domain (pink), histidine kinase domain (purple), dimer interface domain (highlighted in yellow), HATPase_c (green), Mg^{2+} binding site (red asteric), G-X-G motif (red arrows), phosphorylation site (blue triangle), ATP binding site (highlighted in orange). The percentage identities of each pair of ethylene receptors are also indicated. The alignment depicts high conservation amongst the lace plant ethylene receptors and in comparison with the maize ERS receptors. The alignment was carried out using BioEdit Sequence Alignment Editor (Carlsbad, Ottawa, Ontario, Canada). Accession numbers: KR349966 (AmERS1a), KR349967 (AmERS1b), KR349968 (AmERS1c), AAR25566 (ZmERS1a) and NP_001137032 (ZmERS1b).

Figure 2.2 Amino Acid Sequences of the Lace Plant Ethylene Receptors and Their Alignment to *Z. Mays* ERS1a and ERS1b

Figure 2.3 The Structure of *Z. mays*, *O. sativa* and Lace Plant Ethylene Receptors

ZmERS1a, ZmERS1b, OsERS1 and OsERS2 share a similar structure consisting of an ethylene binding domain, a GAF domain and a functional histidine kinase domain. The lace plant ethylene receptors, AmERS1a, AmERS1b and AmERS1c also share this similar structure. The lace plant ethylene receptors also posses the conserved essential residues (H, N, G1, F and G2), within the histidine kinase domain, that are necessary for kinase activity. ZmETR2a, ZmETR2b and OsETR2, lack these essential residues within their histidine kinase domain, posses an additional hydrophobic transmembrane region within the ethylene-binding domain and has a C-terminal receiver domain. The receiver domain has a conserved phosphorylated aspartate (D) residue. OsETR3 and OsETR4 also have a receiver domain, and lack all essential or some of the essential residues within the histidine kinase domain. ZmERS1a, ZmERS1b, OsERS1 and OsERS2 are subfamily I, while ZmETR2a, ZmETR2b, OsETR2, OsETR3 and OsETR4 are subfamily II ethylene receptors.

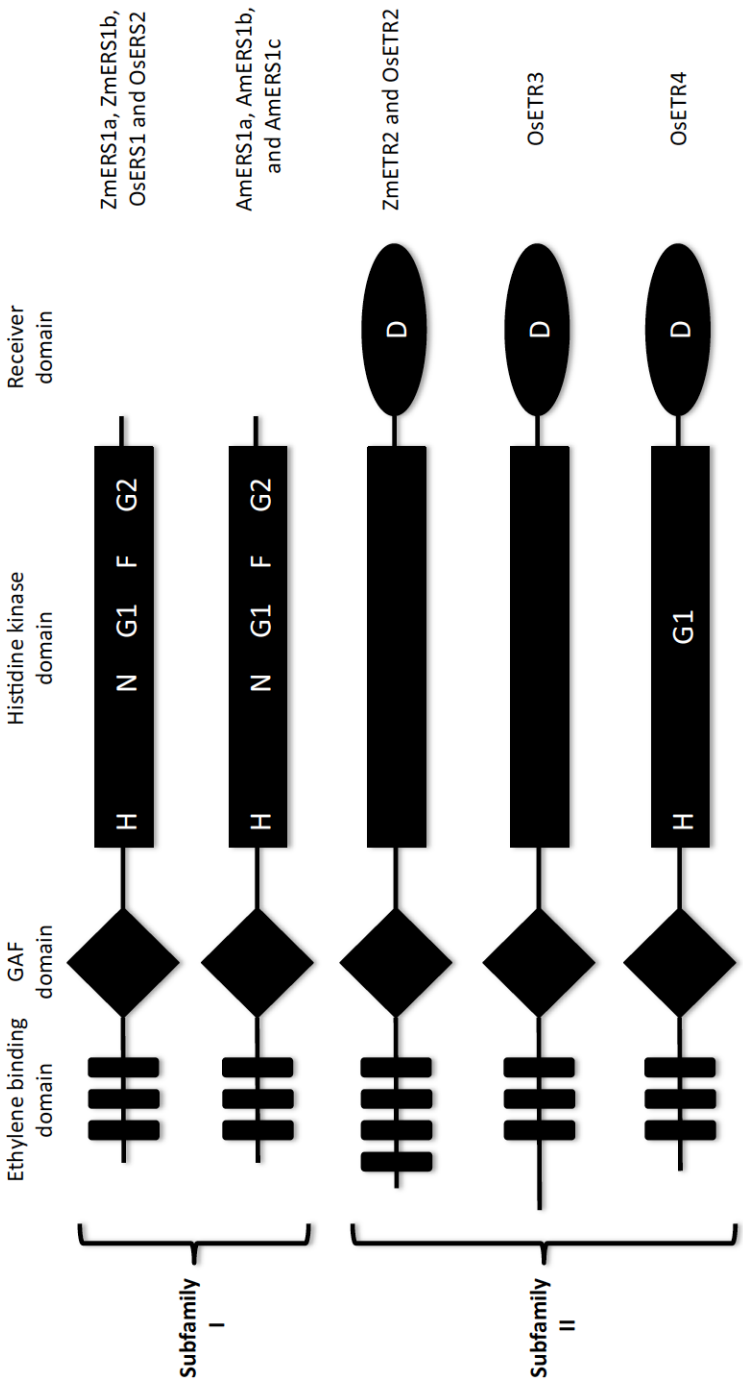

Figure 2.3 The Structure of *Z. mays, O. sativa* and Lace Plant Ethylene Receptors

Figure 2.4 A Phylogenetic Tree Composed of Lace Plant, Rice and Maize Ethylene Receptors

The GenBank accession numbers of the amino acid sequences used are KR349966 (AmERS1a), KR349967 (AmERS1b), KR349968 (AmERS1c), AAR25566 (ZmERS1a), NP_001137032 (ZmERS1b), NP_001104852 (ZmETR2a), XP_008667201 (ZmETR2b), AAB72193 (OsERS1), AAL66363 (OsERS2), CAD39679 (OsETR2), AAL29303 (OsETR3), AAQ07254 (OsETR4) and NP_187108.1 (AtEIN4). Bar represents the gap separation distance.

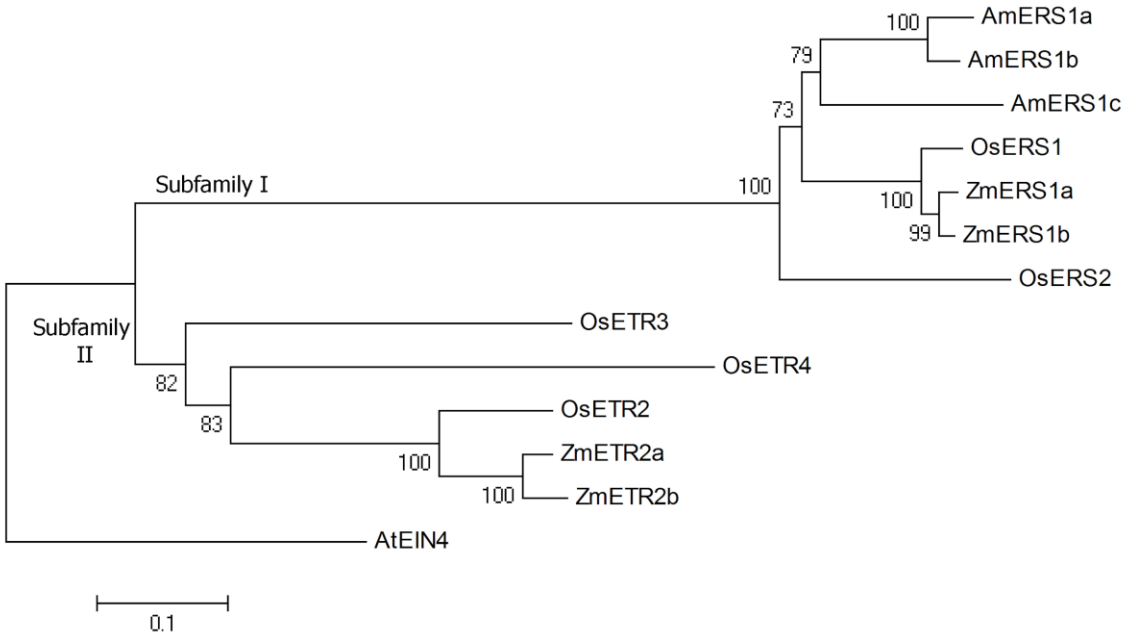

Figure 2.4 A Phylogenetic Tree Composed of Lace Plant, Rice and Maize Ethylene Receptors

Figure 2.5 Seven Stages of Leaf Development in the Lace Plant

Lace plant leaf development. For experimental purposes, lace plant leaf development was divided into seven stages; early preperforation (EPP), preperforation (PP), early window (EW), window (W), late window (LW), mature (M) and senescence (S). Perforation formation and PCD are occurring during early window, window and late window stages. PCD is also occurring in senescent stage leaves. Bars = 0.7 cm (EPP-LW) and 1.3 cm (M and S).

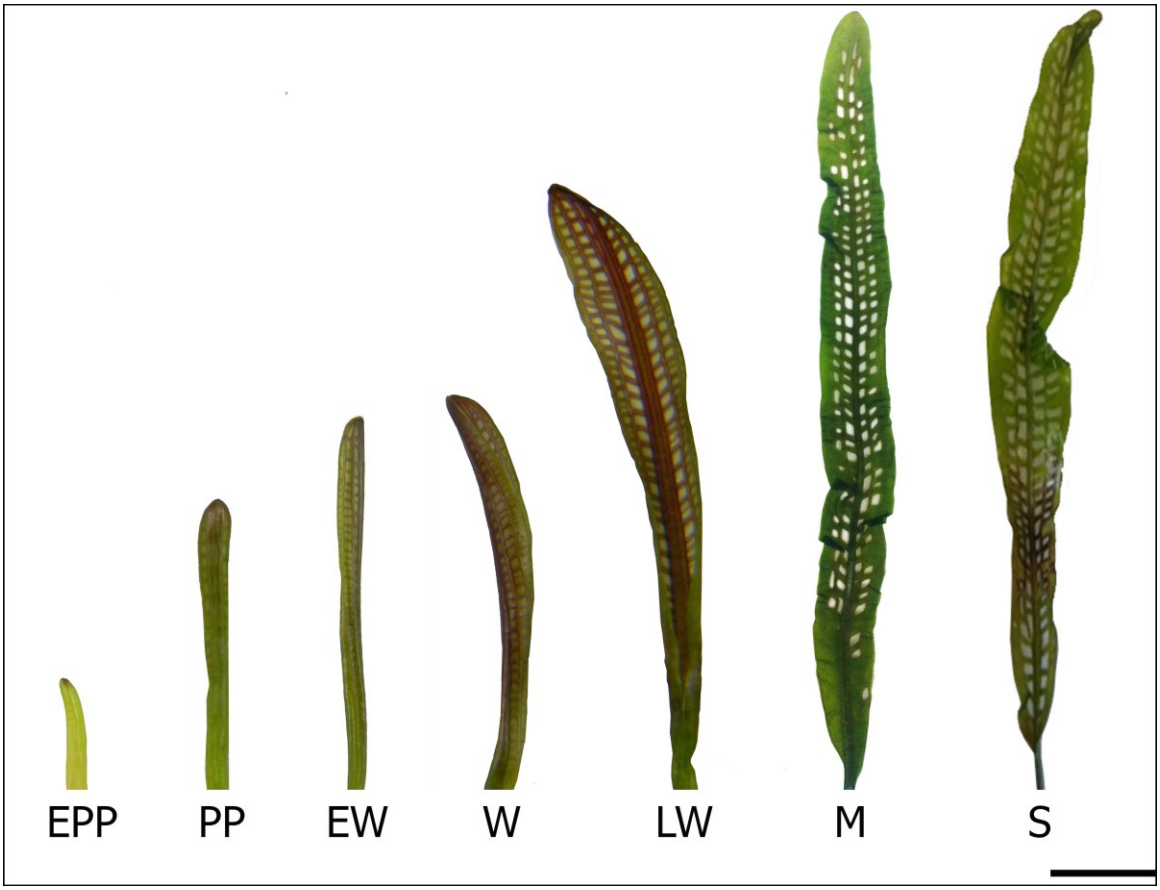

Figure 2.5 Seven Stages of Leaf Development in the Lace Plant

Figure 2.6 Normalized Ethylene Receptor Transcript Levels at Different Stages of Leaf Development

(A**)** Normalized AmERS1a transcript levels at different stages of leaf development. Window stage leaves, in which PCD is occurring, had significantly lower transcript levels AmERS1a than all other leaf developmental stages. Mature leaves, in which perforation formation is complete, had significantly higher AmERS1a transcript levels than all the other developmental stages. The transcript levels declined during leaf senescence. There was no significant difference in AmERS1b transcript levels throughout leaf development (B). AmERS1c had the highest transcript levels during the mature stage, while senescent leaves (in which PCD is occurring) had the lowest transcript levels (C). (D**)** Actin transcripts were constitutively expressed in all stages of lace plant leaf development. Bars represent SE ($n \geq 12$). Means with the same letters are not significantly different ($P > 0.05$).

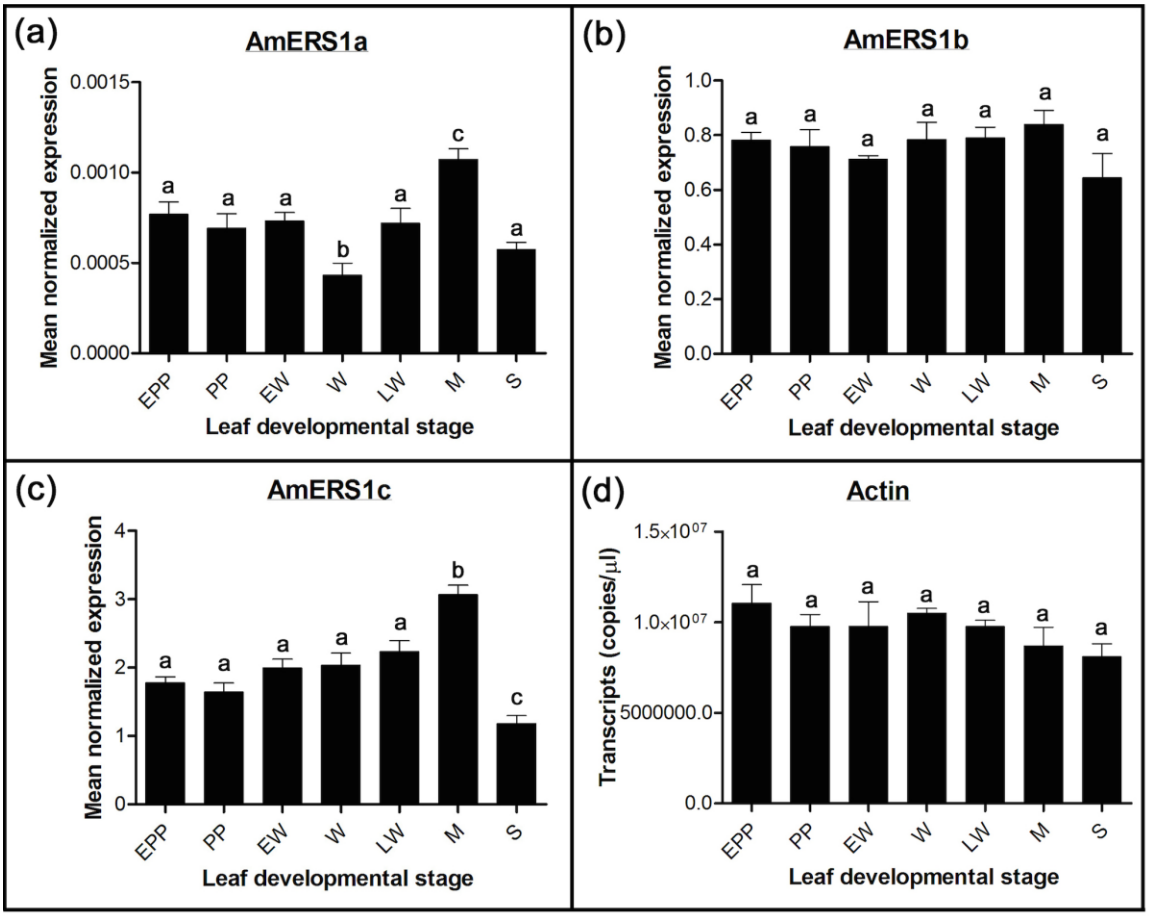

Figure 2.6 Normalized Ethylene Receptor Transcript Levels at Different Stages of Leaf Development

Figure 2.7 Ethylene Receptor Levels Between PCD and NPCD Cells

AmERS1a and AmERS1b did not have significant difference in transcript expression between PCD and NPCD cells (A and B respectively). Transcript levels for AmERS1c, the most abundant ethylene receptor, were significantly higher in NPCD than in PCD cells (C). (D**)** Reference gene, actin, did not show significant difference in transcription expression between the two types of cells. Bars represent SE ($n \geq 12$). Means with the same letters are not significantly different ($P > 0.05$).

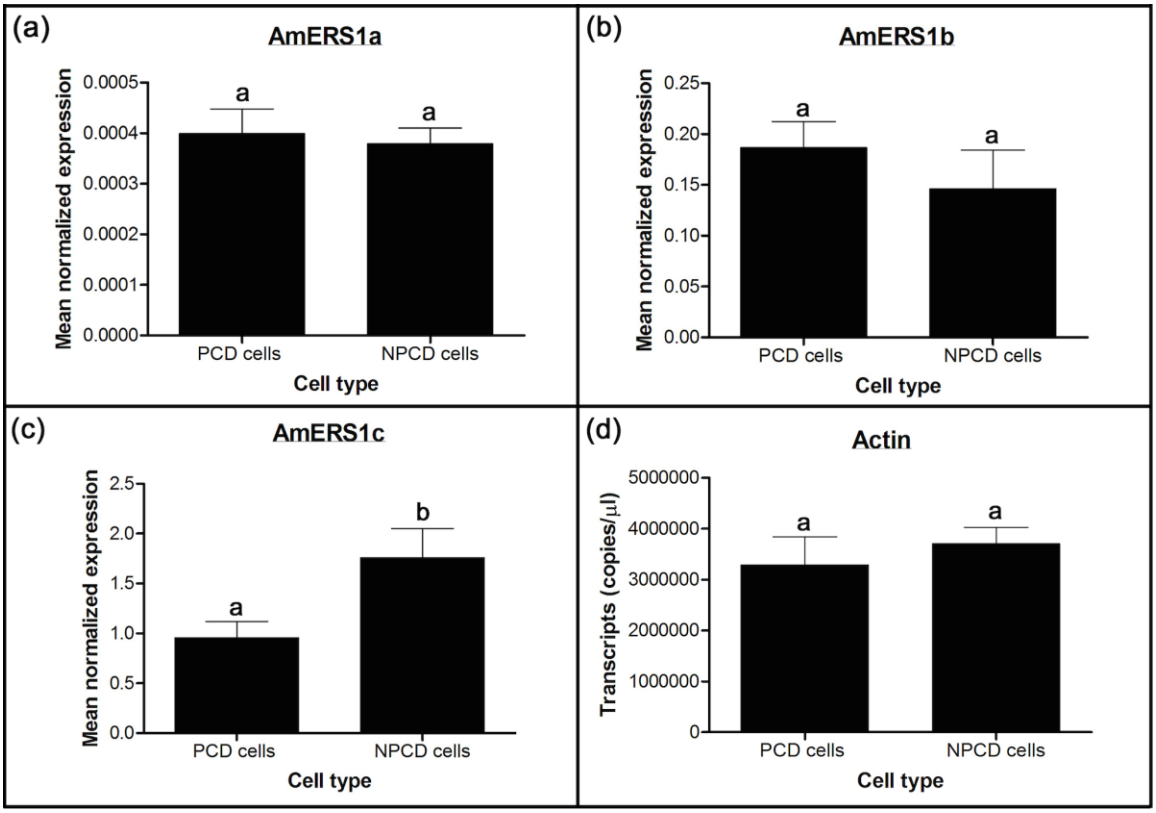

Figure 2.7 Ethylene Receptor Levels Between PCD and NPCD Cells

Figure 2.8 Proposed Ethylene Receptor Expression Pattern Model During Lace Plant Leaf Development

(A) Illustration of the proposed AmERS1a and AmERS1c expression pattern model in window stage lace plant leaves. Non-PCD cells (NPCD) are outside the perforation site while PCD cells are within the perforation site. The diagram illustrates how each cell type responds to high ethylene levels in window stage leaves during PCD. (B**)** Illustration of the proposed ethylene receptor expression pattern model in mature stage lace plant leaves. At this stage ethylene levels are normal and no cells are undergoing PCD. The only cells remaining at this stage are NPCD cells and they have high AmERS1a and AmERS1c transcript levels. **(**C**)** During the senescence stage, when the entire leaf tissue dies, there is a peak in ethylene production. AmERS1a and AmERS1c levels are significantly low during this ethylene-induced PCD process. Scale bars = 100 μm in A, and 150 μm in B and C.

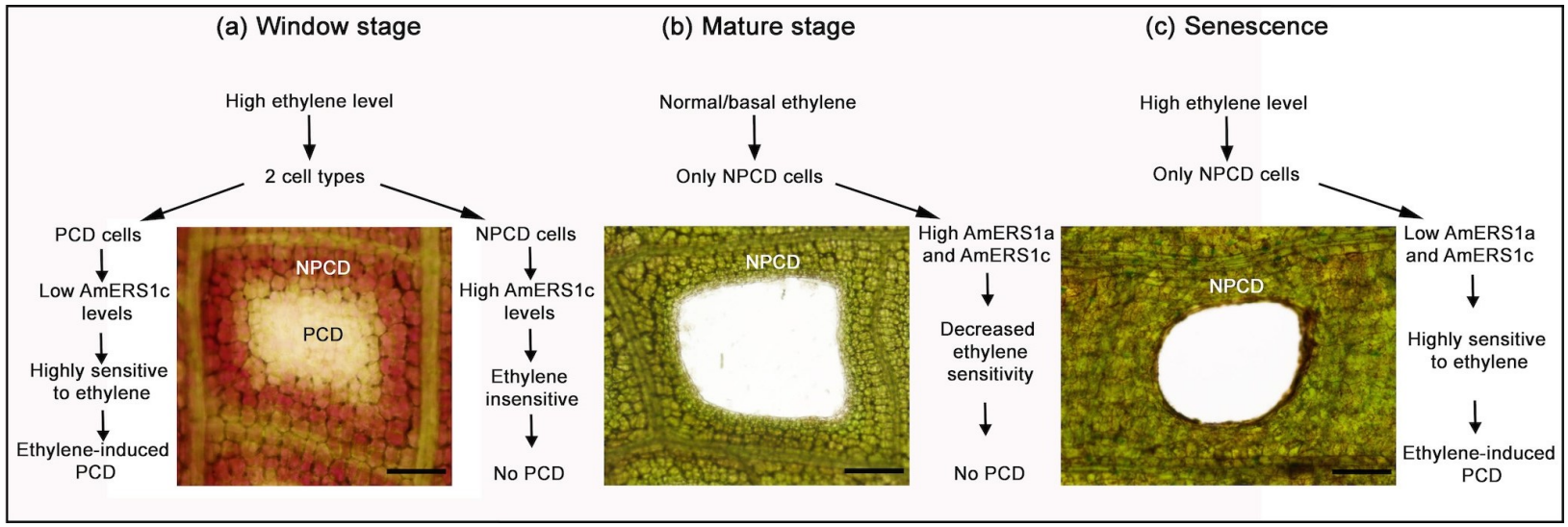
(a) Window stage
High ethylene level
2 cell types
PCD cells
Low AmERS1c levels
Highly sensitive to ethylene
Ethylene-induced PCD
NPCD
PCD
NPCD cells
High AmERS1c levels
Ethylene insensitive
No PCD
(b) Mature stage
Normal/basal ethylene
Only NPCD cells
NPCD
High AmERS1a and AmERS1c
Decreased ethylene sensitivity
No PCD
(c) Senescence
High ethylene level
Only NPCD cells
NPCD
Low AmERS1a and AmERS1c
Highly sensitive to ethylene
Ethylene-induced PCD

Figure 2.8 Proposed Ethylene Receptor Expression Pattern Model During Lace Plant Leaf Development

Chapter 3 Vacuolar Processing Enzymes as Executors of Programmed Cell Death During Leaf Morphogenesis in the Lace Plant (*Aponogeton madagascariensis*)

Will be submitted as:

Rantong G, Gunawardena AHLAN. Vacuolar processing enzymes, AmVPE1 and AmVPE2, as potential executors of ethylene regulated programmed cell death in the lace plant (*Aponogeton madagascariensis*)

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Author contributions

GR designed and carried out all experiments. GR wrote the first draft of the manuscript. AHLANG, conceived the study, participated in its design and coordination, and helped in drafting and revising the manuscript as well as supervised all experimental work.

3.1 Abstract

Programmed cell death (PCD) is genetically regulated cell demise and is essential for adaptation, development and survival of multicellular organisms. *Aponogeton madagascariensis* (lace plant) employs developmentally regulated PCD to form perforations during normal leaf morphogenesis. Developmental signals involved in the regulation and execution of lace plant PCD are unclear. We investigated the involvement of plant caspase-like cysteine proteinases, vacuolar processing enzymes (VPEs), in the regulation and execution of lace plant PCD during leaf development. VPEs share numerous enzymatic properties with caspase-1, which play a role in the execution of PCD in animals. We isolated two lace plant vegetative type VPEs, *AmVPE1* and *AmVPE1*, and studied their transcript levels throughout lace plant leaf development. VPE transcript levels and activity were also determined between cells that die during perforation formation (PCD cells) and cells that did not undergo PCD during perforation formation (NPCD cells). Quantitative PCR data showed that AmVPE1 had higher transcript levels during a preperforation stage of leaf development (immediately prior to visible signs of PCD). AmVPE2 transcripts were higher during window and late window stages of leaf development (where PCD was actively occurring). In window stage leaves, where perforation formation occurs via PCD, both lace plant VPEs had higher transcript levels in PCD compared to NPCD cells. A VPE activity probe (AMS101) detected more VPE activity in protoplasts from PCD cells than NPCD cell protoplasts. These results suggested that both lace plant VPEs are involved in regulation and execution of lace plant PCD during perforation formation. AmVPE1 is involved early in the PCD process, while AmVPE2 is involved in the later stages where rapid cell disintegration is occurring.

3.2 Introduction

Programmed cell death (PCD) is regulated cell demise necessary for elimination of specific cells in most multicellular organisms, which is necessary for development, adaptation and response to stress (Greenberg 1996; Lam 2004). It is conserved among multicellular organisms, from fungi, to plants and animals. In mammals, apoptosis (a form of PCD) is modulated and executed by cysteine-aspartic proteases (caspases). Although the execution of PCD through caspases is well understood in animals, execution of PCD in plants is still unclear. Plant species whose complete genomes have been sequenced lack genes encoding true caspases.

Despite the lack of conservation of caspases between plants and animals, some of the morphological features of PCD, which are directly or indirectly effects of caspases, are also observed in plants. These include chromatin condensation, DNA laddering, release of Cytochrome c, shrinkage of the cytopalasm, and activation of death proteases (Wang et al. 1996, Adrain and Martin 2001, reviewed in Reape and McCabe 2008; reviewed in Elmore 2007). This suggested the presence of genes that encode proteins that perform caspase-like duties during plant PCD. Identification of such proteins led to isolation of saspases (Dodson and Wlodawer 1998; Coffeen and Wolper 2004), phytaspases (Chichkova et al. 2010), metacaspases (Uren et al. 2000), and vacuolar processing enzymes (VPEs; Kinoshita et al. 1995a, 1995b).

Like caspases, saspases are synthesized as proenzymes, are aspartate specific and cleave some synthetic caspase substrates (Coffeen and Wolper 2004; Vertapetian et al. 2011). However, unlike caspases, saspases are serine-dependent (instead of cysteine), are

structurally different (reviewed in Tripathi and Sowdhamini 2006), active in the extracellular fluid (instead of cytoplasm; Vertapetian et al. 2011) and do not cleave general caspase substrates (Coffeen and Wolpert 2004). Therefore, their role as caspase-like enzymes during plant PCD is still questionable. Phytaspases are also aspartate specific, synthesized as proenzymes, cleave some synthetic caspase substrates (Chichkova et al. 2004; Chichkova et al. 2010) and display caspase 3-like activity in tobacco (Chichkova et al. 2004). However, they are serine proteases, structurally different from caspases and are mainly active in the apoplast until PCD is initiated (Chichkova et al. 2010; Chichkova et al. 2012). Like caspases, metacaspases are cysteine proteases, have a similar secondary structure, synthesized as proenzymes (Piszeczek and Gutman 2007), inhibited by caspase-inhibitors (Bozhkov et al. 2004), and are mainly found in the cytoplasm (Woltering 2004; Bozkov et al. 2005b). However, they prefer cleaving substrates at basic residues, such as arginine and lysine (Vercammen et al. 2004; González et al. 2007). Also, whether or not they cleave natural caspase-substrates during plant PCD is unknown.

VPEs share many characteristics with animal caspases. They are both cysteine proteases, have a His-Cys catalytic dyad and are synthesized as inactive proenzymes. Despite preferably cleaving substrates after an asparaginyl residue (Crawford and Wells 2011; Tsiatsiani et al. 2011), VPEs can also cleave substrates after the caspase-preferred Asp residue (Hatsugai et al. 2004; Rojo et al. 2004; Misas-Villamil et al. 2013). Several caspase inhibitors, especially caspase-1 inhibitors, also inhibit VPE activity in plants (Hatsugai et al. 2004; Rojo et al. 2004; Misas-Villamil et al. 2013). VPEs have also been shown to cleave caspase-1 substrates. They share other similarities with caspase-1; the

residues within their catalytic dyad are comparable (Cohen 1997; Hiraiwa et al. 1999; Nicholson 1999; Hara-Nishimura et al. 2005), the pentapeptides within the active sites are similar (Sanmartin et al. 2005) and amino acids within the substrate binding pockets are conserved (Wilson et al. 1994; Nicholson 1999; Hara-Nishimura et al. 2005).

Due to the similarities between caspases and VPEs, they have been isolated in several species and their role in plant PCD is under investigation. In tobacco, caspase-1 activity has been detected during hypersensitive (HR) response to TMV (del Pozo and Lam 1998; Hatsugai et al. 2004). This caspase-1 activity was attributed to VPEs and was required for PCD to occur during the HR. VPEs have also been shown to play an important role in other types of plant PCD such as embryogenesis (Hara-Nishimura et al. 2005), seed development (Nakaune et al. 2005), and leaf senescence (Kinoshita et al. 1999). VPEs seem to be mainly required in PCD involving vacuolar collapse, which marks the beginning of rapid cell demise.

In *Arabidopsis*, a total of 4 VPEs have been isolated (Kinoshita et al. 1995a, 1995b; Nakaune et al. 2005; Yamada et al. 2005).). They have been divided into 2 types based on expression pattern. Seed type VPEs, delta-VPE and beta-VPE, are mostly expressed in seeds. Vegetative type VPEs, alpha-VPE and gamma-VPE, are mainly expressed in vegetative tissue. VPEs have been isolated in other plant species like tobacco (Hatsugai et al. 2004), tomato (Lemaire-Chamley et al. 1999), soybean (Shimada et al. 1994), rice (Kumamaru et al. 2002), and maize (Schnable et al. 2009).

In the lace plant (*Aponogeton madagascariensis*), a caspase-1 inhibitor (Ac-YVAD-CMK) stopped PCD and perforation formation, which provided indirect evidence for the involvement of VPEs in lace plant PCD during leaf morphogenesis (Lord et al. 2013).

The lace plant is a submerged aquatic monocot belonging to the family Aponogetonaceae. Lace plant forms perforations on its leaves during normal development and these perforations form through developmentally regulated PCD (Figure 3.1A-D; Gunawardena et al. 2004). Lace plant is one of the few plant species known to employ PCD during leaf morphogenesis. Unlike in the other plant species such as *Monstera obliqua*, lace plant PCD regions are easily visible and occur at highly predictable locations (Figure 3.1C, D and E). The lace plant can also be propagated in microbe-free conditions in magenta boxes for experimental purposes (Figure 3.1B; Gunawardena et al. 2006). In addition, lace plant is more suitable for light microscopy due to its thin and transparent leaves.

In the lace plant, tonoplast rupture is one of the characteristic features of PCD and it does mark the beginning of rapid cell death (Wright et al. 2009). Even though the whole process of PCD takes approximately 2 days in the lace plant, after tonoplast rupture the cell is completely dead within minutes (Wright et al. 2009). This highlights the importance of tonoplast integrity during PCD. VPEs are thought to be responsible for tonoplast degradation and rupture during vacuole dependent PCD (Hatsugai et al. 2004). They are localized in the vacuole and are thought to compromise tonoplast integrity when active, or activate hydrolases that compromise tonoplast integrity. However, nothing is known about lace plant VPEs and their role in PCD during leaf morphogenesis.

Therefore, the objective of this study was to isolate lace plant vegetative type VPEs and study their role in developmental PCD during perforation formation in the lace plant.

3.3 Materials and Methods

3.3.1 Plant Propagation

Lace plants were propagated as described by Gunawardena et al. (2006), under sterile conditions, and in Magenta GA7 boxes. Plants were kept in 12 h light/12 h dark cycles. The light was approximately 125 μmol m^{-2} s^{-1} and was provided through daylight simulating fluorescent bulbs (Philips, Daylight Deluxe, F40T12/DX, Markham, Ontario, Canada). The plants were maintained at 24ºC.

3.3.2 Isolation of Lace Plant VPEs

Initial fragments of the lace plant VPEs were amplified using a degenerate primers method. The method involves RNA extraction, cDNA synthesis, PCR amplification, cloning, and sequencing. The 3′ end (including 3′ UTR) of each of the VPEs was isolated through 3′-RACE.

3.3.2.1 RNA Extraction

RNA extraction was performed using TRI-reagent (Sigma, Oakville, Ontario, Canada), with a few modifications to the standard method. These modifications were introduced since the phenolic compounds primarily found in lace plant vascular tissue was interfering with RNA extraction. The RNA pellet was not allowed to air-dry and twice the recommended volume of TRI-reagent was used. The midrib was also removed from leaf tissue before RNA extraction. Approximately 200 mg of leaf tissue was used in

RNA extraction. RNA was extracted from leaves divided into 7 stages of leaf development. Four RNA samples were collected from each leaf developmental stage. In total, 28 independent RNA samples were analyzed. Each RNA sample consisted of tissue from at least 3 leaves from different plants.

3.3.2.2 cDNA Synthesis

M-MuLV reverse transcriptase (New England Biolabs, Pickering, Ontario, Canada) was used for cDNA synthesis. Two µg of RNA was treated with DNase (Fermentas, Burlington, Ontario, Canada), and was added to nuclease free tube containing 1 µl of 10µM dT primer and 1 µl of 10 mM dNTP mix. A hot water bath was used to incubate the mixture at 65ºC for 5 minutes. The mixture was then chilled quickly on ice, and 4 µl of 5X First Strand Buffer (Invitrogen, Burlington, Ontario, Canada), 1 µl of RNase inhibitor (40u/µl) (New England Biolabs, Pickering, Ontario, Canada) and 2 µl of 0.1 M DTT (Invitrogen, Burlington, Ontario, Canada) were added. On a water bath, the mixture was incubated at 37ºC for 2 minutes, after which 2 µl of the M-MuLV reverse transcriptase (200 U/µl) was added. Tube contents were then mixed by pipetting and incubated at 37ºC for 1 hour in a water bath. The reaction was heat inactivated at 70ºC for 15 minutes and the mixture diluted to 50 µl with nuclease free water.

3.3.2.3 PCR Amplification of Lace Plant VPE cDNA

Initial VPE fragments were amplified degenerate primers. The degenerate primers were designed using alignments of VPE sequences from different species, performed through CLC combined workbench (CLC Bio-Qiagen, Aarhus, Denmark). The sequences used in the alignment were from *Arabidopsis thaliana* (NM_128154), *Ricinus communis*

(D17401), *Vitis vinifera* (XM_002276723), *Populus trichocarpa* (XM_006371798), *Populus tomentosa* (FJ461342), *Malus hupehensis* (FJ891065), *Solanum tuberosum* (NM_001288343), *Zea mays* (NM_001111649 and AJ131719), *Hordeum vulgare* (AM941114), *Beta vulgaris* (AJ309173), *Nicotiana tabacum* (AB075947 and AB075948), and *Solanum tuberosum* (EU605871). The forward and reverse degenerate primers used for amplification of the initial fragments of *AmVPE1* are Alpha deg VPE F3 and Alpha deg VPE R3, respectively (Table 3.1). Forward (Gamma deg VPE F3; Table 3.2) and reverse (Gamma deg VPE R3; Table 3.2) degenerate primers were used to amplify a fragment of *AmVPE2*. The PCR reaction consisted of 3.5 µl of cDNA, 11.15 µl nuclease free water, 1 µl of 10 mM dNTP mix, 1 µl of 10 mM forward and reverse primer, 2 µl 10X Thermobuffer, and 0.35 µl of 5 U/ µl Taq DNA polymerase. For PCR amplification, conditions consisted of initial denaturing at 94ºC for 5 minutes, 40 cycles at 94ºC for 30 seconds (denaturing), primer annealing at 48ºC for 30 seconds, elongation at 72ºC for 1 minute, and final elongation at 72ºC for 10 minutes.

Table 3.1 Primers used for isolation, amplification and quantification of *AmVPE1* mRNA

Primer name	**Type of primer**	**Sequence**
Alpha deg VPE F3	Degenerate	TGGCYRTGCTCATYGCCGGCTC
Alpha deg VPE R3	Degenerate	ACCARGCTTTTDTAGGTYCC
AmVPE1 R2 deg	Degenerate	CCAAACAAAAGSTTYCCAATGAG
AmVPE1 nested F1	Nested primer	TCTAGCTGTTCTCCTAGGTGAT
AmVPE1 F; 877-899	Nested primer	GCTGTGAGACCATCAGGACAACC
AmVPE1 F3-QPCRE/I	RT-PCR and qPCR primer	GCATTGTTAAGGAGCGGACA
AmVPE1 R1-QPCRF3	RT-PCR and qPCR primer	TTGTACCCATAAACAAGGCAAT
AP	3′-RACE	GGCCACGCGTCGACTAGTACTTT-TTTTTTTTTTTTT
AUAP	3′-RACE	GTACTAGTCGACGCGTGGCC

Table 3.2 Primers used for isolation, amplification and quantification of *AmVPE2* mRNA

Primer name	**Type**	**Sequence**
Gamma deg VPE F3	Degenerate	TGGGCYGTCCTSMTCGCCGG
Gamma deg VPE R3	Degenerate	GGCATCCCAAGRACHCCAGGTCC
AmVPE2 R2 deg	Degenerate	CCACARTGYGTCTCAAATGTCCTC
AmVPE2 nested F1	Nested primer	CTAAGGATTATGTTGGGAAG
AmVPE2 F; 915-937	Nested primer	ACTGGCACAAGTTCAAGAAGGCG
AmVPE2 F4 -QPCR E/I	RT-PCR and qPCR primer	CCGAGTGGTTAAAGAACGAA
LpVPE1 R2 –QPCR F4	RT-PCR and qPCR primer	CTGAACCAATGTACAAGGCAAGT

3.3.2.4 Cloning and Sequencing

The PCR products were separated on 1.5% agarose gels stained with ethidium bromide. The gel was visualized in a DNR F- ChemiBIs 3.2M Pro (Bio- imaging Systems, Montreal, Quebec, Canada). Amplified products were excised from the gels and purified using a GENECLEAN Turbo Kit (Qbiogene, Carlsbad, California, USA), following manufacturer's instructions. The purified fragments were cloned into the pGEM-T Easy Vector System (Promega, Nepean, Ontario, Canada) also following manufacturer's instructions. Plasmids were purified using a GenElute plasmid miniprep kit (Sigma, Oakville, Ontario, Canada), and the purified plasmids were sent to Macrogen Corp (Rockville, Maryland, USA) for sequencing.

3.3.2.5 3′-RACE

After sequencing of the initial lace plant VPE fragments, a nested forward primer for each VPE was designed and used along with 3′-RACE to isolate the rest of the 3′ end, including 3′ UTR. The forward primers AmVPE1 F 877-899 (Table 3.1) and AmVPE2 F 915-937 (Table 3.2) were used to amplify fragments of *AmVPE1* and *AmVPE2* respectively. In the 3′-RACE technique, an anchored primer (AP; Table 3.1) was used in cDNA synthesis. An abridged universal amplification primer (AUAP; Table 3.1) was later used as a reverse primer in PCR reactions, along with the respective forward primers, to amplify 3′ ends of the lace plant VPEs. The PCR products were run through an agarose gel, bands selected and purified, cloned into a vector and sent for sequencing.

3.3.3 Sequence Analysis

BioEdit Sequence Alignment Editor (Carlsbad, Ottawa, Ontario, Canada) was used to trim low quality portions and vector sequences. It was also used to deduce the amino acid sequences from the provided nucleotide data. Both nucleotide and amino acid sequences were then compared with National Center for Biotechnology Information (NCBI) nucleotide collection (blastn) and nonredundant protein (blastx) database sequences, respectively. ClustalW2 (Larkin et al. 2007) was used to calculate sequence identities for both nucleotide and amino acid sequences.

3.3.4 Phylogenetic Analysis

The lace plant VPE sequences were aligned with 35 known VPE sequences from other plant species using CLUSTALW (Thompson et al. 1997). The names and accession numbers of the sequences used in this analysis are listed in Table 3.3. A bootstrap neighbor-joining phylogenetic tree was created using MEGA version 4 (Tamura et al. 2007). The nonparametric bootstrap test performed consisted of a thousand replicates. The rice putative asparagine-specific endopeptidase precursor (NP_910213) was used as an out-group.

Table 3.3 GenBank accession numbers of VPE amino acid sequences used for phylogenetic analysis

VPE name	**Species**	**Accession number**
Mung Bean VmPE1a	*Vigna mungo*	BAA76745.1
Kidney Bean LLP (VPE)	*Phaseolus vulgaris*	O24325.1
Vetch Protease B (VPE)	*Vicia sativa*	P49044.1
Mung Bean VmPE1	*Vigna mungo*	BAA76744.1
Orange Cit VPE	*Citrus sinensis*	P49043.1
Sweet potato SPAE (cysteine protease)	*Ipomoea batatas*	AAF69014.1
Arabidopsis alpha-VPE	*Arabidopsis thaliana*	BAA09614.2
Tobacco NtVPE3	*Nicotiana tabacum*	BAC54830.1
Tobacco NtVPE2	*Nicotiana tabacum*	BAC54829.1
Sugar Beet VPL vacuolar processing enzyme	*Beta vulgaris*	CAC43295.1
Tobacco NtVPE1a	*Nicotiana tabacum*	BAC54827.1
Tobacco NtVPE1b	*Nicotiana tabacum*	BAC54828.1
Rice VPE	*Oryza sativa*	NP 918390.1
Maize See2a (legumain-like protease)	*Zea mays*	CAB64544.1
Maize C13 endopeptidase NP1 precursor	*Zea mays*	AAD04883.1
Rice C13 cysteine proteinase precursor NP1	*Oryza sativa*	AAL40390.1
Rice asparaginyl endopeptidase REP-2	*Oryza sativa*	BAC41386.1
Barley C13 endopeptidase NP1 precursor	*Hordeum vulgare*	AAD04882.1
Rice Glup3 (vacuolar processing enzyme)	*Oryza sativa*	BAC76418.1
Arabidopsis beta-VPE	*Arabidopsis thaliana*	BAA09615.1
Tobacco putative preprolegumain	*Nicotiana tabacum*	CAB42651.2
Sesami asparaginyl endopeptidase	*Sesamum indicum*	AAF89679.1

VPE name	**Species**	**Accession number**
Castor Bean VPE	*Ricinus communis*	P49042.1
Kidney Bean PvVPE	*Phaseolus vulgaris*	O24326.1
Soy Bean seed maturation protein PM40	*Glycine max*	AAF89646.1
Soy Bean VPE	*Glycine max*	P49045.1
Jack Bean asparaginyl endopeptidase	*Canavalia ensiformis*	P49046.1
Narbon bean cysteine proteinase precursor	*Vicia narbonensis*	CAB16318.1
Vetch cysteine proteinase precursor	*Vicia sativa*	O82102
Tobacco NtPB3 putative legumain	*Nicotiana tabacum*	CAE84598
Tobacco NtPB1 putative preprolegumain	*Nicotiana tabacum*	CAB42650.2
Tomato LeVPE1	*Solanum lycopersicum*	CAB51545.1
Arabidopsis delta-VPE	*Arabidopsis thaliana*	BAC65233.1
Rice asparagine-specific endopeptidase	*Oryza sativa*	NP_910213
Arabidopsis gamma-VPE	*Arabidopsis thaliana*	BAA018924.1

3.3.5 Analysis of VPE Transcript Levels Throughout Leaf Development

VPE transcript levels were determined in seven different stages of lace plant leaf development. Reverse transcription polymerase chain reaction (RT-PCR) and quantitative PCR were used to detect and quantify VPE transcript levels during the developmental stages. For RT-PCR, the reaction consisted of 1 µl of cDNA, 13.65 µl nuclease free water, 1 µl of 10 mM dNTP mix, 1 µl of 10 mM forward and reverse primer, 2 µl 10X Thermobuffer, and 0.35 µl of 5 U/ µl Taq DNA polymerase. For PCR amplification, conditions consisted of 94ºC for 5 minutes (initial denaturing), 40 cycles at 94ºC for 30 seconds (denaturing), primer annealing at 60ºC for 30 seconds, elongation at 72ºC for 1 minute, and final elongation at 72ºC for 10 minutes. The forward and reverse primers used for amplifying *AmVPE1* fragments are AmVPE1 F3-QPCRE/I and AmVPE1 R1-QPCRF3 respectively (Table 3.1). The forward and reverse primers used for amplifying *AmVPE2* fragments are AmVPE2 F4-QPCRE/I and LpVPE1 R2–QPCRF4 respectively (Table 3.2). To test for the quality of cDNA synthesized from RNA extracted from each sample, Actin fragments were amplified through PCR using forward primer (5´-CCCAAGGCTAATCGTGAAAA-3´) and reverse primer (5´-CAAGCACGATACCTGTCGTA-3´). For RT-qPCR, 20 µl reactions were performed in a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) using QuantiTect SYBR Green PCR Kit (Qiagen, Mississauga, Ontario, Canada), following the manufacturer's instructions and using the same primers used in RT-PCR. The PCR conditions used consisted of 95ºC for 15 min (initial denaturing), 40 cycles of 94ºC for 15 s (denaturing), primer annealing temperature (dependent on primer used) for 20 s, and 72ºC for 30 s (elongation). PCR product purity was determined by observing the melting temperature

curve. A standard curve of copy number was generated from quantified target sequences for each gene. The standard curves were used to determine mRNA copy numbers. mRNA copy numbers for each VPE were divided by the copy number of *AmActin* mRNA to obtain mean normalized expression values.

3.3.6 Analysis of VPE Transcript Levels Between Dying (PCD) and Non-PCD (NPCD) Cells

A Zeiss PALM Laser Capture Microdissection and Imaging System was used to separate PCD and NPCD cells (Figure 3.2). Cells were collected from at least three different leaves per sample and a total of 8 different samples (4 samples per cell type) were used for RNA extraction. A ReliaPrep RNA Cell Miniprep kit (Promega, Nepean, Ontario, Canada) was used for RNA extraction, following manufacture's instructions. A Protoscript M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs, Pickering, Ontario, Canada) was used for cDNA synthesis, following manufacturer's instructions.

3.3.7 Isolation of Lace Plant Protoplasts

Protoplasts were isolated as described in Lord and Gunawardena (2010). Window and mature stage leaves were harvested from plants grown in magenta boxes and rinsed in water. The midrib was removed from the leaves and the leaf tissue was dissected into narrow strips about 1 cm wide. One gram of tissue was then put in a protoplast buffer solution (100 mL) containing 0.005 M 2-N-morpholino-ethanesulfonic acid (Sigma-Aldrich, Oakville, Ontario, Canada) and 0.6 M sorbitol. The pH of the buffer had been adjusted to pH 5.5 using potassium hydroxide. The tissue was incubated in the buffer for 20 min and transferred into an enzyme solution containing 10 mL protoplast solution, 2

% (w/v) cellulose R10 and 0.5 % Pectolyase Y-23 (Yakult Pharmaceutical Ind, Tokyo, Japan), in a Petri dish. The Petri dish containing the tissue was incubated at 27ºC for 4 hr in the dark, and shaken during the last 30 min of incubation at 50 rpm. The protoplast suspension (liquid) was then filtered into a 50 mL falcon tube using a 7 μm mesh. The protoplast suspension was then centrifuged at room temperature for 20 min at 100 x g, with no brake. Pelleted protoplasts were washed with 15 mL of protoplast buffer solution. They were centrifuged again and then suspended in 500 μl of clean protoplast buffer solution.

3.3.8 In vivo Labeling of Lace Plant Protoplasts

Protoplasts in the protoplast buffer solution (pH 5.5) were exposed to a VPE activity probe AMS101 (kindly donated by Dr. Renier van der Hoorn, Max Planck Institute for Plant Breeding Research, 50829 Cologne, Germany) as described in Misas-Villamil et al. (2013). They were incubated in 2 μM of the probe, at room temperature with constant gentle shaking for six hours. Incubation was performed in the dark. The control was incubated under the same conditions, without exposure to the probe. After incubation, the protoplasts were washed five times with water prior to analysis, to remove extra probe. A Zeiss LSM 510 confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA) was used to detect the fluorescence of the BOPIDY fluorescence tag at excitation 532 nm/emission 580 nm, using a HeNe1 laser with excitation of 534 nm and emission of 580 nm. The experiment was repeated twice.

3.3.9 Statistical Analysis

GraphPad Prism version 5.00 (San Diego, California, USA) was used to analyze Quantitative PCR data. Significant differences in the number of transcripts were determined through a general linear model of variance. Means of individual treatments were compared using a Tukey post-test, if the overall relationships were significant. Data was determined to be statistically significant if $P < 0.05$.

3.4 Results

3.4.1 Lace Plant VPEs

Two vegetative type lace plant VPEs were isolated through the degenerate primer, nested PCR and 3´-RACE techniques. They were annotated AmVPE1 (KR779002) and AmVPE2 (KR779003). Their isolated fragments were 1436 and 1603 base pairs, respectively. The entire 3´ end of their cDNA was isolated, including the untranslated region. The VPE fragments translated to 437 amino acids (Figure 3.3). The lace plant VPEs are 83.6 % and 88.3 % identical at the nucleotide and amino acid level respectively. They are also both more identical to the Arabidopsis vegetative type VPEs than to the seed type. AmVPE1 is 69 % and 71 % identical to Arabidopsis gamma and alpha-VPEs respectively. It is only 58 and 53 % identical to the seed type Arabidopsis VPEs (beta and delta-VPE, respectively). AmVPE2 shares 68 % and 70 % identity to Arabidopsis gamma and alpha-VPEs respectively, while it is only 60 % and 52 % identical to the Arabidopsis beta and delta-VPEs, respectively.

The domains and sites identified within the lace plant VPEs and conserved in the Arabidopsis vegetative VPEs include mature protease domain, essential amino acids

(Cys102, His191 and Cys241), N-glycosylation site (Asn360), and a C-terminal propeptide. Most of these are also conserved in the Arabidopsis seed-type VPEs, except His191, which is an essential amino acid, and is instead replaced by an Asn in delta-VPE. Also, less of the C-terminal propeptide region is conserved between lace plant VPEs and Arabidopsis seed-type VPEs, than between the lace plant VPEs and vegetative type Arabidopsis VPEs.

Phylogenetic analysis comparing the amino acid sequence lace plant VPEs with 34 VPEs in other plant species, revealed that the lace plant VPEs share more in common with the vegetative type VPEs than seed type VPEs (Figure 3.4). The accession numbers of the VPEs used in this analysis are listed in Table 3.3. They were from both monocotyledonous (monocot) and dicotyledonues (dicot) species. The lace plant VPEs are more closely related to the monocot (rice and maize) vegetative VPEs than dicot vegetative type VPEs.

3.4.2 AmVPE1 and AmVPE2 Transcript Expression Levels In Different Stages of Lace Plant Leaf Development

AmVPE1 and *AmVPE2* transcript levels were determined throughout seven stages of lace plant leaf development, to determine the involvement of VPEs in lace plant leaf developmental PCD. Through RT-PCR, VPE transcripts were detected in RNA extracted from all the seven stages of leaf development (Figure 3.5). To determine the quality of RNA from each sample, the constitutively expressed actin was assayed throughout leaf development as well, and was detected during all leaf developmental stages (Figure 3.5). *AmVPE1* and *AmVPE2* were also detected in cDNA from all leaf developmental stages

(Figure 3.5). Primers used for qPCR were also shown not to amplify any products from genomic DNA, but amplified expected-size products in cDNA samples (Figure 3.6). Through the more sensitive qPCR, the amounts of VPE transcripts were quantified in the RNA from all the seven leaf developmental stages (Figure 3.7 and 3.8). Actin was used as a reference gene, and its transcript levels were also quantified throughout leaf development (Figure 3.9). Transcript level results demonstrated that the lace plant VPE transcripts are differentially expressed during leaf development. AmVPE1 transcripts were significantly higher ($P < 0.05$) during the preperforation stage of leaf development (Figure 3.7), than all the other stages of leaf development. This stage of leaf development is immediately prior to obvious visible signs of PCD and perforation formation. During the early window and window stage, the *AmVPE1* transcript levels decreased to significantly lower ($P < 0.05$) levels than in preperforation (Figure 3.7). During the late stages of PCD, in late window stage, the *AmVPE1* transcript levels declined to significantly lower ($P < 0.05$) levels than in the early window and window stages. In mature and senescence stage, the *AmVPE1* transcripts were at their lowest levels as compared to all the other stages of leaf development. *AmVPE2* transcript levels were significantly higher ($P < 0.05$) in window and late window stage leaves, in which rapid cell death was occurring (Figure 3.8). There were no significant differences in *AmVPE2* transcript levels among all the other stages (early preperforation, preperforation, early window, mature and senescence) of leaf development. In leaf tissue, *AmVPE2* had higher transcript levels than *AmVPE1*. Its transcript levels were approximately 1000-fold higher than *AmVPE1* transcript levels.

3.4.3 AmVPE1 and AmVPE2 Transcript Expression Levels in PCD Versus NPCD cells

To further examine the role of VPEs in lace plant PCD, transcript levels were determined in PCD cells and those that are not destined to die during perforation formation (NPCD cells). The cells were obtained from window stage leaves and separated through a laser capture microscope (Figure 3.2). RT-PCR was used to detect *AmVPE1*, *AmVPE2* and *AmActin* cDNA from total RNA extracted from the two cell types (Figure 3.10). Transcript levels were determined through the more sensitive qPCR. PCD cells had significantly higher ($P < 0.05$) transcript levels of both lace plant VPEs than the NPCD cells (Figure 3.11). *AmVPE1* transcript levels were about 4-fold the in PCD than in NPCD cells. The amount of *AmVPE2* transcripts was more than double in PCD than in NPCD cells. *AmVPE2* transcripts were higher than *AmVPE1* transcripts at the cell level as well.

3.4.4 VPE Activity is Higher in PCD Compared to NPCD Cells

Lace plant protoplasts were successfully isolated and they remained viable for approximately 10 hours. The isolated protoplasts were exposed to 2 μM of a VPE activity-based probe AMS-101 for 6 hours. VPE activity within protoplast from PCD cells and NPCD cells was inferred from probe-based fluorescence. The activity was observed in the vacuoles of the protoplasts (Figure 3.12), where VPEs are known to be active. The VPE activity and fluorescence was higher in PCD cell protoplasts compared to NPCD protoplasts. No probe-associated fluorescence was detected in the no-probe controls.

3.5 Discussion

The two lace plant VPEs isolated showed higher percentage identity and similarity towards vegetative type VPEs in Arabidopsis than the seed type (Figure 3.3). The domains and essential amino acids identified within the lace plant VPEs are also more identical to those in the *Arabidopsis* vegetative type VPEs. Phylogenetic analysis is also consistent with the alignment analysis since they grouped the lace plant VPEs with vegetative type VPEs in other plant species (Figure 3.4). The lace plant VPEs are also more evolutionarily similar to the other monocot (maize CAB64544.1 and AAD0488.1; rice NP 918390.1) vegetative type VPEs than dicot VPEs.

Transcript level data suggest that both lace plant VPEs are involved in PCD during perforation formation in the lace plant. AmVPE1 seems to be involved in the very early stages of PCD, before any obvious visible signs of cell degradation (in preperforation stage leaves). AmVPE2 seems to be involved during the later stages of PCD (in window and late window stage leaves), where cellular degradation signs are obvious. Its higher transcript levels during the stages where PCD is actively occurring suggest that AmVPE2 is involved in the execution of PCD during perforation formation. VPEs are sometimes known to display increases in transcript levels prior to and during visible signs of PCD (Iakimova et al. 2009). In apple leaves inoculated with *Erwinia amylovora,* increases in VPE transcript levels were detected early, prior to any visible of lesion-associated PCD and also later after obvious signs of lesion formation (Iakimova et al. 2009). The early-expressed VPEs are thought to be involved in early stages of PCD, in signaling/regulation prior to obvious signs of cell death. They may also activate proteases, hydrolases, enzymes responsible for cell degradation and death. In addition,

they may be responsible for early invisible signs of PCD or initiate the execution of processes leading to obvious signs of PCD. The higher transcript levels of lace plant VPEs in preperforation (*AmVPE1*) and window stages (*AmVPE2*) compared to mature stage leaves, are consistent with the Lord et al. (2011) findings that showed higher caspase-1 activity in preperforation and window stage leaves compared to mature stage leaves. Taken together, the findings suggest that the observed transcriptional increases of VPEs in the preperforation and window stage leaves also translate to increased proteolytic activity within these leaves.

Developmentally regulated PCD also occurs during leaf senescence. Therefore, it was expected that both *AmVPE1* and *AmVPE2* transcript levels would be significantly higher during this leaf developmental stage compared to stages in which PCD is not occurring. However, *AmVPE1* transcript levels were at their lowest of all leaf developmental stages while *AmVPE2* transcripts were not significantly higher than the other stages where PCD is not occurring (Figure 3.7 and 3.8). Based on this expression pattern, even though AmVPE1 may be involved in the early stages of PCD during perforation formation, it may not play a role in PCD during senescence. During perforation formation specific cells are supposed to undergo PCD, while during leaf senescence all the leaf cells die, AmVPE1 may be involved in regulation of PCD where only specific cells are supposed to die. Alternatively, since senescent stage leaves that were used in these experiments already displayed visible signs of PCD (appearance of yellow and brown spots on the leaves), a potential increase in *AmVPE1* transcript levels may have happened prior to the PCD signs, as observed during perforation formation. Therefore, it is still likely that AmVPE1 is involved in leaf senescence. Similar to

AmVPE1, AmVPE2 also doesn't seem to be involved in PCD during leaf senescence. Its transcript levels did not increase during the stage of leaf senescence that we used. However, leaf senescence happens over an extended time period, and since the leaves selected were at a specific stage of senescence, AmVPE2 could be involved during the other stages (possibly later stages) of leaf senescence where PCD is more rampant. Therefore, to determine whether both AmVPE1 and AmVPE2 play a role in leaf senescence, we have to track changes in their transcript levels throughout the process of leaf senescence, instead of a specific senescence stage. Since whole leaves typically have a combination of cells undergoing senescence-associated PCD and those that are still healthy, only portions of the leaves that are displaying visible signs of senescence/PCD could be used in the future research.

VPEs in other plant species have previously been shown to play a role in tissue senescence (Kinoshita et al. 1999; De Michele et al. 2009; Hoeberichts et al. 2007; Doorn et al. 2009). In *Arabidopsis*, both vegetative VPEs (gamma and alpha-VPE) had increased transcript levels during leaf senescence (Kinoshita et al. 1999). A *Medicago truncatula* VPE was also highly expressed during leaf senescence (De Michele et al. 2009), suggesting that generally VPEs (not all) are involved in leaf senescence. VPEs have also been shown to be involved in senescence of other plant organs such as flowers (Hoeberichts et al. 2007; Doorn et al. 2009). In *Lilium longiflorum*, VPEs do not seem to be involved in the early stages of flower senescence, but are involved in regulation during the final stages of flower senescence (Battelli et al. 2011). Therefore, the timing of the involvement during senescence is paramount, as was observed even during perforation

formation in the lace plant. In the lace plant, further experiments are required to determine the involvement of VPEs in leaf senescence.

Generally, AmVPE2 had higher transcript levels in leaves than AmVPE1. Therefore, it seems to be the more dominant VPE in lace plant leaf tissue, and its transcript expression pattern suggests it plays a more significant role during execution of perforation formation-associated PCD in the lace plant. Even though AmVPE1 transcripts were mainly significantly higher during the preperforation stage of leaf development, during the window stage its transcript levels were higher in PCD cells than NPCD cells (Figure 3.11). Suggesting that it still plays a role during the later stages of PCD. AmVPE2 transcript levels were also higher in the PCD cells compared to NPCD cells. Given its significantly higher ($P < 0.05$) transcript levels in window stage leaves and its possible involvement during late stages of PCD, its higher transcript levels in PCD cells was expected.

Although the transcript level evidence is compelling, due to the potential post-translational regulation of VPEs, we investigated the activity levels of the VPEs within the two cell types. VPE activity during lace plant PCD was detected using a VPE-activity based probe AMS-101. The activity-based probe is an inhibitor tagged with a reporter (BOPIDY fluorescent reporter) and reacts with VPE active-site residues (Cravatt et al. 2008; Edgington et al. 2011; Misas-Villami et al. 2013). VPE activity is determined by fluorescent labeling, because it implies the availability and reactivity of VPE active sites (Misas-Villami et al. 2013). Misas-Villami et al. (2013) demonstrated that AMS101 does label VPE-like proteins in leaf tissue from various species, including various dicots

(tobacco, tomato, lettuce and parsley) and monocots (maize and barley). They discussed that the signal intensities and label efficiencies varied among plant species.

Initially, whole lace plant leaves at the pre-perforation, window and mature stages were exposed to probe for 24-48 hours and assayed. Due to permeability issues, the probe did not penetrate whole leaves (results not shown). Dissecting leaf tissue to 1 cm^2 squares also did not significantly improve labeling results. Tissue could not be ground since it was important for tissue to be alive during incubation. Even though AMS101 is cell permeable, it is not known whether the process is passive or active. Lace plant protoplasts were isolated and exposed to 2 μM of the probe for 6 hours. VPE activity was observed in protoplasts from PCD and NPCD (Figure 3.12), through probe fluorescence. The activity was observed in the vacuoles of the protoplasts, where VPEs are known to be active. The VPE activity and fluorescence was higher in PCD cell protoplasts compared to NPCD protoplasts (Figure 3.12). This provided more evidence for the involvement of VPEs and VPE activity in lace plant PCD during perforation formation, and more support for the possible central role of VPEs in mediating PCD involving vacuolar rupture. VPEs are thought to mediate the maturation of hydrolytic enzymes within the vacuole, which degrade the tonoplast and initiate a proteolytic executing-PCD cascade (Guicciardi et al. 2004; reviewed in Hatsugai et al. 2015). Tonoplast rupture is one of the hallmarks of PCD in the lace plant, and it marks the beginning of rapid cell deterioration (Wright et al. 2009). The identification of high VPE activity in PCD cells supports the aforementioned role of VPEs during lace plant PCD. It also highlights their importance as key regulators and executors of PCD during perforation formation in the lace plant. Within the vacuole, anthocyanin also emits autoflourescence that is similar to that produced by the probe,

therefore the VPE probe results could include anthocyanin flourescence too. Therefore, more replicates with and without anthocyanin are needed to confirm the VPE activity results. The flourescence should also be quantified. In addition, VPE protein levels could be measured to determine if protein levels differ during the different stages of leaf development or between PCD versus NPCD cells.

3.6 Conclusions and Further Work

The isolated lace plant VPEs are part of a multi-gene family, the rest of the lace plant VPEs need to be isolated. Lace plant genome most likely posseses seed type VPEs as well. Lace plant vegetative type VPEs appear to play a role in developmentally regulated PCD during leaf morphogenesis. This developmental PCD example provides new insight regarding involvement of VPEs in the sculpting of leaves. AmVPE1 plays a role during this early PCD process, while AmVPE2 plays a role during later stages. They possibly cleave, activate or degrade different substrates as required during different stages of PCD. It would be of interest to determine the natural substrates cleaved by each of these VPEs and determine the role of these substrates during PCD. The natural substrates would provide insight into whether AmVPE1 is also involved during the signaling or regulation phase of PCD, since its transcript levels increase early. The ability to transform the lace plant would also be important in order to study the effects of changes in VPE expression through knockout or over-expression lines. It would provide more understanding of the regulation and execution of PCD in the unique developmental PCD system of the lace plant. Studying the interaction of VPEs and other components involved within the plant PCD cascade such as plant hormones and transcription factors

would also provide much needed insight into how the components work together to orchestrate PCD.

Figure 3.1 The Lace Plant

The lace plant (A) is an aquatic monocot that forms perforations on its leaves through developmentally regulated PCD. It can be grown in sterile conditions in Magenta boxes (B). Early in development, lace plant leaves do not show any signs of perforation formation (C). Eventually cells at the center of a perforation site (PCD cells) undergo PCD, while cells 4-5 layers from the vascular tissue (NPCD cells) remain alive (D). Once the PCD cells have disintegrated, actual holes are formed and perforations result (E). Scale bars (A) = 1.25 cm, B = 0.92 cm, C = 40 μm, D = 100 μm and E = 150 μm.

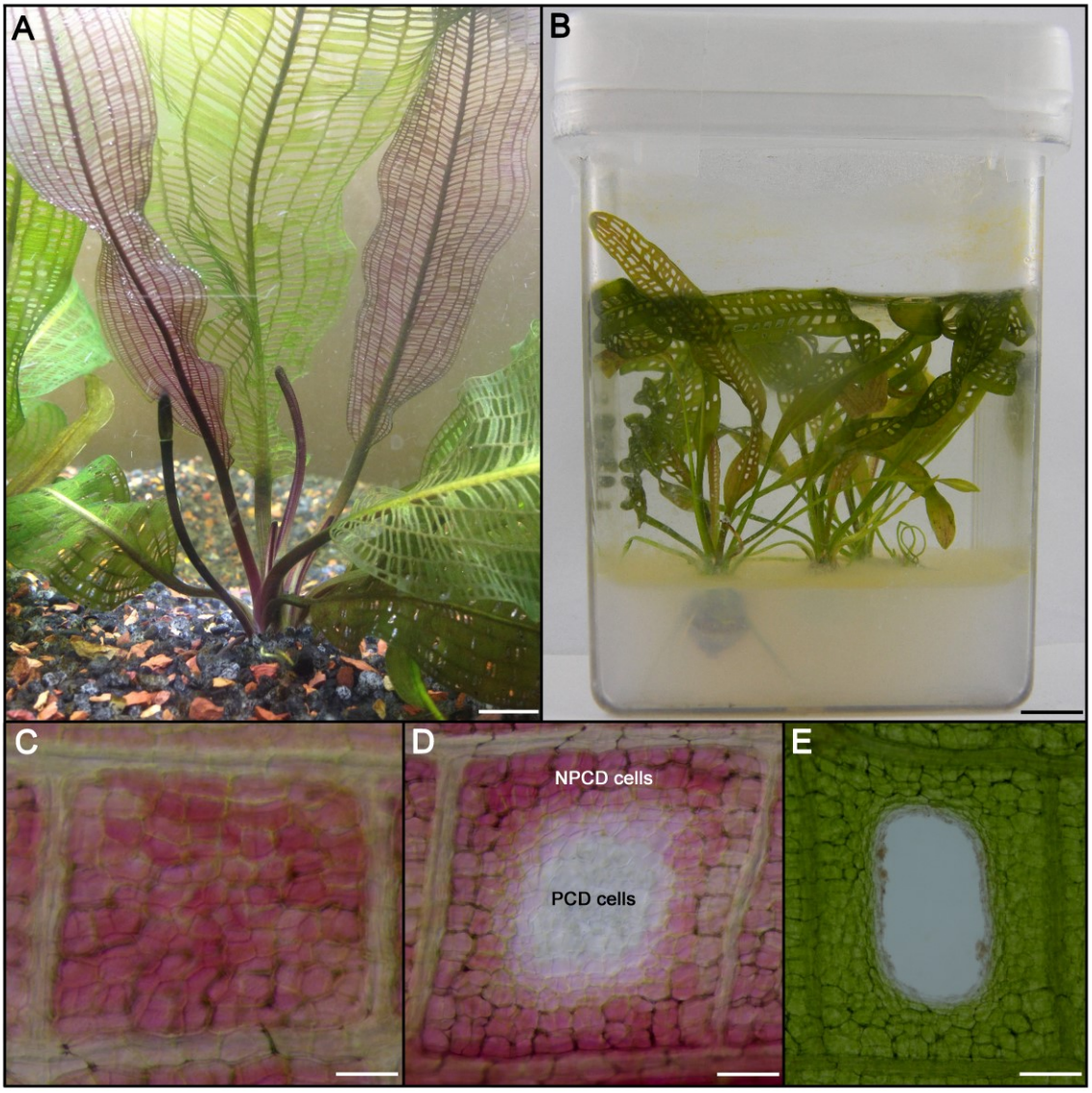

Figure 3.1 The Lace Plant

Figure 3.2 Separating PCD and NPCD Cells Using a Zeiss PALM Laser Capture Microdissection and Imaging System

A free hand line was drawn between the two types of cells (A). The line was followed by a laser, which cut through the leaf blade (B) and separated PCD cells from NPCD cells. NPCD cells were also collected in a similar manner (C).

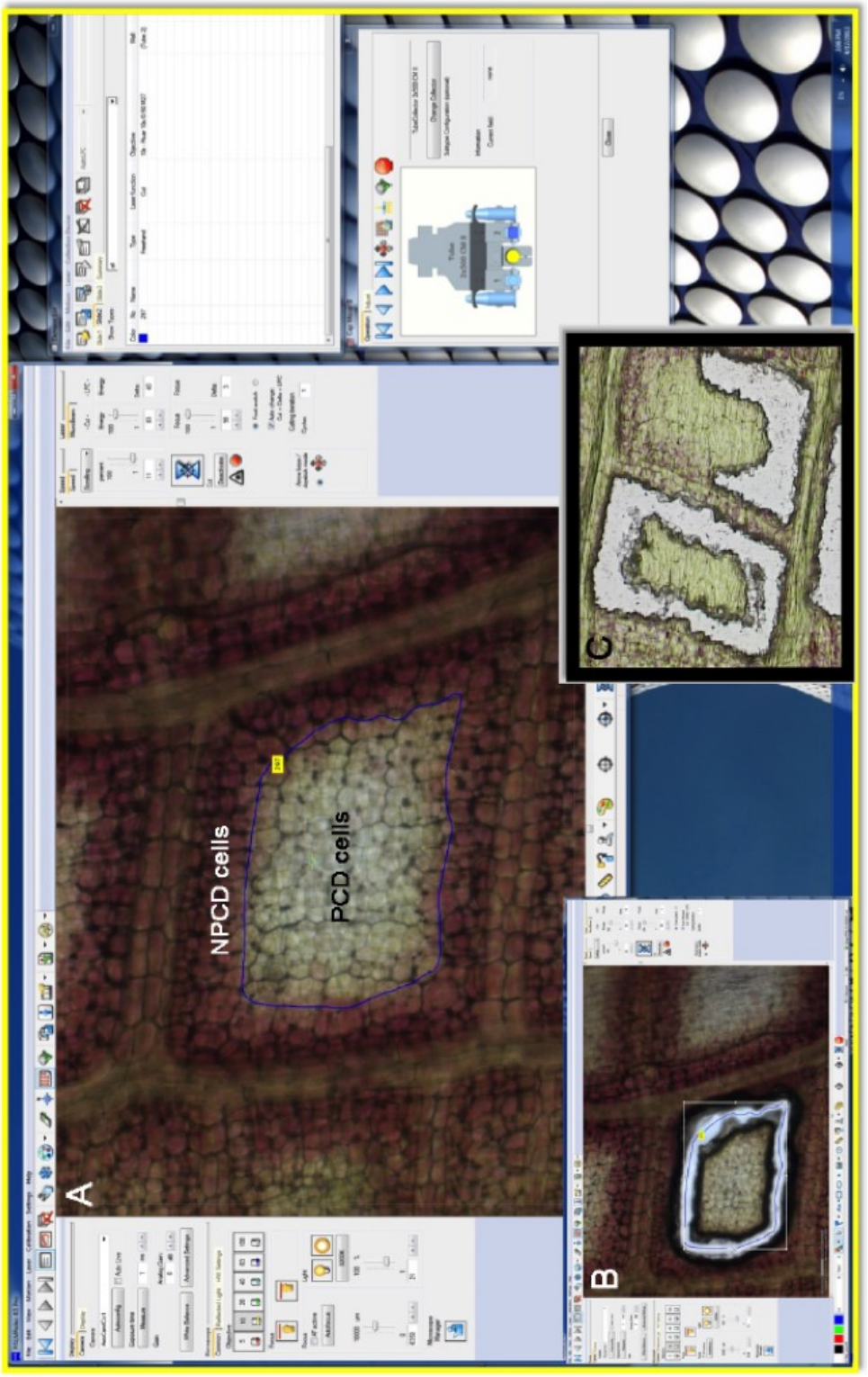

Figure 3.2 Separating PCD and NPCD Cells Using a Zeiss PALM Laser Capture Microdissection and Imaging System

Figure 3.3 Lace Plant VPE Amino Acid Fragments (AmVPE1 and AmVPE2) Aligned With *Arabidopsis* Vegetative (AtVPE-gamma and AtVPE-alpha) and Seed (AtVPE-beta and AtVPE-delta) Type VPEs

Domains and essential amino acids such as the mature protease domain (blue), essential amino acids (Cys76, His165 and Cys215; red), N-glycosylation site (spring green) and the C-terminal propeptide (orange) were identified. Dots represent amino acid identity and dashes indicate gaps. At the end of each sequence, its percentage identity and similarity with either AmVPE1 or AmVPE2 are indicated. Accession numbers: AtVPE-gamma (BAA018924.1), AtVPE-alpha (BAA09614.2), AtVPE-beta (BAA09615.1) and AtVPE-delta (BAC65233.1).

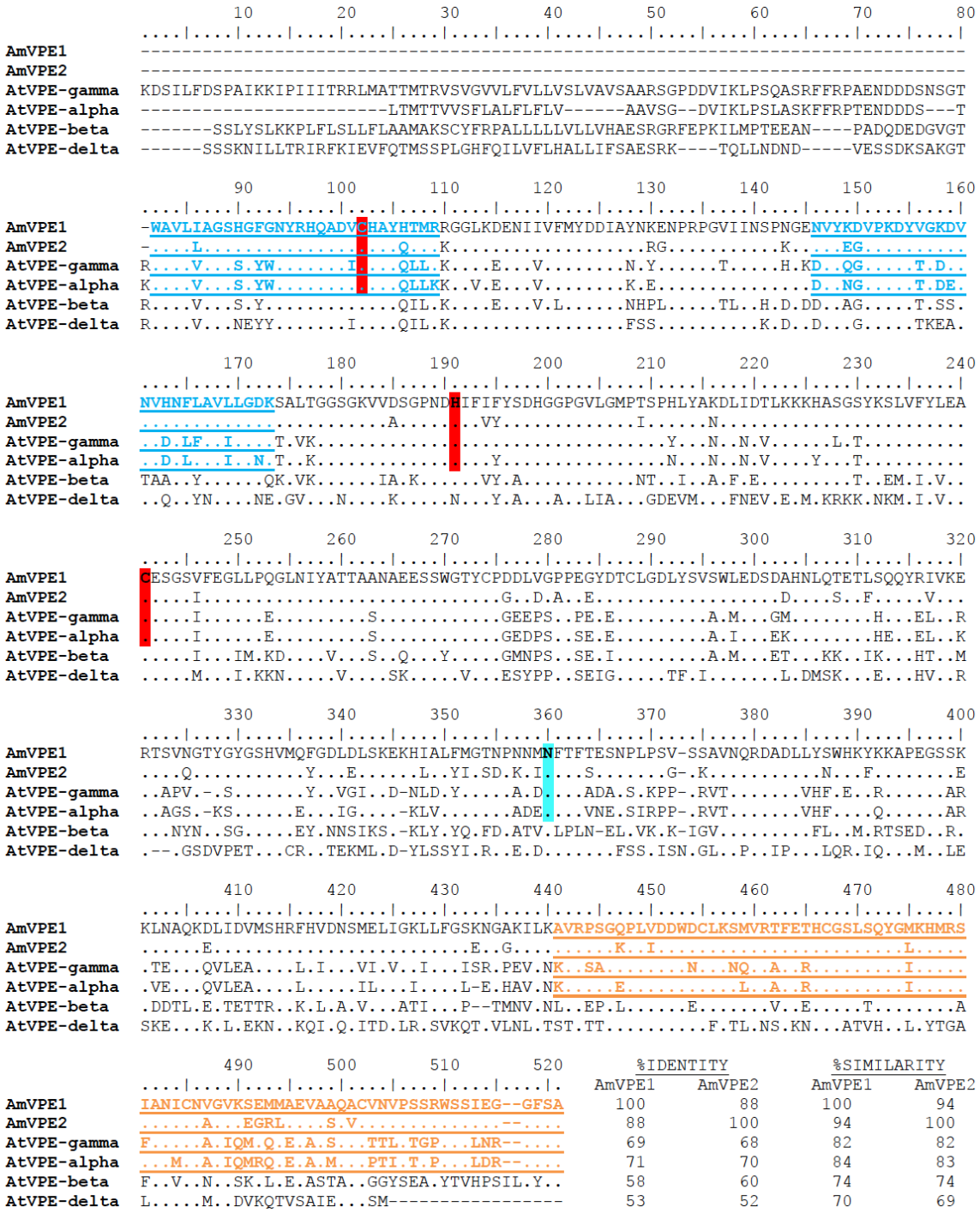

Figure 3.3 Lace Plant VPE Amino Acid Fragments (AmVPE1 and AmVPE2) Aligned With *Arabidopsis* Vegetative (AtVPE-gamma and AtVPE-alpha) and Seed (AtVPE-beta and AtVPE-delta) Type VPEs

Figure 3.4 Phylogenetic Analysis of Vegetative and Seed Type VPEs From Different Plant Species Highlighting the Relationship of the VPEs With Lace Plant VPEs

Rice asparagine-specific endopeptidase (NP_910213) was used as out-group in the neighbor-joining tree. The vegetative VPEs from the different species (including lace plant) formed their own clade (highlighted in orange) away from the seed type VPEs (blue). Bar represents the gap separation distance, and the bootstrap values (from a thousand replicates) are indicated above or below each node. Accession numbers of each amino acid sequence are provided in parentheses.

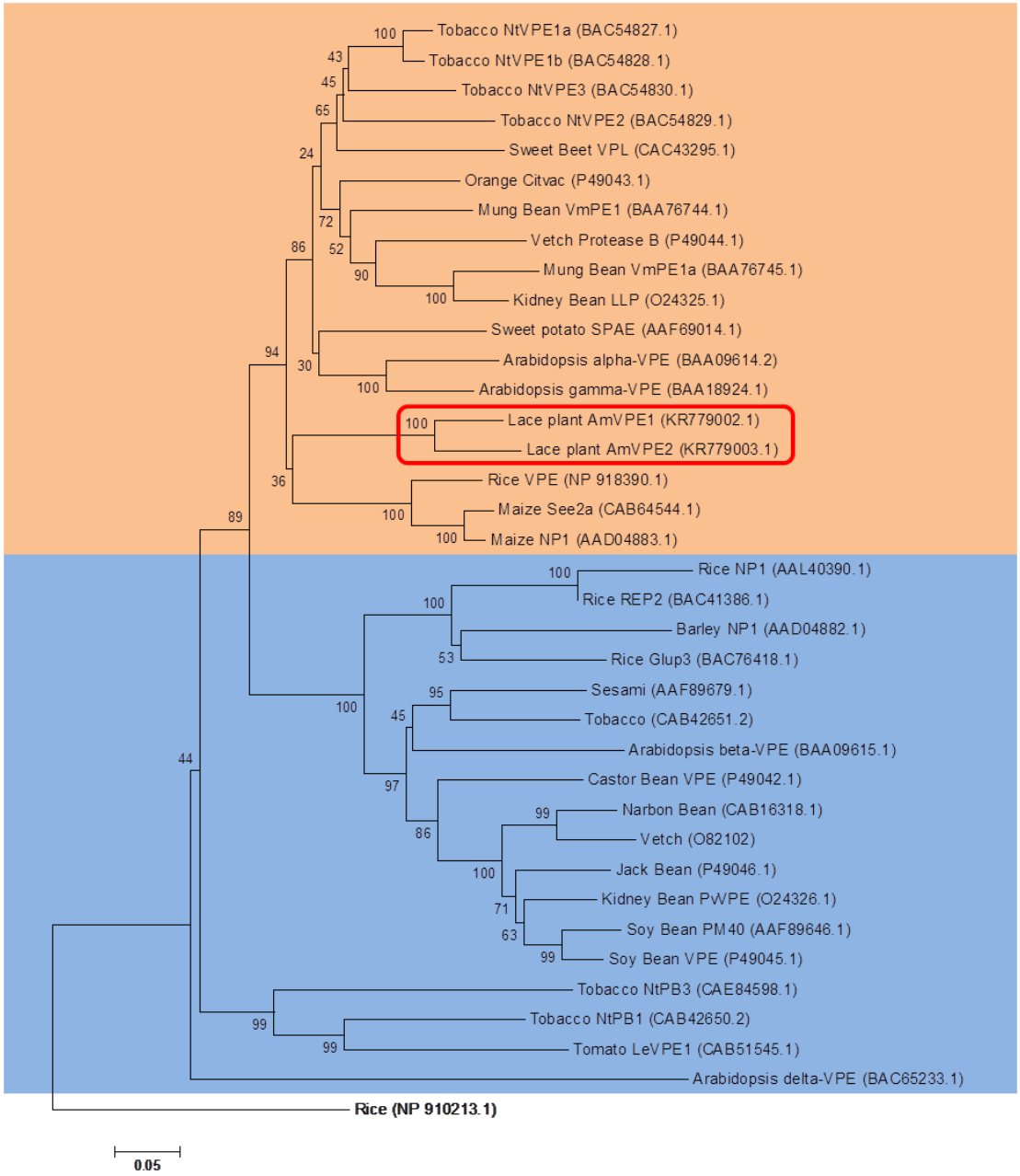

Figure 3.4 Phylogenetic Analysis of Vegetative and Seed Type VPEs From Different Plant Species Highlighting the Relationship of the VPEs With Lace Plant VPEs

Figure 3.5 Detection of *AmVPE1* and *AmVPE2* Transcripts Through RT-PCR

The transcripts were detected in RNA extracted from leaves at different stages of development. Lace plant Actin, *AmActin*, was used as a control and was detected in RNA from all leaf developmental stages. Early preperforation (EPP), preperforation (PP), early window (EW), window (W), late window (LW), mature (M) and senescence (S).

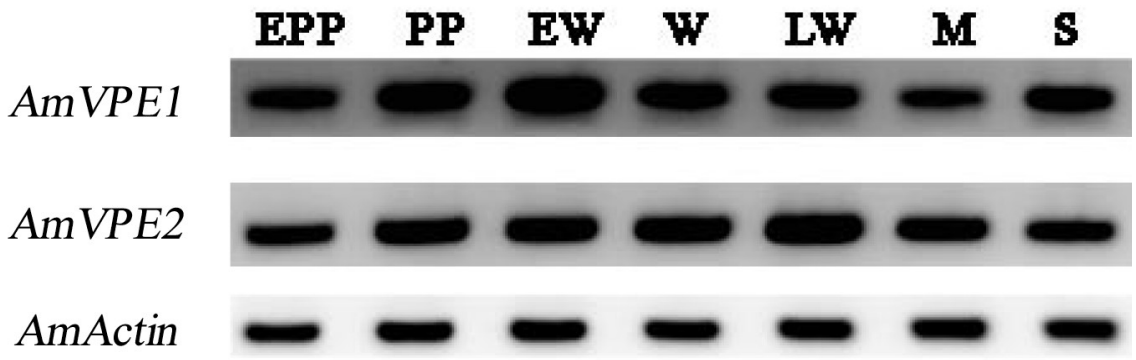

Figure 3.5 Detection of *AmVPE1* and *AmVPE2* Transcripts Through RT-PCR

Figure 3.6 *AmVPE1* and *AmVPE2* Transcripts Detected Through Gradient RT-PCR, Using Exon-Intron Primers

Primer annealing was performed at a gradient (52ºC – 60ºC) of temperatures. The *AmVPE1* and *AmVPE2* transcripts were detected in cDNA (C) but not in genomic DNA (G).

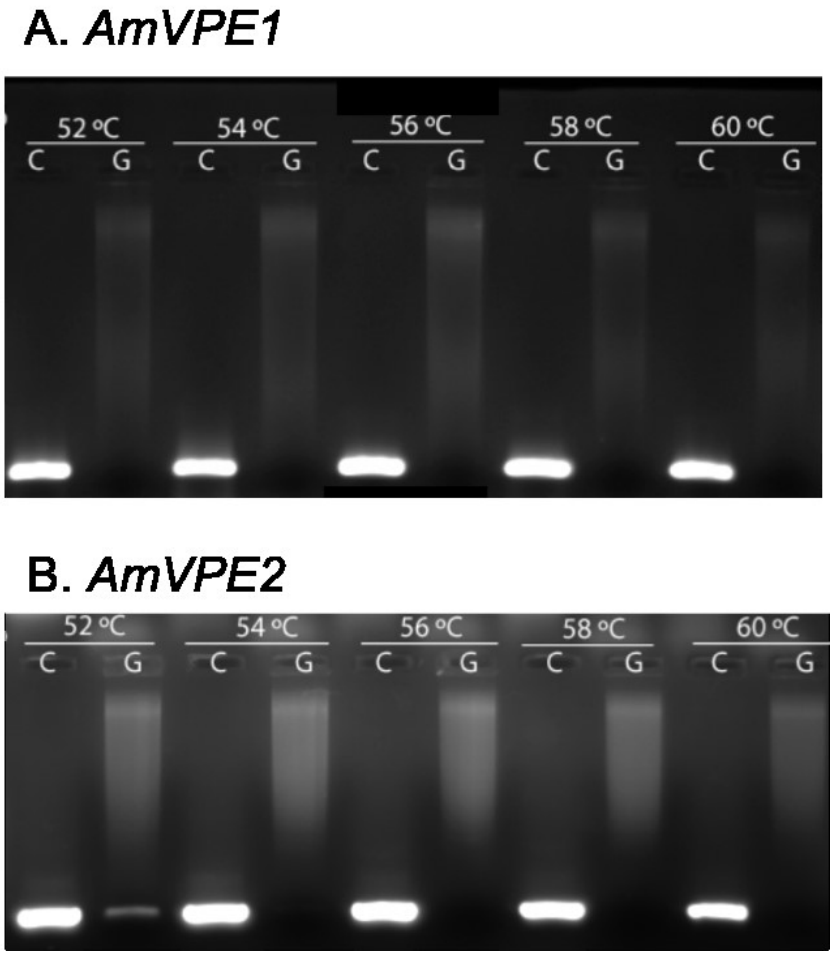

Figure 3.6 *AmVPE1* and *AmVPE2* Transcripts Detected Through Gradient RT-PCR, Using Exon-Intron Primers

Figure 3.7 Mean Normalized *AmVPE1* Transcript Levels in Different Stages of Leaf Development.

AmVPE1 transcript levels were significantly higher ($P<0.05$) in preperforation stage leaves (PP) compared to all the other stages of leaf development. This leaf developmental stage is immediately prior to visible signs of perforation formation and PCD. Late window (LW), mature (M) and senescence stages had the lowest *AmVPE1* transcript levels. Means with the same letters are not significantly different ($P > 0.05$). Bars represent SE ($n \geq 12$). Early preperforation (EPP), preperforation (PP), early window (EW), window (W), late window (LW), mature (M) and senescence (S).

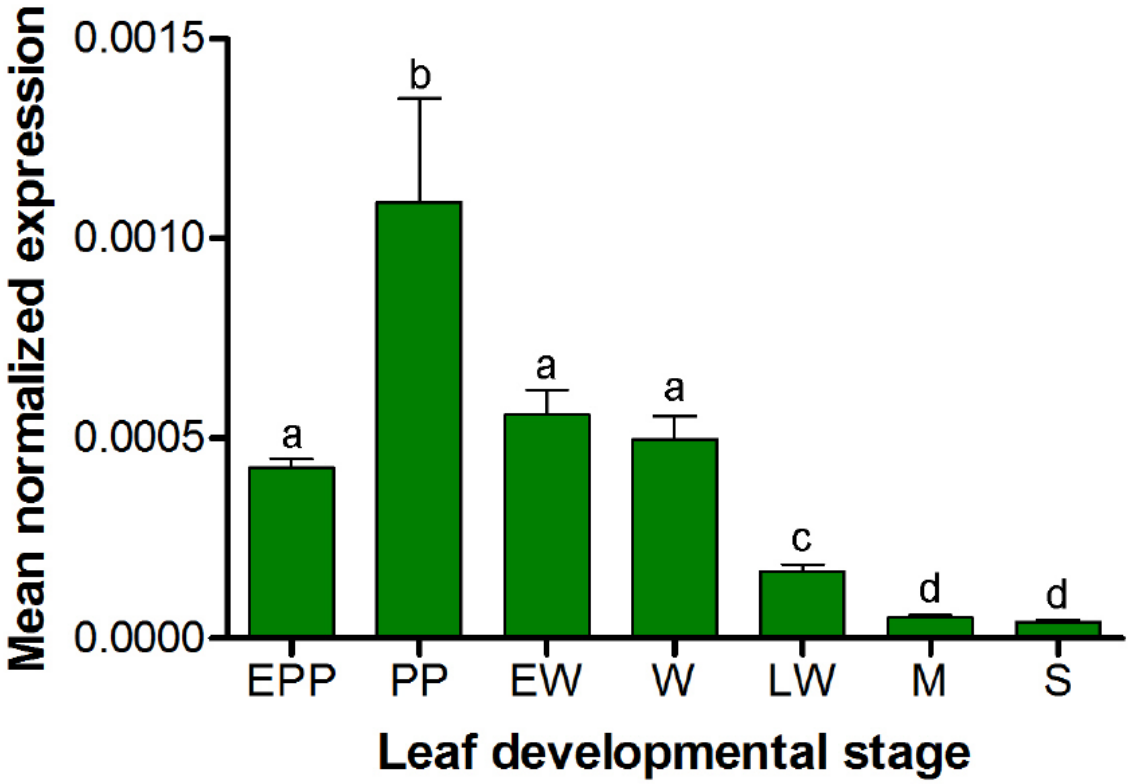

Figure 3.7 Mean Normalized *AmVPE1* Transcript Levels in Different Stages of Leaf Development

Figure 3.8 Mean Normalized *AmVPE2* Transcript Levels in Different Stages of Leaf Development.

The AmVPE2 transcripts were significantly higher ($P<0.05$) in window (W) and late window (LW) stages of leaf development, compared to the other stages. Perforation formation and PCD are occurring during the window and late window stages of leaf development. Means with the same letters are not significantly different ($P > 0.05$). Bars represent SE ($n \geq 12$). Early preperforation (EPP), preperforation (PP), early window (EW), window (W), late window (LW), mature (M) and senescence (S).

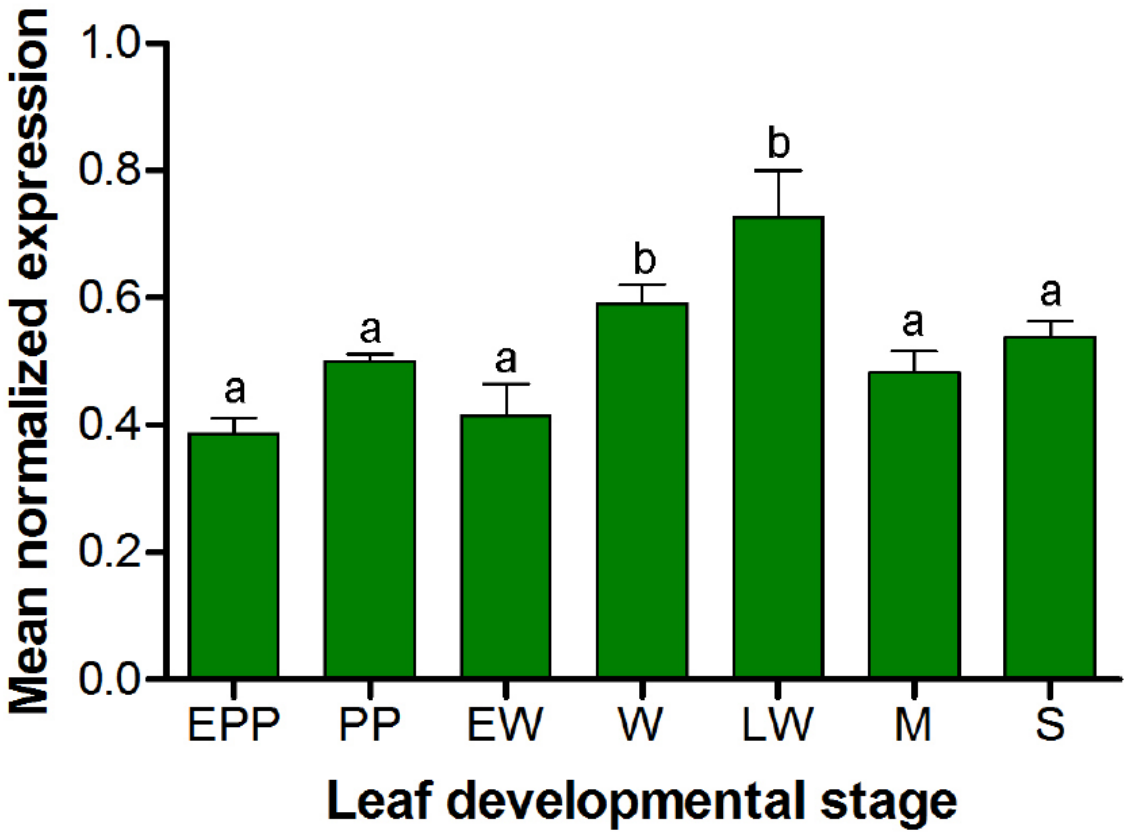

Figure 3.8 Mean Normalized *AmVPE2* Transcript Levels in Different Stages of Leaf Development.

Figure 3.9 *AmActin* Transcript Levels in Different Stages of Leaf Development

AmActin transcripts were constitutively expressed throughout leaf development. Means with the same letters are not significantly different ($P > 0.05$). Bars represent SE ($n \geq 12$). Early preperforation (EPP), preperforation (PP), early window (EW), window (W), late window (LW), mature (M) and senescence (S).

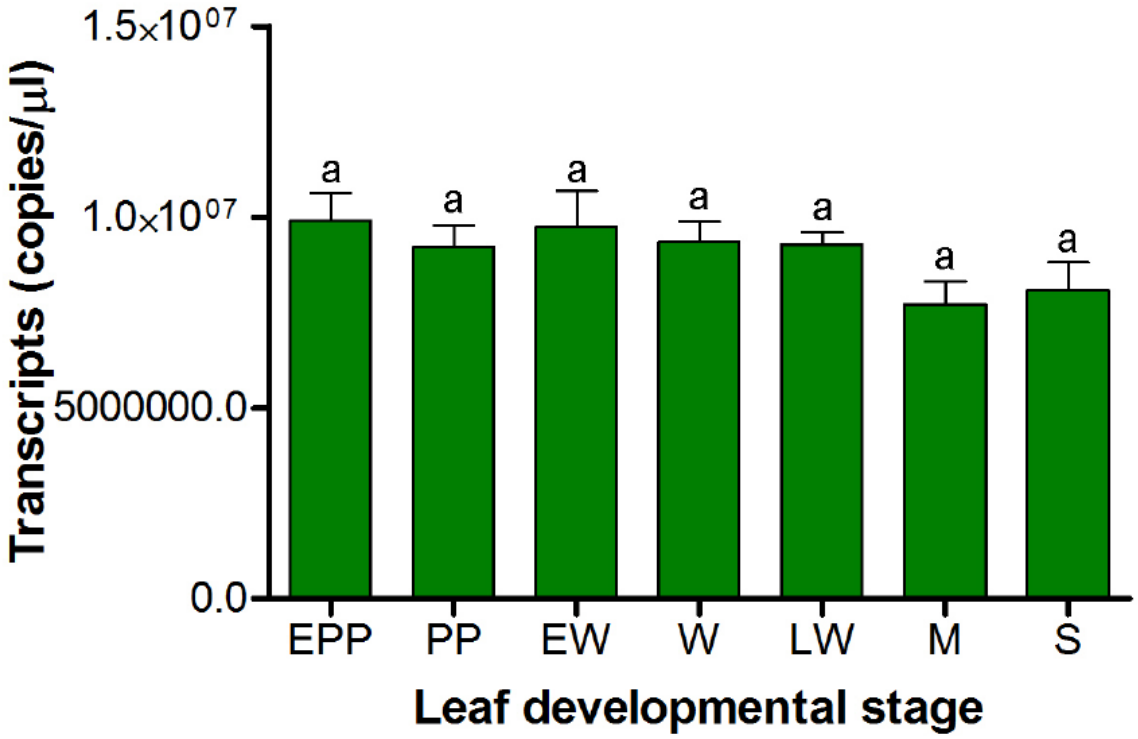

Figure 3.9 *AmActin* Transcript Levels in Different Stages of Leaf Development

Figure 3.10 RT-PCR Used to Detect *AmVPE1* and *AmVPE2* Transcripts

The transcripts were detected in RNA extracted from dying cells (PCD cells; P) and cells not undergoing PCD (NPCD cells; N). Lace plant Actin, *AmActin*, was also detected in the RNA from the two types of cells.

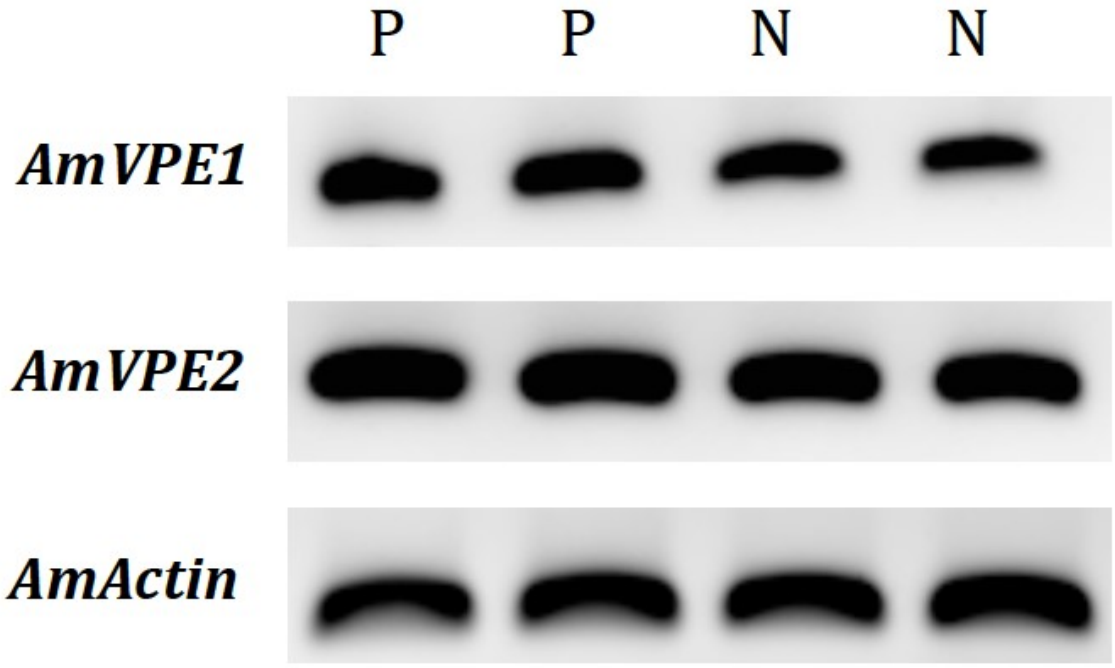

Figure 3.10 RT-PCR Used to Detect *AmVPE1* and *AmVPE2* Transcripts

Figure 3.11 Mean Normalized *AmVPE1* and *AmVPE2* Transcript Levels in Dying (PCD) Versus Cells not Undergoing PCD (NPCD Cells)

Both *AmVPE1* and *AmVPE2* had significantly higher ($P<0.05$) transcript levels in PCD than NPCD cells. *AmActin* transcript levels were not significantly different between PCD and NPCD cells. Means with the same letters are not significantly different ($P > 0.05$). Bars represent SE ($n \geq 8$).

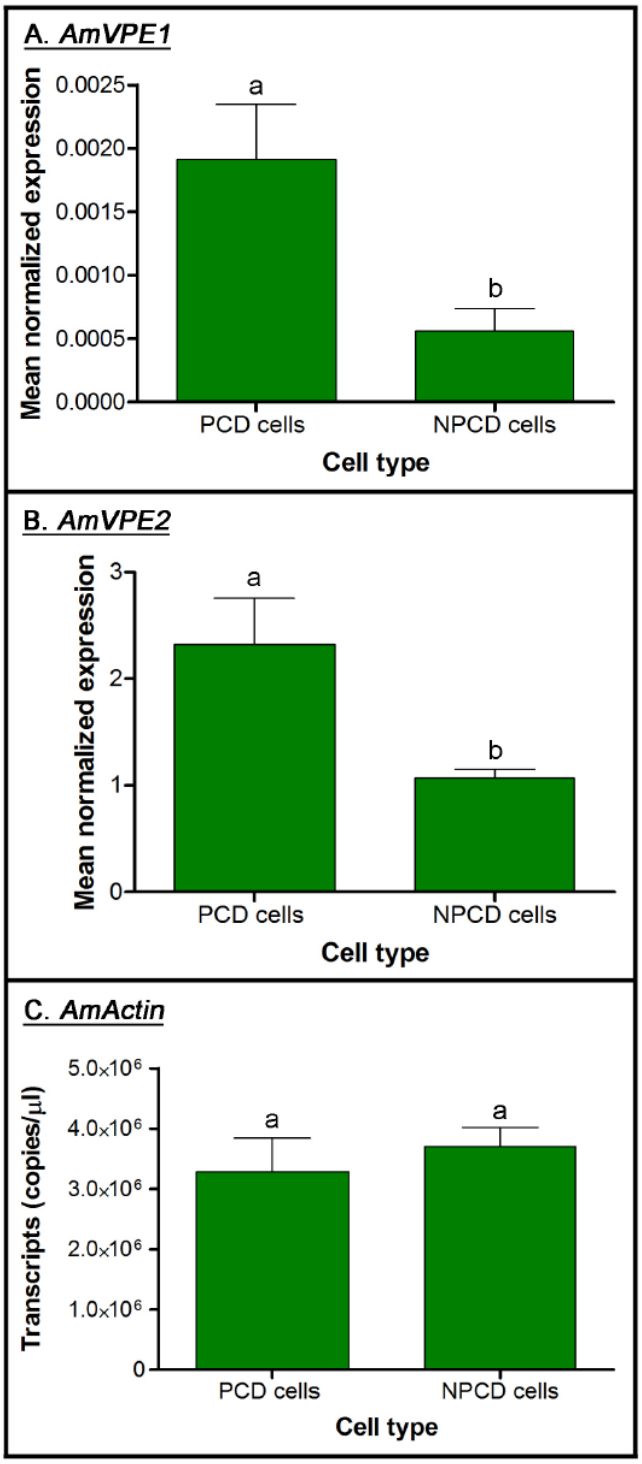

Figure 3.11 Mean Normalized *AmVPE1* and *AmVPE2* Transcript Levels in Dying (PCD) Versus Cells not Undergoing PCD (NPCD Cells)

Figure 3.12 VPE Activity Analysis in Protoplasts From PCD and NPCD Cells

VPE activity was detected through an activity-based probe AMS-101. More fluorescence and VPE activity was detected in PCD protoplast compared to the NPCD protoplasts from early window stage leaves. The fluorescence is localized within the vacuole, where active VPEs are localized. Chloroplasts are produced autoflourescence that is unrelated to the probe. BF = Bright field and FL = Fluorescence. All scale bars = 10 μm.

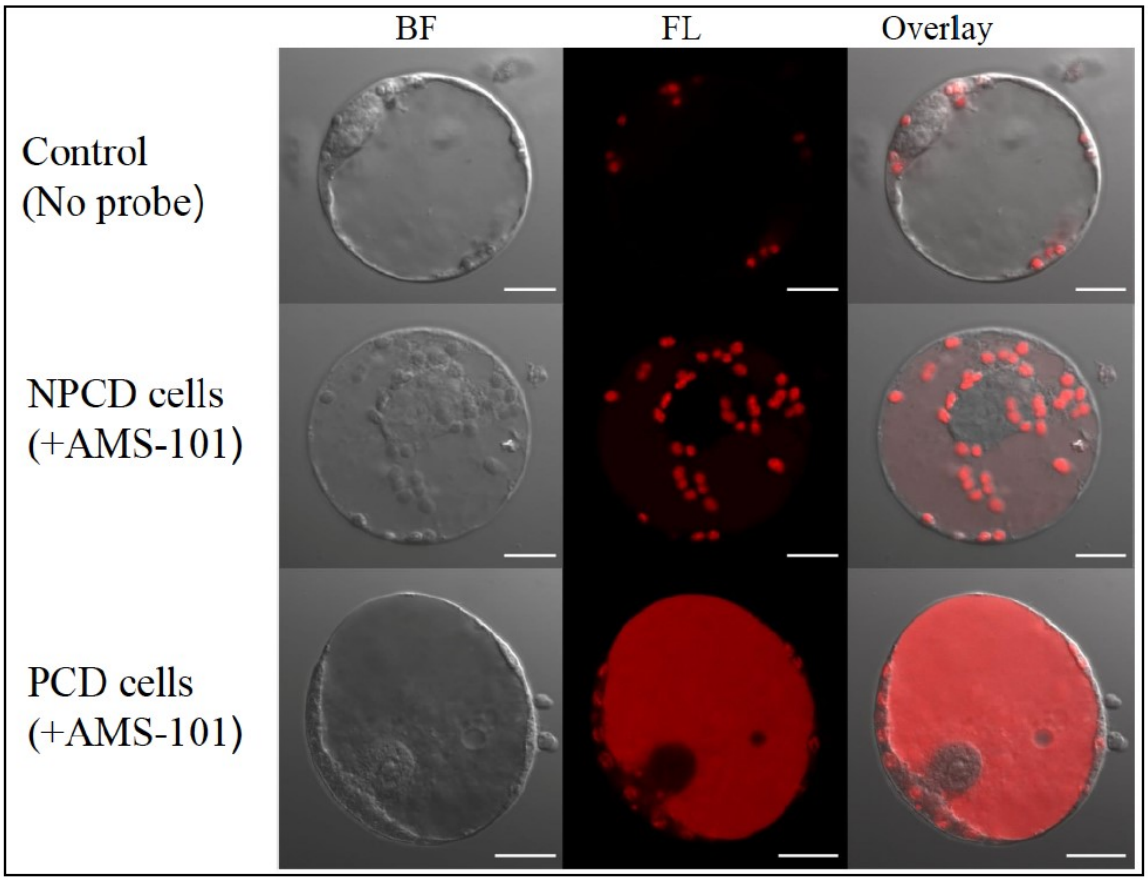

Figure 3.12 VPE Activity Analysis in Protoplasts From PCD and NPCD Cells

Chapter 4 The Effect of Ethylene on Vacuolar Processing Enzyme Transcript Expression During Developmentally Regulated Programmed Cell Death in Lace Plant *(Aponogeton Madagascariensis)*

Most of the content in this chapter is included in the MS that will be submitted as:

Rantong G, Gunawardena AHLAN. Vacuolar processing enzymes, AmVPE1 and AmVPE2, as potential executors of ethylene regulated programmed cell death in the lace plant (*Aponogeton madagascariensis*). *Journal of Experimental Botany*

Author contributions

GR designed and carried out all experiments. GR wrote the first draft of the manuscript. AHLANG, conceived the study, participated in its design and coordination, and helped in drafting and revising the manuscript as well as supervised all experimental work.

4.1 Abstract

Aponogeton madagascariensis (lace plant), an aquatic monocot, forms perforations on its leaves during normal development. Formation of these perforations occurs through developmentally regulated programmed cell death (PCD). The lace plant is an excellent model for studying developmentally regulated PCD in plants because the process occurs at highly predictable areas, and in cells that are easily distinguishable. In addition, the nearly transparent leaves of the lace plant are only 4-5 cell layers thick and ideal for microscopy. Morphological changes that occur during PCD in the lace plant are well studied, but the molecular mechanisms involved are still elusive. Ethylene is involved in a respiratory climacteric-like pattern during signalling in this PCD system, and its receptors play a role in determining cell fate during developmentally regulated PCD in the lace plant. Two lace plant vacuolar processing enzymes (VPEs; *AmVPE1* and *AmVPE2*) appear to be involved in execution of PCD in the lace plant. VPEs are plant caspase-1 like enzymes that play a role during the execution phase of plant PCD. We investigated the effect of ethylene on the transcript expression of the lace plant VPEs to determine their relationship within the lace plant PCD cascade. Lace plants were treated with an ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG), which has been shown to inhibit PCD and perforation formation. VPE transcript levels were monitored during 6 stages of leaf development in AVG treated and control plants to determine the effect of limited ethylene biosynthesis on VPE transcript and execution of PCD. As previously observed, treatment of lace plants with ethylene biosynthesis inhibitor AVG inhibited perforation formation and PCD. AVG treatment also inhibited increases in VPE transcript levels, which appear to be necessary for PCD and perforation formation to

occur. In control plants, VPE transcript levels increase immediately prior to and during visible signs of PCD and perforation formation. In AVG treated plants however, the VPE transcript levels do not increase and perforations do not form. These results suggest that ethylene is required to stimulate the increases in transcription of VPEs during the execution of PCD in perforation formation.

4.2 Introduction

Programmed cell death (PCD) is a physiological cell demise mechanism coordinated genetically in multicellular organisms for development, survival and response to stress conditions. In plants, PCD is involved during response to biotic and abiotic stresses and development. Developmental processes that employ PCD include death of the embryonic suspensor (Lombardi et al. 2007), root development (Wang et al. 1996; Shishkova and Dubrovsky 2005), xylem differentiation (Groover and Jones 1999; Fukuda, 2000), reproductive development (Senatore et al. 2009; Zhang et al. 2013), perforation formation (Gunawardena et al. 2004; Wright et al. 2009; Wertman et al. 2012), aerenchyma formation (Gunawardena et al. 2001; Lenochová et al. 2009), leaf senescence (Kinoshita et al. 1999), and flower senescence (Wang et al. 2013). Some of the components involved in signaling, regulation and execution of plant PCD have been identified (reviewed in Rantong and Gunawardena 2015). Ethylene, a phytohormone, has been shown to play a regulatory role in several plant PCD processes such as the hypersensitive response (HR; Liu et al. 2008; Mur et al. 2009), endosperm development (Young et al. 1997), tissue senescence (Reid and Wu 1992; Jing et al. 2002), and perforation formation in the lace plant (Dauphinee et al. 2012). Of the above mentioned ethylene-regulated PCD processes, plant cysteine proteinases possessing caspase-1 like

activities known as vacuolar processing enzymes (VPEs) have been shown to be involved in the HR (Iakimova et al. 2013), embryogenesis (Hara-Nishimura et al. 2005) leaf and flower senescence (Kinoshita et al. 1999; Wang et al. 2013), and floral bud abortion (Zang et al. 2013; Cerveny and Miller 2010). Other plant processes that involve both ethylene and VPEs include fruit ripening (Alonso and Cranell 1999), and response to stress conditions such as wounding (Kinoshita et al. 1999), water stress (Apelbaum and Yang 1981; Albertini et al. 2014) and salt stress (Deng et al. 2011; Kim et al. 2014; reviewed in Cao et al. 2008).

In the lace plant (*Aponogeton madagascariensis*) ethylene has been shown to play a role in leaf development via PCD during perforation formation (Dauphinee et al. 2012). The lace plant forms leaf perforations at highly predictable areas and during a predictable stage of leaf development (Gunawardena et al. 2004). The perforations form through developmentally regulated PCD. Ethylene production peaks during the formation of perforations, and treatment of plants with ethylene biosynthesis inhibitors aminoethoxyvinylglycine (AVG) inhibits PCD and therefore reduces the number of perforations on leaves. The inhibition of PCD and perforation formation in AVG treated plants is reversible through the addition of an ethylene precursor and biosynthesis enhancer 1-aminocyclopropane-1-carboxylic acid (ACC; Dauphinee et al. 2012). The results suggest that ethylene is required for PCD during perforation formation in the lace plant. VPEs also appear to be involved in lace plant PCD. Prior to visible signs of PCD and perforation formation, transcript levels of lace plant VPE, AmVPE1 increase (unpublished data, discussed in section 3.4.2 of Chapter 3). Transcript levels of another lace plant VPE, AmVPE2 increase later (window and late window stage) during

formation of perforations through PCD. Transcript levels of both lace plant VPEs are also higher in cells that are undergoing PCD during perforation formation (PCD cells) compared to those that remain alive (NPCD cells). These results correlate with findings by Lord et al. (2013), who found that caspase-1 activity (which is attributed to VPEs in other plant species) is required for PCD to occur in the lace plant, and that this activity is high immediately prior to visible signs of PCD (preperforation stage) and also during occurrence of observable PCD characteristics (window stage).

In the plant PCD cascade, ethylene is thought to be within the regulation phase of PCD and upstream of VPEs (reviewed in Rantong and Gunawardena 2015). If this is the case in the lace plant PCD cascade, changes in ethylene biosynthesis should affect VPE transcript levels. During ripening in citrus fruits, has been shown to increase VPE transcript levels (Kinoshita et al. 1999). Similarly, apple leaves that were infected and treated with ethylene biosynthesis inhibitor had a reduced ethylene production and an inhibited HR associated PCD (Iakimova et al. 2013). Iakimova et al. (2013) hypothesized that ethylene might be required to stimulate transcription of VPEs during HR-associated PCD. Treatment with ethylene has been shown to result in increases in VPE transcript level during plant defense against microbes (Liu et al. 2005). These VPE mRNA increases are thought to be necessary for maturation of defense-related proteins and their accumulation within lytic vacuoles (Liu et al. 2005; Iakimova et al. 2013). Even though links between ethylene and VPEs during PCD have been studied in the HR, to our knowledge no studies have been performed investigating their relationship during developmentally regulated PCD. In this study we aimed to investigate if ethylene affects VPE transcript expression during developmentally regulated PCD in the lace plant.

4.3 Materials and Methods

4.3.1 Plant Material

All plants used in this study were gown under sterile conditions in Magenta G47 boxes, according to Gunawardena et al. (2006). Corms of similar sizes were embedded in microbe-free Murashige and Skoog (MS) solid medium (50 mL) and covered with 200 mL of liquid MS. Plants were grown under a 12 h light/12 h dark cycle and room temperature of 24 °C. The light of 125 μmol m^{-2} s^{-1} intensity was provided by daylight simulating fluorescent light bulbs (F32T8/DX; Philips Electronics Ltd., Markham, Ontario).

4.3.2 AVG Treatment

AVG treatments were performed as described in Dauphinee et al. (2012) (Appendix B). In addition to the tissue collected from Dauphinee et al. (2012) experiments, fifty more plants grown in the Magenta boxes for 4 to 6 weeks (with similar corm sizes, and about 3-4 perforated leaves) were used for AVG experiments. Twenty-five of the plants were randomly selected and treated with 5 μmol/L AVG (Sigma Aldrich, St. Louis, Missouri, USA). An equivalent amount of water was added in the other 25 plants as controls. The plants were grown under the above-mentioned conditions for an additional 3 weeks. Experimental progress and leaves produced after treatments were monitored through photography. Leaf tissues were harvested from the healthy looking plants at the end of the 3 weeks for RNA extraction. The leaves were separated into the 6 stages of leaf development; early preperforation (EPP), preperforation (PP),

early window (EW), window (W), late window (LW) and mature (M). They possessed the same characteristics as described in section 2.2.5 of Chapter 2.

4.3.3 RNA Extraction

RNA from each sample was extracted as described in section 3.3.2.1 of Chapter 3. RNA was extracted from samples collected from 2 independent experiments. At least 3 different leaves were used in each RNA sample. A minimum of 4 independent RNA samples per leaf developmental stage (24 independent samples in total) was used. Half of the RNA samples were extracted from tissue collected from Dauphinee et al. 2012 (Appendix B) experiments. The RNA was treated with DNase 1 before used in cDNA synthesis.

4.3.4 cDNA Synthesis

cDNA was synthesized was carried out as described in section 3.3.2.2 of Chapter 3.

4.3.5 VPE Transcript Levels Throughout Leaf Development

RT-PCR was used to confirm the quality of cDNA produced and to amplify the expected fragment of *AmActin*. It was performed as described in section 3.3.5 of Chapter 3 and using the same primer pair. Transcript levels of *AmVPE1*, *AmVPE2* and *AmActin* were determined through q-PCR as described in section 3.3.5 of Chapter 3.

4.3.6 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 5.00 (San Diego, California, USA. A 2-way ANOVA with no repeated measures, followed by

Bonferroni post-test, was used to determine significant differences in transcript levels. Data was determined to be statistically significant if $P < 0.05$.

4.4 Results

4.4.1 Leaf Morphology

The leaf morphology results were consistent with those obtained in Dauphinee et al. 2012 (Appendix B). Plants treated with AVG and control plants produced about 3-4 new leaves after treatment (Figure 4.1A and B). At the time of treatment they had 3-4 already perforated leaves. All leaves produced after treatment were excised, harvested and categorized into one of the 6 leaf developmental stages (Figure 4.1C). Representative leaf layouts were assembled to highlight leaf morphological differences at the 6 stages of development and between treatments (Figure 4.1D and E). Within the leaf layouts, leaf numbers 1-6 were produced after treatments, while leaves 7 and 8 were produced (and already perforated) prior to treatment (Figure 4.1D and E). As observed in Dauphinee et al. 2012, leaves from the AVG treated plants had fewer perforations as compared to the leaves from the controls. In most cases, the leaves from AVG treated plants had no perforations at all. The leaves from the control and AVG treatment looked identical in all aspects, expect for the absence of (or fewer) perforations in leaves from the AVG treated plants. The two leaves produced prior to treatment (leaves 7 and 8) had a similar number of perforations in both control and AVG treated plants (Figure 4.1D and E).

4.4.2 Detection of Transcripts Through RT-PCR

The RNA extracted from the leaves of AVG treated and control plants was intact (Figure 4.2). *AmActin* was detected in all cDNA synthesized using the RNA extracted

from both control and AVG plants (Figure 4.3). *AmActin* was amplified to determine the quality of cDNA produced, and it was successfully amplified in all samples.

4.4.3 AmVPE1 Transcript Levels in AVG Treated and Control Plants

Through qPCR, AmVPE1 transcript levels were determined in plants treated with AVG and in control plants. The transcript levels were compared in both groups during 6 stages of leaf development. In control plants, AmVPE1 transcript levels were significantly higher ($P < 0.05$) in preperforation and early window stage leaves as compared to the other stages (Figure 4.4). The AmVPE1 transcript levels declined during the window, late window and mature stage in control plants.

In AVG treated plants, *AmVPE1* transcript levels were not significantly different ($P < 0.05$) in early preperforation, preperforation, early window and window stage leaves. However, they declined significantly during the late window and mature stages ($P < 0.05$). During preperforation and early window stages, control plants had significantly higher ($P < 0.05$) *AmVPE1* transcript levels than AVG treated plants. There was no significant difference in *AmVPE1* transcript levels between AVG treated and control plants in early preperforation, window, late window and mature stages.

4.4.4 AmVPE2 Transcript Levels in AVG Treated and Control Plants

In control plants, *AmVPE2* transcript levels were significantly higher ($P < 0.05$) during the late window stage than all the other stages of leaf development (Figure 4.5). There were no significant differences ($P > 0.05$) in transcript levels among early preperforation, preperforation, early window, window and mature stages. In AVG treated plants, *AmVPE2* transcripts were constitutively expressed throughout leaf development.

There were no significant differences in *AmVPE2* transcripts levels between AVG and control plants in all stages of leaf development except during the late window stage. During the late window stage, control plants had significantly higher ($P < 0.05$) *AmVPE2* transcript levels than AVG treated plants.

4.4.5 AmActin Transcript Levels in AVG Treated and Control Plants

AmActin transcripts were constitutively expressed in both AVG and control plants (Figure 4.6). Even though there was no significant differences ($P > 0.05$) in *AmActin* transcript levels within treatments (AVG and control), AVG treated plants generally had lower *AmActin* transcript levels than control plants. The *AmActin* transcript levels were significantly lower ($P < 0.05$) in AVG treated plants during early preperforation, early window and mature stages compared to the same developmental stages in control plants.

4.5 Discussion

4.5.1 Leaf Morphology

Plants treated with AVG produced leaves with fewer or no perforations compared to controls, which had the normal number of perforations. This result is consistent with findings of Dauphinee et al. (2012) (Appendix B). The AVG treated plants still produced leaves that looked healthy and normal (except for the lack of perorations). The plants also produced approximately the same number of leaves as the control plants (Dauphinee et al. 2012). Therefore, AVG does not seem to affect the plant's ability to produce new leaves. Early preperforation stage leaves looked identical in both AVG treated and control plants (Figure 4.1A and B, leaf 1). They both had just emerged from a corm, were light green, tightly furled and had no signs of perforation formation and PCD.

Preperforation leaves from both AVG treated and control plants also looked similar (Figure 4.1A and B, leaf 2). The leaves were still furled, and still did not show obvious signs of perforation formation or PCD. They were also of approximately the same size. At the early window stage, leaves in control plants started to show signs of perforation formation. The perforation sites started to become somewhat transparent as PCD cells in these leaves started to lose their pigmentation (Figure 4.1A, leaf 3). In the AVG treated plants, however, the signs of perforation formation were minimal or inexistent in early window stage leaves. There was minimal/no difference between the putative PCD cells and NPCD cells, therefore the leaves did not show any signs of PCD (Figure 4.1B, leaf 3). The leaves in both the AVG treated and control plants were about half-unfurled at this stage. During the window stage, perforation formation had progressed in control plants, and signs of PCD within perforation sites were visible. The leaves were actively forming perforations (Figure 4.1A, leaf 4). In the AVG treated plants however, most of the window stage leaves did not have any signs of perforation formation. They were completely whole and the PCD cells did not look any different from NPCD cells (Figure 4.1B, leaf 4). During the late window stage, holes had formed within the perforation sites in control plants (Figure 4.1A, leaf 5). The holes were continuously getting larger as more cells within the perforation sites were undergoing PCD and disintegrating. The late window stage leaves in AVG treated plants, lacked holes and or any signs of PCD (Figure 4.1B, leaf 5). Mature stage leaves were fully perforated within control plants (Figure 4.1B, leaf 6), while in AVG treated plants they had few or no perforations (Figure 4.1B, leaf 5). Leaves that were produced before treatment (Figure 4.1A and B, leaves 7 and 8) looked similar and had approximately the same number of perforations, suggesting

that the effects on perforation number observed were due to treatment. Generally, at the same stage of leaf development, the leaves within control and AVG treated plants were of similar sizes.

The morphological results suggest that treatment of the plants with the ethylene biosynthesis inhibitor, AVG, does inhibit perforation formation and PCD. Suggesting that ethylene is an important component necessary for PCD and perforation formation in the lace plant. These results were consistent with conclusions from Dauphinee et al. (2012). The AVG treated plants were probably not producing enough ethylene required for regulation and subsequent execution of PCD, as ethylene is known to be involved in the regulatory phase of PCD in the lace plant and other plants (reviewed in Rantong and Gunawardena 2015).

4.5.2 Effect of AVG Treatment on VPE Transcript Levels

It is thought that ethylene is upstream of VPEs within the plant PCD cascade. While ethylene is thought to be within the regulation phase, VPEs are thought to be executors of plant PCD. In earlier Chapters (Chapter 2 and 3), I showed that both ethylene and VPEs play significant roles during PCD and perforation formation in the lace plant. In this Chapter, through AVG treatment experiments, we studied the effect of reduced ethylene production on the execution of PCD through VPEs. Leaf morphological experiments above have already showed that a lack of ethylene results in a lack of execution of PCD, therefore, VPE transcript levels were measured to determine if the lack of PCD execution is due to lack of downstream execution of PCD.

4.5.3 Effect of AVG Treatment on AmVPE1 Transcript Expression

Transcript levels of *AmVPE1* in control plants increased in preperforation and early window stage leaves, which are early stages in the PCD and perforation formation process (Figure 4.4). However, AVG treated plants did not display the increase in *AmVPE1* transcript levels as observed in the preperforation and early window stage leaves of controls. Instead, their *AmVPE1* transcript levels in preperforation and early window stage leaves were not significantly different ($P > 0.05$) from those in the early preperforation stage. Preperforation and window stage leaves in AVG treated plants had significantly lower ($P < 0.05$) transcript levels than in control plants. Therefore, treatment of plants with AVG and inhibiting ethylene production seems to prevent the increase in *AmVPE1* transcript levels during the preperforation and early window stage. The increase in *AmVPE1* levels in control plants coincides with the beginning of perforation formation and PCD. Therefore, inhibiting ethylene biosynthesis seems to hinder the increase in *AmVPE1* transcript levels during developmental stages where PCD and perforation formation usually occurs. The lack of increase in *AmVPE1* transcript levels seems to result in inhibition of PCD and no perforation formation.

4.5.4 Effect of AVG Treatment on AmVPE2 Transcript Expression

In control plants, *AmVPE2* transcript levels increase significantly ($P < 0.05$) during the late window stage (Figure 4.5). This increase is not evident in AVG treated plants. Therefore, the limited ethylene production due to AVG seems to be inhibiting the increase in AmVPE2 transcript level during the late window stage. This is the stage of leaf development where PCD is rampantly occurring and cells are disintegrating to give rise to holes at perforation sites. AmVPE2 appears to be the main executioner of PCD

during perforation formation and lack of its transcript level increase during the PCD execution stage possibly led to no PCD, and subsequently no perforations. Ethylene seems to be involved in the signaling leading to the increase in transcriptional upregulation of AmVPE2.

4.5.5 AmActin Transcript Levels in AVG and Control Plants

AmActin was constitutionally expressed in both AVG treated and control plants. However, control plants generally had higher transcript levels throughout leaf development than AVG treated plants. Ethylene is involved in many other cellular processes and its limited production may be affecting other metabolic processes unrelated to PCD. At higher AVG concentrations (higher than 5 μmol/L), the lace plant produced deformed leaves (Dauphinee et al. 2012). Transcription of housekeeping genes like *AmActin* could also be affected. However, since the leaves in 5 μmol/L AVG treated plants looked healthy and did not show any developmental defects (except for lack of perforations), the limited ethylene was not enough to adversely affect the plants.

4.5 Conclusions and Future Work

The data presented here suggests that ethylene is upstream of VPEs in the plant PCD cascade. Changes in ethylene levels affect transcriptional regulation of VPEs. In the case of the lace plant, the inhibition of ethylene production seems to result in the abolishment of increases in VPE transcript levels that are necessary for PCD and perforation formation to occur. Both ethylene and VPE may be necessary for PCD in the lace plant and together they play a role during leaf morphogenesis. The interconnection between ethylene and VPEs is not unique to the lace plant. Kinoshita et al. (1999)

showed that during ripening, treating citrus fruits with ethylene increased VPE transcript levels. This shows that ethylene can affect VPE transcript levels, even in other processes unrelated to PCD. Increased ethylene levels may affect transcription factors that regulate VPE expression. Also, VPEs may be involved in execution of many of the ethylene-induced processes. Within the plant PCD cascade, ethylene is thought to stimulate (through an ethylene signal transduction pathway involving ethylene receptors) transcription factors that regulate the expression of plant caspase-like enzymes (reviewed in Rantong and Gunawardena 2015). The effect of changes in ethylene receptor levels would be interesting to investigate, since they seem to play a major role in regulation of PCD during perforation formation in the lace plant (model in Chapter 2 section 2.5). It has been shown that treatment of lace plant with ethylene receptor inhibitor, silver ions, stopped perforation formation (Gunawardena et al. 2006). Insights into whether blocking the ethylene receptors also hinders increases in VPE transcript levels, would be useful to elucidate the mechanism involved in ethylene regulation of VPE transcript levels. The effect of other components within the ethylene signal transduction pathway, such as ethylene insensitive 3 (EIN3, a transcription factor that induces expression of other transcription factors responsible for regulating the execution of ethylene induced responses) on transcript levels of VPEs need to be investigated. EIN3 could be regulating the induction of transcription factors that regulate the transcription of VPEs. This insight would be important in understanding the direct link between ethylene production, perception, signal transduction and execution of the ethylene induced responses, especially PCD.

Figure 4.1 Morphology of AVG Treated Lace Plant Compared to Control Plants

Morphology of AVG treated lace plant compared to control plants. A leaf layout representing the different developmental stage leaves from control and AVG treated plant (A and B, respectively) was assembled. It highlighted the differences in number of perforations in control and AVG treated plants, especially after treatment (leaves 1-6). Control plants had the normal morphology with perforated leaves, while in AVG treated plants most of the leaves produced after treatment lacked perforations. Upon harvesting, leaves from both AVG treated and control plants were separated into 6 stages of leaf development (leaves 1-6). The leaf developmental stages were early preperforation (EPP; leaf 1), preperforation (PP; leaf 2), early window (EW; leaf 3), window (W; leaf 4), late window (LW; leaf 5) and mature (M; leaf 6). Leaves 7 and 8 developed before treatment. Bars = 1.8 cm.

Figure 4.1 Morphology of AVG Treated Lace Plant Compared to Control Plants

Figure 4.2 RNA Extracted From Control and AVG Treated Plants

Total RNA was extracted from leaves at different stages of development (EPP-M). At least 4 RNA samples were obtained per leaf developmental stage. All the RNA extracted from control and AVG treated plants was intact and displayed well-defined ribosomal RNA bands. EPP = Early preperforation, PP = preperforation, EW = early window, W = window, LW = late window and M = mature.

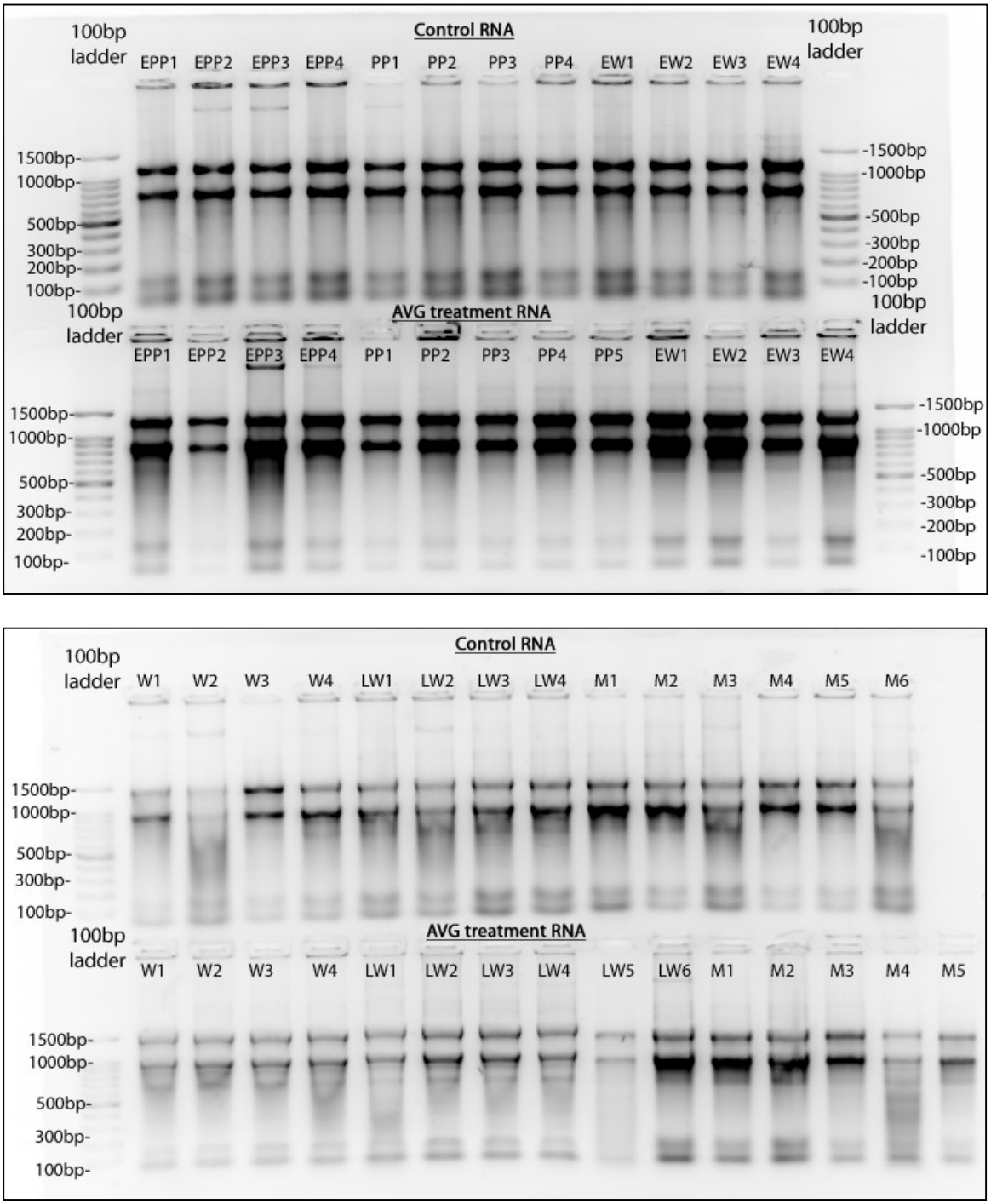

Figure 4.2 RNA Extracted From Control and AVG Treated Plants

Figure 4.3 Detection of *AmActin* Transcripts Through RT-PCR

AmActin transcripts were detected in all cDNA synthesized from control and AVG treated plants. EPP = Early preperforation, PP = preperforation, EW = early window, W = window, LW = late window and M = mature.

Figure 4.3 Detection of *AmActin* Transcripts Through RT-PCR

Figure 4.4 Quantification of *AmVPE1* Transcripts Through qPCR

Mean normalized *AmVPE1* transcript levels were determined in leaves at different stages of leaf development, in both control and AVG treated plants. During the preperforation and early window stages, AVG treated plants had significantly lower ($P < 0.05$) transcript levels than control plants. Asterisks highlight developmental stages displaying significant differences ($P < 0.05$) between treatments. Bars represent SE ($n \geq 12$). EPP = Early preperforation, PP = preperforation, EW = early window, W = window, LW = late window and M = mature.

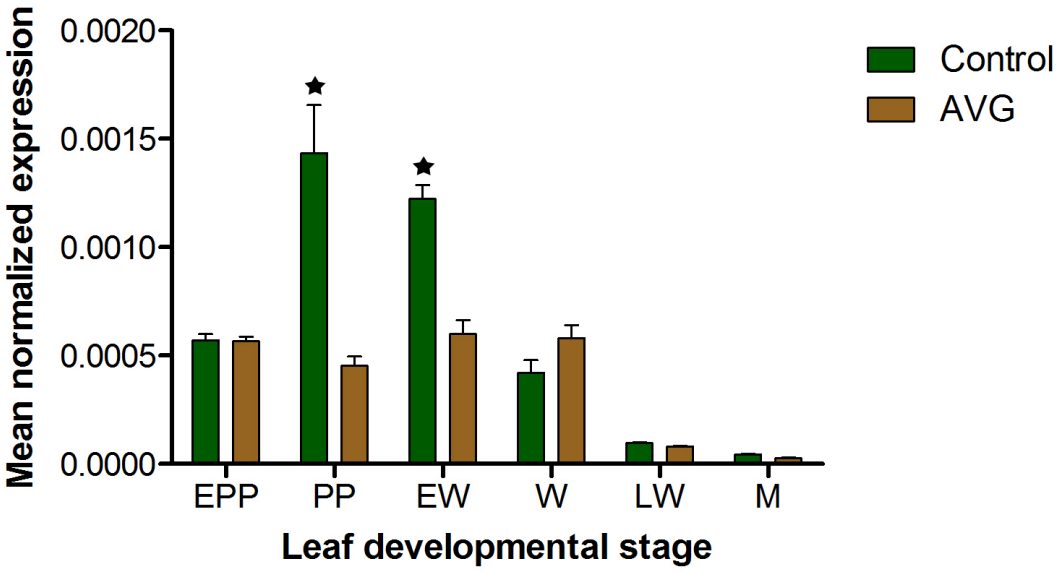

Figure 4.4 Quantification of *AmVPE1* Transcripts Through qPCR

Figure 4.5 Quantification of *AmVPE2* Transcripts Through qPCR

Mean normalized *AmVPE2* transcript levels in leaves at different stages of leaf development, in control and AVG treated plants. During the late window stage, Control plants had significantly higher ($P < 0.05$) transcript levels than AVG treated plants. Asterisks highlight developmental stages displaying significant differences ($P < 0.05$) between treatments. Bars represent SE ($n \geq 12$). EPP = Early preperforation, PP = preperforation, EW = early window, W = window, LW = late window and M = mature.

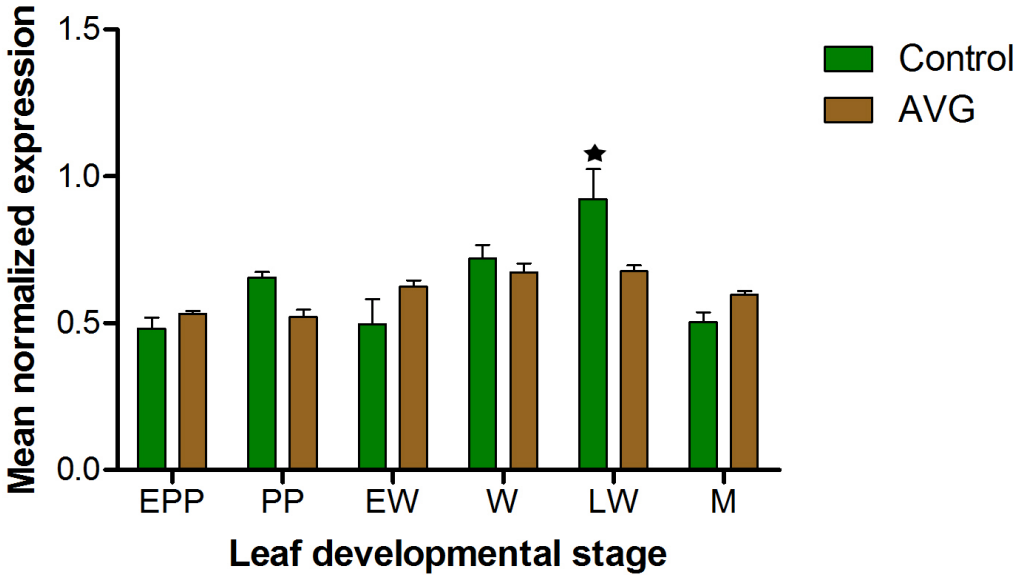

Figure 4.5 Quantification of *AmVPE2* Transcripts Through qPCR

Figure 4.6 Quantification of *AmActin* Transcripts Through qPCR

AmActin transcript levels in different developmental stage leaves from control and AVG treated plants. *AmActin* was constitutively expressed throughout leaf development in control and AVG treated plants. However, control plants generally had significantly higher ($P < 0.05$) *AmActin* transcript levels throughout development. Asterisks highlight developmental stages displaying significant differences ($P < 0.05$) between treatments. Bars represent SE ($n \geq 12$). EPP = Early preperforation, PP = preperforation, EW = early window, W = window, LW = late window and M = mature.

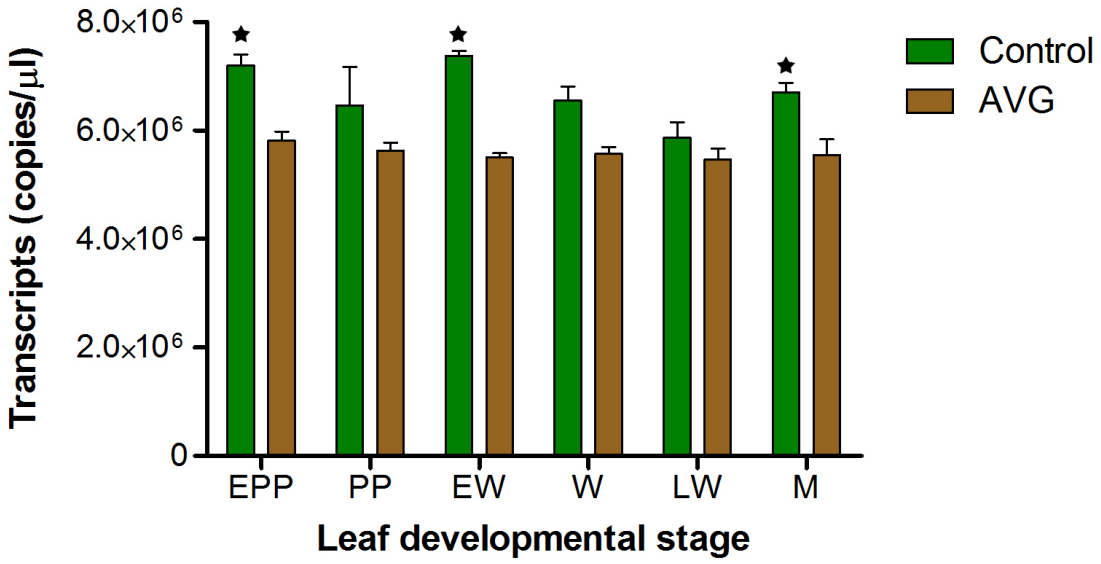

Figure 4.6 Quantification of *AmActin* Transcripts Through qPCR

Chapter 5 Conclusions

The lace plant is an excellent model system to study developmentally regulated PCD in plants. The predictability (in terms of timing and location) and easy accessibility of the cells undergoing developmentally regulated PCD in the lace plant are valuable. The presence of a developed sterile propagation method without interference from microbes makes it a suitable study organism for developmentally regulated PCD in plants. An extensive amount of morphological studies of PCD have been carried out in the lace plant. The morphological cellular dynamics and their chronological order during PCD are well documented. Even though a significant amount of knowledge has been gathered in terms of the morphological characteristics of PCD, relatively less is known about the developmental signals that initiate, regulate and execute PCD in the lace plant. This study was mainly focused on elucidating some of the molecular components involved in lace plant PCD pathways.

So far, we know that lace plant PCD is developmentally regulated, but the signals that initiate the process are still unknown. There is indirect evidence for the involvement of reactive oxygen species in signaling in the dying cells (PCD cells) and a potential involvement of anti-oxidants such as anthocyanin in helping NPCD cells (cells not destined to die during perforation formation) to resist PCD during perforation formation. The involvement of calcium as a potential signaling molecule has also been studied in the lace plant. Ethylene has also been shown to be involved in hormonal signaling and regulation of PCD in the lace plant. Dauphinee et al. 2012 (and Chapter 2 of this dissertation) demonstrated that ethylene is involved in a climacteric-like pattern (due to a

peak in production during the window stage) during leaf morphogenesis via PCD in the lace plant. The peaks in ethylene production coincided with leaf developmental stages involving PCD. The involvement of ethylene in a climacteric-like pattern during leaf morphogenesis had not been reported before; therefore this study provided new insights. Even though it was demonstrated that ethylene regulates PCD in the lace plant, the mechanism that allows for PCD in only a subset of cells within the leaves was unknown until this study was carried out. As a gaseous phytohormone, ethylene diffuses easily through tissues, therefore all the cells in the leaf tissue are exposed to the PCD-inducing ethylene levels. Yet, the NPCD cells within 4-5 cell layers from the vascular tissue and in close proximity with the PCD cells remained resistant to ethylene-induced PCD. It was clear that there was an underlying mechanism that closely regulated and determined cell fate in conjunction with ethylene levels. In this study I demonstrated that ethylene receptors (along with ethylene levels) determine cell fate in a concentration-dependent manner. I demonstrated the model in Chapter 2 and to our knowledge this new model is novel information of the use of ethylene and receptor concentrations to sculpt plant leaves. This could be further investigated using in situ hybriziation.

The executors of PCD in the lace plant were unknown until this study was carried out, and in Chapter 3, I isolated two lace plant vacuolar processing enzymes (VPEs) and demonstrated their involvement in the execution of lace plant PCD. VPEs are caspase-1 like enzymes and their increased transcript levels and activity is observed during PCD and perforation formation in the lace plant. They are known to play a role during PCD in other plant species, especially in PCD examples in which the vacuole and its membrane rupture play a central role. In the lace plant, studies on morphological cellular dynamics

during PCD have shown that rupture of the vacuolar membrane signals the beginning of rapid degradation of cell components. This highlighted the importance of tonoplast rupture during lace plant PCD. VPEs are localized within the vacuole and are thought to be responsible for degrading or compromising the integrity of the tonoplast during PCD. I showed that in the lace plant, VPE transcript levels increase during perforation formation and PCD. One of the lace plant VPEs, AmVPE1, is involved during the early stages before any visible signs of PCD. It may play a role in the activation other PCD executors, or play a regulatory role within PCD. The other VPE, AmVPE2, is highly expressed during the late stages of PCD when degradation of cellular components is rampant. VPE activity experiments showed that there is increased VPE activity in the vacuole during PCD, providing more support for the involvement of VPEs in degradation of the tonoplast, or activation of hydrolases that compromise tonoplast integrity. The timing of increases in transcript expression levels and increased VPE activity within the vacuole reconcile the observed tonoplast rupture and subsequent rapid degradation of cellular components with the molecular mechanism behind it. Therefore, Chapter 3 provides the molecular insight that possibly explains some of the observed and well-recorded morphological changes during PCD in the lace plant.

It is still unclear how increases in transcription rates of VPEs are initiated during PCD. I therefore studied the effect of ethylene, a known PCD regulator, on the rate of VPE transcript expression. In Chapter 4, I demonstrated that ethylene is required to observe the stimulation of increases in VPE transcript levels that appear to be required for the occurance of PCD. Ethylene has been shown to increase VPE transcript levels during HR-related PCD, but to our knowledge its effect on VPE transcription rates during

developmentally regulated PCD had not been demonstrated before this study. I showed that during leaf morphogenesis via PCD in the lace plant, increases in ethylene levels stimulate the increased transcription of VPEs in specific cells (PCD cells) within the leaves, and this may initiate the execution of PCD. The ethylene signal only results in PCD in these cells because their levels of ethylene receptors (which are negative regulators of ethylene induced responses) are not enough to inhibit activation of ethylene-induced PCD. The cells not destined to die during perforation formation (NPCD cells) increase their ethylene receptor levels to hinder the transduction of the ethylene signal to downstream components, which would initiate PCD. The signals that initiate increases in receptor levels within NPCD cells are still unknown. Identifying these signals is a good area for future research. In PCD cells, the ethylene signal (most likely through stimulating transcription factors that regulate the expression of VPEs) activates the increased transcription and expression of VPEs. VPEs then mediate the execution of PCD in a vacuole-dependent mechanism. The transcription factors that are involved in regulating VPE transcript levels remain unknown. However, candidates include WRKY transcription factors, which are known to regulate some PCD related genes and have also been isolated in the lace plant. Figure 5.1 summarizes and reconciles the findings of this study with what is currently known about lace plant PCD, and explains the overall mechanism thought to be involved during sculpting of lace plant leaves via PCD.

Overall, many of the molecular mechanisms involved in plant PCD are still unclear, and studies of the PCD pathways within suitable developmental PCD systems like in the lace plant are essential to provide more insight into how PCD is regulated in plants. This study provides a foundation for future potential studies in elucidating more

molecular components of the developmental PCD pathway and reconciling the molecular data with the already known morphological data of lace plant PCD. Future work including isolation of other PCD candidate genes through transcriptomics (using RNA seq), genome sequencing and the use of mutants and transgenics would provide more understanding of molecular mechanisms involved in lace plant PCD. A predictable, well-controlled and easily accessible example of developmentally regulated PCD as in the lace plant provides a perfect opportunity to understand plant PCD in more detail to fill in the informational gaps that exist currently about its regulatory pathways.

Figure 5.1 Summary of the developmentally regulated PCD pathway in the lace plant

A developmental cue initiates the increase in ethylene production within leaves, through signaling molecules and cytosolic calcium (Ca^{2+}). Ethylene is perceived by ethylene receptor within two cell types (PCD cells and NPCD cells). In NPCD cells, ethylene receptor transcription and expression are upregulated to a level that inhibits the relaying of a signal to the downstream components of the signal transduction pathway. Therefore, ethylene-induced PCD does not occur in these cells. In PCD cells however, ethylene receptor levels are downregulated (or stay at the same levels) and the high ethylene environment within the cells overrides the inhibitor effect of the receptors, allowing for the ethylene signal transduction to continue downstream to components such as the MAPK cascade, EIN2, and the transcription factor EIN3. EIN3 is known to activate ethylene-induced responses, including PCD. Hormonal crosstalks with hormones such as salicylic acid will occur, and ultimately transcription factors (such as WRKY transcription factors) may be activated. These transcription factors may be responsible for stimulating an increased expression of VPEs, which will execute PCD or cleave hydrolases, nucleases, cell modifiers or other proteases involving in execution of PCD. Components studied (or implicated by indirect evidence) in the lace plant are highlighted in green. Also highlighted are some of the components whose partial cDNA sequences have been identified in the lace plant and could potentially play a role.

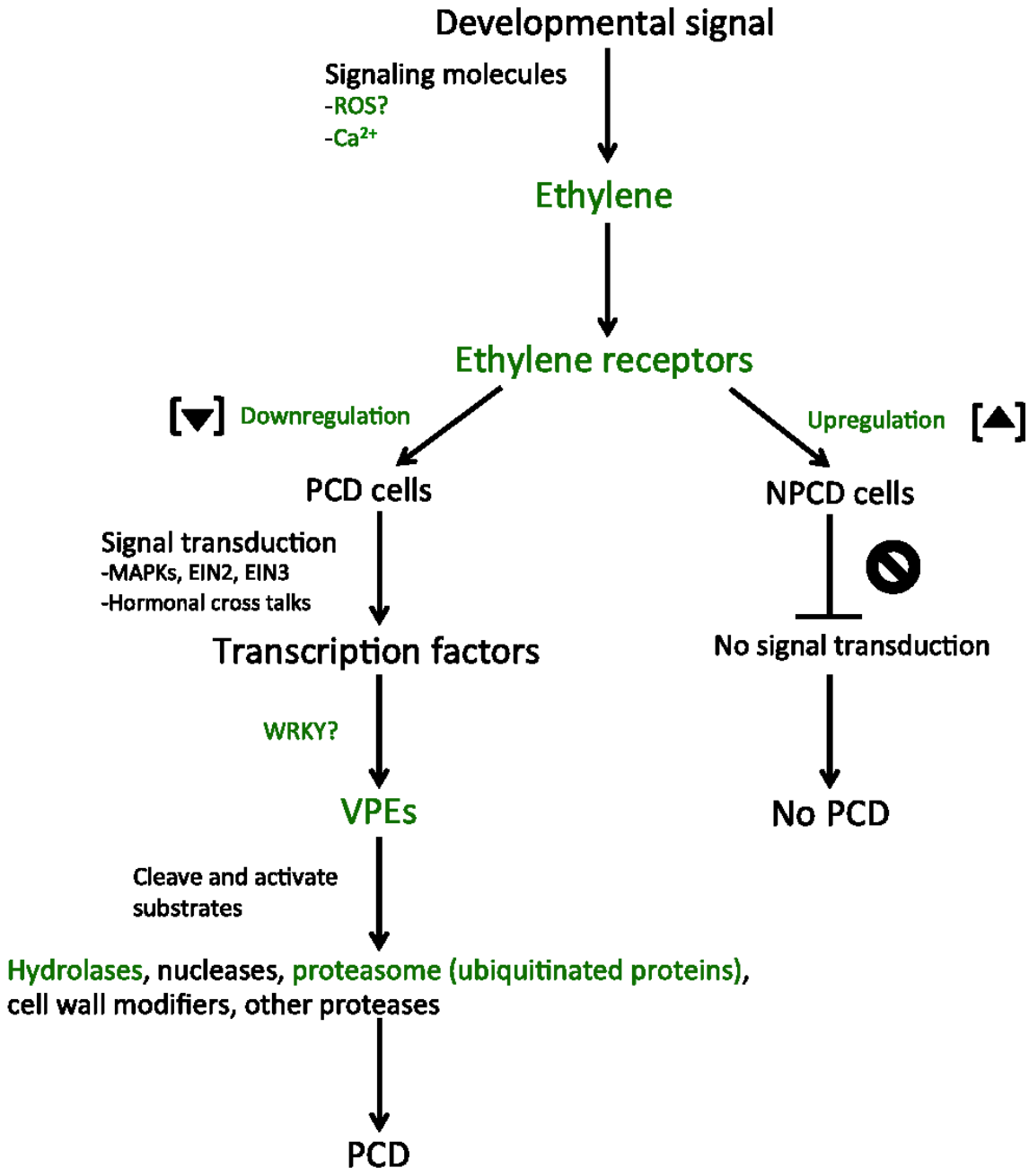

Figure 5.1 Summary of the Developmentally Regulated PCD Pathway in the Lace Plant

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Appendix B Dauphinee et al. 2012

The involvement of ethylene in programmed cell death and climacteric-like behaviour during the remodelling of lace plant (*Aponogeton madagascariensis*) leaves

A.N. Dauphinee, H. Wright, G. Rantong, and A.H.L.A.N. Gunawardena

Abstract: Programmed cell death (PCD) plays an important role in several plant developmental processes. The phytohormone ethylene has been implicated in PCD signalling in many plant systems, but it is also important in developmental processes such as seed germination, flowering, and climacteric fruit ripening. Lace plant (*Aponogeton madagascariensis* (Mirbel) H. Bruggen) is an aquatic monocot that develops perforated leaves via the deletion of cells through developmentally regulated PCD. The plant is ideal for studying PCD; however, little is known about the regulation of cellular death involved in this system. The current study examines ethylene as a potential signalling molecule in lace plant PCD and investigates climacteric-like behaviour during lace plant leaf development. Whole plants were treated with the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG), the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), or a combination of both. Subsequently, ethylene levels were monitored, and leaf development was analyzed. The results indicate that ethylene is involved in lace plant PCD signalling. AVG-treated plants had significantly lower ethylene outputs and a significant reduction in perforation formation. The inhibitory effect of AVG was recovered when AVG and ACC were applied simultaneously. The data presented here show for the first time, to our knowledge, climacteric-like behaviour during the remodelling of leaves.

Key words: PCD, aminoethoxyvinylglycine, 1-aminocyclopropane-1-carboxylic acid, leaf development, leaf senescence, ethylene.

Résumé : La mort cellulaire programmée (MCP) joue un rôle important dans plusieurs processus développementaux chez les plantes. La phytohormone éthylène est impliquées dans la signalisation chez plusieurs systèmes végétaux, mais influence également de façon importante des processus tels que la germination des graines, la floraison et la maturation climatérique des fruits. La plante en dentelle (*Aponogeton madagascariensis* (Mirbel) H. Bruggen), une monocotyle aquatique, développe des feuilles perforées via l'élimination de cellules par une MCP développementale régulée. Cette plante est idéale pour l'étude de la MCP; cependant, on connait peu de chose à propos de la régulation de la mort des cellules dans ce système. Les auteurs ont examiné l'éthylène comme molécule de signalisation potentielle dans la MCP chez la plante dentelle et ont recherché un comportement de nature climatérique au cours du développement foliaire de cette plante. Ils ont traité des plantes entières avec l'inhibiteur de la biosynthèse de l'éthylène, l'aminoéthoxyvinylglycine (AVG), le précurseur de l'éthylène l'acide 1-aminocyclopropane-1-carboxylique (ACC), ou une combinaison des deux. Par la suite, ils ont suivi les teneurs en éthylène et ont analysé le développement foliaire. Les résultats indiquent l'implication de l'éthylène dans la signalisation de la MCP chez cette plante. Les plantes traitées avec l'AVG produisent significativement moins d'éthylène et on observe une diminution significative de la formation des perforations. L'effet inhibiteur de l'AVG s'estompe lorsqu'on applique simultanément l'AVG et l'ACC. Les données montrent pour la première fois, selon les auteurs, un comportement de type climatérique au cours du remodelage des feuilles.

Mots-clés : MCP, aminoéthoxyvinylglycine, acide 1-aminocyclopropane-1-carboxylique, développement foliaire, sénescence foliaire, éthylène.

[Traduit par la Rédaction]

Introduction

Programmed cell death (PCD) plays an important role in the organization and maintenance of plants and can be divided into two broad categories: environmentally induced and developmentally regulated (Gunawardena 2008). Environmentally induced PCD is caused by external factors, which include flooding, salt stress, heat shock, UV radiation, and pathogens (Kacprzyk et al. 2011). Developmentally regulated PCD differs in that it has an endogenous induction signal, which

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include processes such as xylogenesis, petal senescence, and leaf morphogenesis (van Doorn and Woltering 2005; Gunawardena 2008; Reape et al. 2008). In the past few years, significant progress has been made in understanding the developmental signals and pathways regulating PCD in plants; however, no ubiquitous critical executor of plant PCD has been identified to date. A number of plant-specific hormones have been implicated in PCD signalling, including salicylic acid, jasmonic acid, abscisic acid, gibberellins, and ethylene (Hoeberichts and Woltering 2003), although ethylene has most often been associated with the promotion of cellular death (Noodén 2004). Specific examples of developmentally regulated PCD stimulated by ethylene include the degradation of the nucellus in squash (*Sechium edule*) after fertilization (Lombardi et al. 2012) and the senescence of floral organs in *Nicotiana mutabilis* (Macnish et al. 2010).

The ethylene biosynthetic pathway initiates via the conversion of methionine to S-adenosyl methionine (AdoMet) by AdoMet synthetase. Following this, AdoMet is converted to 1-aminocyclopropane-1-carboxylic acid (ACC), which is the rate-limiting step of the pathway and mediated by the enzyme ACC synthase. The final conversion is that of ACC to ethylene, carbon dioxide, and cyanide by ACC oxidase (Adams and Yang 1979; reviewed by Trobacher 2009). Several studies in the past have employed inhibitors of ethylene biosynthesis such as aminoethoxyvinylglycine (AVG) and aminooxyacetic acid (AOA), which competitively inhibit ACC synthase (Yu and Yang 1979; Bae et al. 1996; Mattoo and Handa 2004). Gladish and Niki (2008) used several ethylene inhibitors, including AOA to suppress PCD during vascular cavity formation in submerged pea (*Pisum sativum*) roots, and were able to increase PCD with the application of exogenous ethylene in non-flooded conditions. Likewise, Chae and Lee (2001) used AOA and AVG to demonstrate the involvement of ethylene during PCD signalling in carrot (*Daucus carota*) suspension cells undergoing carbon starvation, and the application of AVG reduced cadmium-induced PCD in tomato suspension cells (Yakimova et al. 2006). Ethylene production can be stimulated by the application of exogenous ACC, which leads to an increase of PCD in epidermal cells of deepwater rice (*Oryza sativa*) at the site of adventitious root emergence (Mergemann and Sauter 2000). Ethylene is also implicated in several other developmental and growth processes including, but not limited to, seed germination, flowering, abscission, and fruit ripening (Noodén 2004).

The involvement of ethylene in the ripening of many fruits has been well established (Hansen 1966; Dhillon and Mahajan 2011). Fruits are classified as climacteric or non-climacteric based on their ethylene production and respiratory behaviour during ripening (Rees 2012). In climacteric fruits, ethylene stimulates ripening (Oeticker and Yang 1995; Rees and Hammond 2002); respiration rates increase during ripening and then decline as fruit senescence progresses. This produces sharp peaks of both ethylene and carbon dioxide production at the beginning of ripening (Biale 1950; Tucker 1993). Climacteric-like behaviour has also been associated with other forms of PCD such as petal senescence (Serrano et al. 1991), leaf abscission (Morgan et al. 1992), and leaf senescence (Katz et al. 2005). Morgan et al. (1992) showed that in cotton leaves, a peak in ethylene production is observed 3–4 days before abscission. In citrus, detached leaves were shown to maintain constitutive amounts of ethylene production before peaking after 9 days, marking the beginning of leaf senescence (Katz et al. 2005).

Lace plant (*Aponogeton madagascariensis* (Mirbel) H. Bruggen) is an aquatic monocot that develops a unique perforated leaf morphology through developmentally regulated PCD. Lace plant provides an excellent system for studying PCD for several reasons, including the predictability of perforation formation, the ability to propagate plants via sterile tissue culturing (Figs. 1A, 1B), and thin, nearly transparent leaves that are ideal for live cell imaging (Gunawardena et al. 2006; Wertman et al. 2012). Newly cultured lace plants form three or four juvenile leaves that do not develop perforations; however, all leaves that emerge subsequently (adult leaves) develop perforations by maturity (Fig. 1C). Gunawardena et al. (2004) classified the development of perforations into five stages. Young leaves emerge from the corm having furled blades with a grid-like pattern of longitudinal and transverse veins; this point of development is known as the preperforation stage in which areoles (located between the vasculature) show no visible cytological indication that PCD will occur (Fig. 1D). Next is the window stage (Fig. 1E) in which cells at the centre of the areole undergo PCD, as indicated by their loss of pigmentation. PCD continues to expand outwards towards the veins. Cells subsequently collapse and cell walls are degraded, giving rise to the perforation formation stage. Perforations enlarge during the perforation expansion stage. The deletion of cells via PCD halts four or five cell layers from the veins by the mature stage (Fig. 1F).

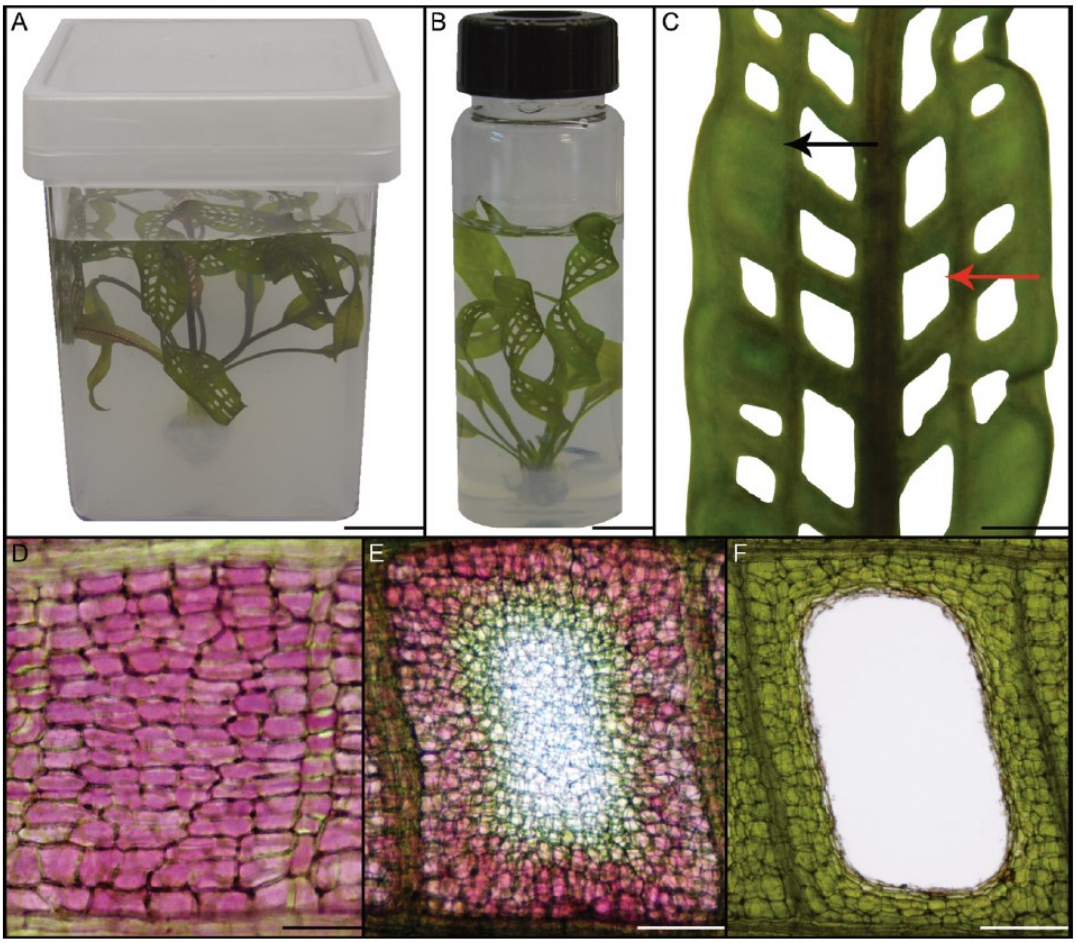
The developmental cues involved in PCD signalling during lace plant leaf morphogenesis are yet to be understood. Because ethylene is involved in many plant developmental processes and has been implicated in PCD signalling, it warrants investigation within the lace plant system. Initial work carried out by Gunawardena et al. (2006) provided some indirect evidence that ethylene may play a role in perforation formation in lace plant. The current study used AVG to inhibit and ACC to enhance ethylene biosynthesis in whole plants in which leaf development and ethylene production was monitored. Furthermore, this study examined ethylene and carbon dioxide evolution at various stages of leaf development in an attempt to elucidate whether or not lace plant leaves exhibit a climacteric pattern.

Materials and methods

Plant material

The axenic lace plant cultures used during this study were propagated according to Gunawardena et al. (2006). Newly cultured corms were placed into autoclaved Magenta G47 boxes and embedded in 50 mL of solid Murashige and Skoog (MS) medium containing 1% agar onto which 200 mL of liquid MS medium (0% agar) was poured (Fig. 1B). The plants were maintained at 24 °C and exposed to fluorescent light (F32T8/DX; Philips Electronics Ltd., Markham, Ontario) at an intensity of 125 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on a 12 h light – 12 h dark cycle. All chemicals used during experimentation were purchased from Sigma Aldrich (St. Louis, Missouri), unless otherwise stated.

Fig. 1. Lace plant (*Aponogeton madagascariensis*) growth and development. Lace plants used for whole-plant experiments in this study were grown under axenic conditions in (A) Magenta boxes or (B) air-tight vials. (C) Adult lace plant leaves develop perforations (bottom arrow) by the time they reach maturity; please note that some areoles do not develop perforations (top arrow). (D) The initial stage of perforation formation is known as the preperforation stage in which there are no visible signs that programmed cell death (PCD) is occurring. During the window stage (E), PCD has initialized at the centre of the areole and is readily observable due to a decrease in pigmentation. In the mature stage (F), PCD has stopped four–five cell layers from the vasculature. Scale bars: A–B, 1 cm; C, 5 mm; D, 40 μm; E, 125 μm; F, 200 μm.



Whole-plant experimentation

Whole-plant experiments were carried out using 4- to 5-week-old lace plants, which had similar corm sizes and three–four perforated leaves, grown in Magenta boxes. The plants were transplanted into 40 mL air-tight vials fitted with septum lids that contained 10 mL of solid and 20 mL of liquid MS medium (Fig. 1B). Lace plants were then randomly assigned into four groups: AVG, ACC, a combination of both AVG and ACC, and control, which received an equal volume of distilled water. To determine optimal treatments, gradients of AVG and ACC concentrations were applied to whole plants and compared with controls in terms of ethylene production and leaf morphology. The AVG applications included 2, 4, 5, 10, and 25 μmol/L, and the ACC concentrations tested were 1, 2, 5, 10, 25, 50, 100, 200, 500, and 1000 μmol/L. At higher concentrations of AVG, there was a complete inhibition of perforation formation; however, the leaves that developed were short and narrow compared with controls (data not shown). At higher concentrations of ACC, leaves became elongated, had dark green and red pigmentation at maturity, and showed early signs of senescence compared with controls (data not shown). The optimal concentrations that significantly altered ethylene production but did not affect normal leaf development were 5 μmol/L AVG, 2 μmol/L ACC, and a

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combination of both 5 μmol/L AVG and 2 μmol/L ACC. Subsequent to treatment, the plants were maintained as mentioned above, and leaf development was monitored through photography and experimenter observations. One week after the initiation of the experiments, ethylene measurements were taken from the plants as described below, and two weeks after the initiation of the experiments, the leaves were harvested. Three independent experiments were carried out with six replicates per treatment (24 plants per experiment, 72 in total). Plants showing visible signs of infection were not considered during statistical analysis.

Detached-leaf experiments

Window-, mature-, and senescent-stage lace plant leaves were selected from aquarium-grown plants. Senescent leaves chosen for experiments comprised a varying proportion of tissues visibly undergoing senescence (observed as yellowing or browning areas). The leaves were carefully excised at the base of the petiole. Approximately 0.56 g of leaf tissues per stage were placed in 40 mL vials with septum-fitted lids. The leaves were then submerged in 30 mL of aquarium water that was supplemented weekly with 0.001g/L monopotassium phosphate, 0.01 g/L potassium nitrate, and 0.003 g/L CSM+B Plantex (Aquarium Fertilizers, Napa, California). Preliminary experiments showed that detached window- and mature-stage leaves continued to grow for up to a week under these conditions without showing any visible signs of senescence. However, preperforation leaves did not continue to grow and unfurl when detached; therefore, they were not used in this study. The vials were maintained as described for whole-plant experiments for three days prior to gas measurements. Five independent experiments were carried out under these conditions with four replicates per stage (12 vials per experiment, 60 in total).

Gas measurements

Ethylene measurements were taken for whole-plant and detached-leaf experiments using a gas chromatograph (Carle Instruments, Anaheim, California) fitted with a 1.9 m × 3.2 mm (o.d.) activated alumina column with a hydrogen carrier flow of 1.17 mL/s and a flame ionization detector. For all samples, 1 mL of the headspace was extracted using a syringe and then immediately injected into the gas chromatograph. For detached-leaf experiments, carbon dioxide measurements were taken following ethylene measurements. To achieve this, 3 mL of the headspace was extracted using a syringe and injected into a gas analyzer (GCS150; Gas Control Systems Inc., Sparta, Michigan). Ethylene and carbon dioxide output values for each replicate were divided by the mass of the sample to obtain the concentration of gas emitted per gram of tissue.

Morphological data collection for whole-plant experiments

Leaves were excised from individual lace plant corms at the base of the petiole and arranged in chronological order. For each replicate, the last adult leaf that had developed perforations prior to treatment was chosen to be a control leaf and was used for a qualitative comparison to all subsequent leaves that emerged following treatment. After the application of treatments, leaves were designated by a number in series of emergence, with the control leaf (0) having the lowest designation. Representative leaf layouts, showing the progression of leaf development from left (control) to right (last leaf to develop) were photographed. The number of newly produced leaves (mature and window stage), the mean mature leaf lengths (base of petiole to tip), and the mean number of perforate and imperforate areoles per mature leaf were recorded for individual plants. The percentage of perforation for individual leaves was calculated by dividing the number of perforate areoles by the total number of areoles (Fig. 1C).

Microscopy, photography, and image preparation

Light micrographs of sections of the detached leaves were taken on a Nikon 90i research microscope (Nikon, Mississauga, Ontario), and the images were captured using a Nikon DXM 1200c digital camera. NIS Elements Advanced Research (3.1) software was used during micrograph acquisition. All photographs were taken with a Nikon L110 digital camera (Nikon, Mississauga, Ontario). Micrographs and photographs were edited and prepared for publication using Adobe Illustrator (13.0) and Adobe Photoshop (10.0) (Adobe Systems Inc., San Jose, California).

Statistical analysis

A general linear model (GLM) analysis of variance (ANOVA) was used to determine statistical significance (Minitab release 15; Minitab Inc., State College, Pennsylvania). Individual treatment means were compared using a Waller–Duncan k ratio t test mean comparison (SAS release 8.0; SAS Institute Inc., Cary, North Carolina). A normal probability plot (NPP) of the residuals was used to test normality, while a fitted values versus residuals plot was used to examine constant variance. When the assumptions of the error term (ε_{ij}) were violated, transformations were used; in those instances, means shown in this paper were back-transformed. Only results significant at $P \leq 0.05$ are discussed, unless noted otherwise.

Results

Whole-plant experimentation

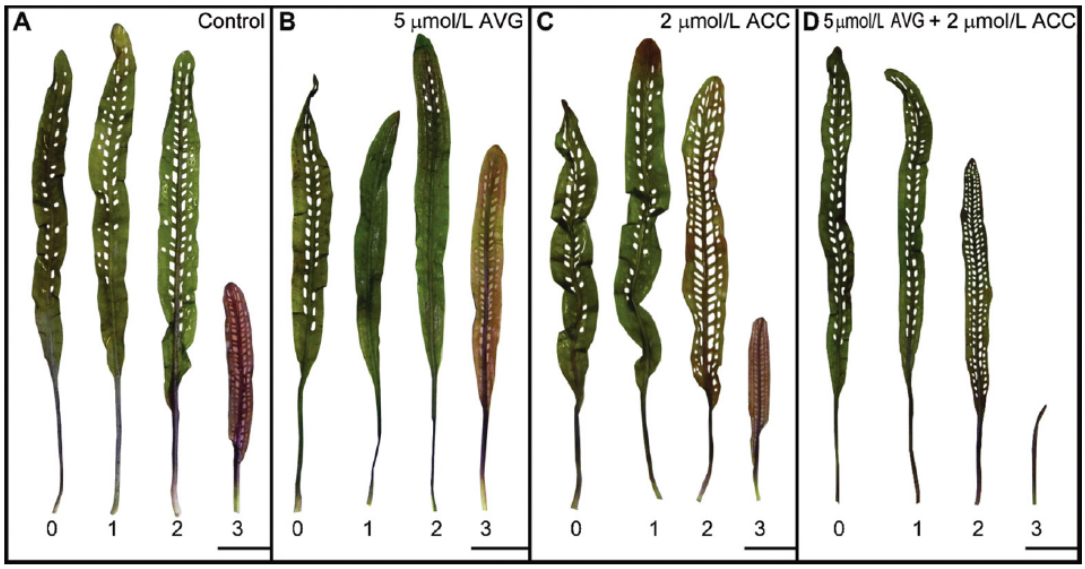
Leaf morphology and ethylene production

For leaf morphology analysis, leaves were excised and representative leaf layouts were collected from control, 5 μmol/L AVG, 2 μmol/L ACC, and the combination of 5 μmol/L AVG and 2 μmol/L ACC treated plants (Fig. 2). A PROC GLM ANOVA showed no significant difference in the number of new leaves formed ($P = 0.063$; Fig. 3A) or the mean mature leaf lengths ($P = 0.997$); however, there was a significant difference in mean percentage of perforations ($P < 0.001$; Fig. 3C). Mean comparison revealed that AVG-treated plants produced significantly fewer perforations compared with controls. There was no difference in number of perforations formed among the leaves of control and ACC- and combination-treated plants. Additionally, at the time of harvest, the latest window-stage leaves to develop in AVG-treated plants appeared as though they would produce more perforations than the leaves that developed to maturity in the two-week experimental period (leaf 3 in Fig. 2B).

Ethylene levels

Ethylene production was determined from whole plants via gas chromatography (Fig. 3D). The ethylene detected from AVG-treated plants was significantly lower compared with

Fig. 2. Leaf growth of experimental lace plants. Leaf layouts for (A) control, (B) 5 μmol/L aminoethoxyvinylglycine (AVG), (C) 2 μmol/L 1-aminocyclopropane-1-carboxylic acid (ACC), and (D) the combination of 5 μmol/L AVG and 2 μmol/L ACC (COMB) treatments. In panels A–D, leaf 0 is a control leaf that had developed prior to the application of treatments. Leaves labeled 1–3 are those that developed subsequently. Scale bars: A–D, 15 mm.



the control plants ($P < 0.001$). Conversely, the ACC- and combination-treated plants produced significantly higher amounts of ethylene than controls, but did not differ from each other.

Detached-leaf experiments

Detached lace plant leaves at the window, mature, and senescent stages of development were put in air-tight vials, and their ethylene and carbon dioxide emissions were measured (Fig. 4). A PROC GLM ANOVA showed a significant difference in both ethylene and carbon dioxide emitted among the developmental stages of lace plant leaves ($P < 0.001$). Mean comparison showed that window-stage leaves produced significantly more ethylene than mature- and senescent-stage leaves. Likewise, senescent-stage leaves emitted significantly higher ethylene levels than mature-stage leaves (Fig. 4A). There was no difference in carbon dioxide produced in window- and senescence-stage leaves; however, both stages produced a significantly higher amount of carbon dioxide than mature leaves (Fig. 4B).

Discussion

The authors of the current study chose to observe the number of leaves formed per plant, as well as the leaf lengths, to determine if chemical treatments had an impact on typical growth and development of the lace plants. Quantitative analysis showed that the number of new leaves formed and leaf lengths did not differ significantly among all four treatments, suggesting that typical lace plant leaf development, excluding perforation formation, had occurred. AVG-treated plants produced significantly fewer perforations compared with all other treatments, indicating that ethylene does play a role in lace plant PCD. It should be noted that the inhibitory effect on perforation formation from 5 μmol/L AVG treatments appeared to subside, as indicated by newly formed leaves at the end of the two-week period (leaf 3 in Fig. 2B). The unabated presence of perforations in the combination treatment indicates that exogenous ACC, the precursor to ethylene, is able to overcome the inhibitory response of AVG. This was expected given that AVG reduces ethylene production by limiting the amount of ACC produced. This observation suggests that reducing ethylene production limits PCD, whereas increasing ethylene output enhances PCD.

Detached-leaf experiments revealed the presence of two ethylene peaks associated with PCD in lace plant leaves: one during the window stage and the other during senescence (Fig. 4A). Ethylene levels are elevated during the window stage (when perforations are being formed), reduced during the mature stage (when perforations are fully formed), and then increase again during senescence of the mature leaves. This suggests the involvement of ethylene climacteric-like behaviour in formation of perforations, as well as leaf senescence. Climacteric behaviour is characterized by low and constant amounts of ethylene (system I pathway) followed by a peak in ethylene production (system II pathway; Katz et al. 2005). It has been shown that ethylene is involved in leaf senescence in other species and that it is produced in a climacteric-like pattern (Aharoni et al. 1979; Aharoni and Lieberman 1979; Jing et al. 2005). The two peaks in ethylene production during the two PCD processes (formation of perforations and leaf senescence) support the involvement of ethylene in lace plant PCD.

Several studies in climacteric behaviour during fruit ripening have shown that carbon dioxide peaks were the result

Fig. 3. Whole-plant experimentation data summary. Data for the control, 5 μmol/L aminoethoxyvinylglycine (AVG), 2 μmol/L 1-aminocyclopropane-1-carboxylic acid (ACC), and the combination of 5 μmol/L AVG and 2 μmol/L ACC (COMB) treatments. The parameters examined include (A) the mean number of leaves formed by plant, (B) the mean mature leaf lengths, (C) the mean percentage of perforation per leaf, and (D) mean detected ethylene. Means represented by different letters are significantly different ($P \leq 0.05$). Error bars represent standard error of $n \geq 14$ plants per treatment.

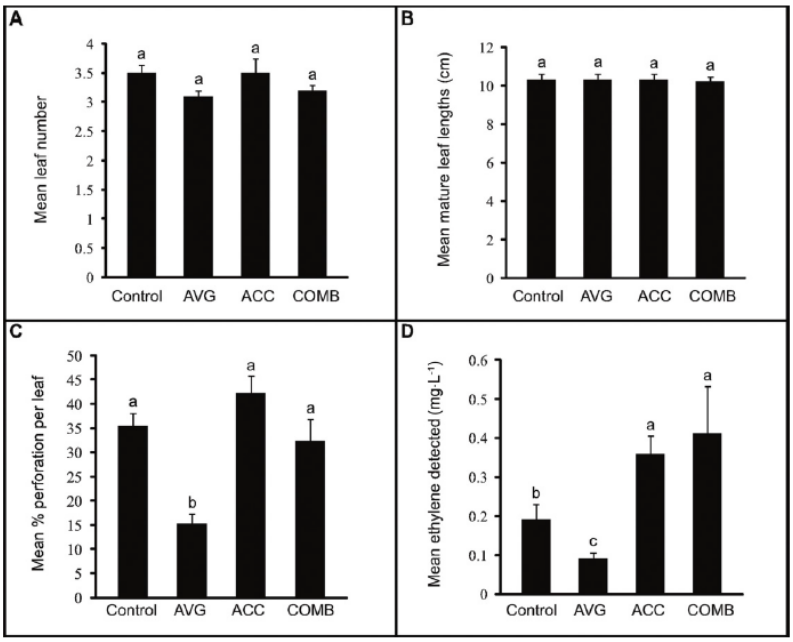
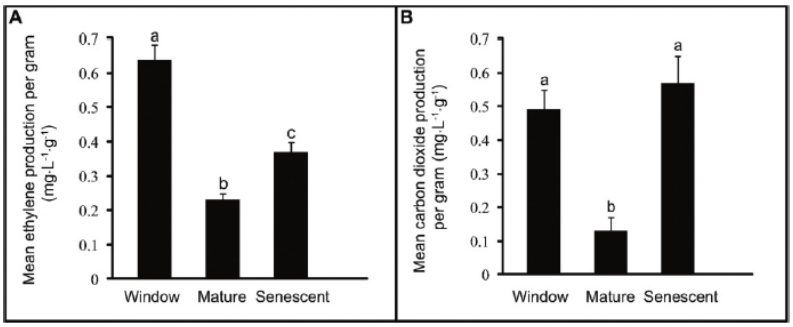


Fig. 4. Gas concentrations detected from detached leaves. (A) Ethylene and (B) carbon dioxide produced per gram of leaf tissue in window-, mature-, or senescent-stage leaves. Means represented by different letters are significantly different ($P \leq 0.05$). Error bars represent standard error of $n \geq 14$ replicates per leaf stage.



of increased cellular respiration rates (McGlasson and Pratt 1964; Hadfield et al. 1995). High ethylene concentrations are thought to initiate an increase in respiration (Brady 1987; Hadfield et al. 1995), and our results in lace plant leaves appear to suggest a similar association. We speculate that fermentation in dying or dead tissues may also be contributing to the increases in carbon dioxide observed. Our study suggests that ethylene and carbon dioxide may be involved in climacteric-like behaviour during lace plant leaf remodelling and senescence.

The data presented here provide evidence that endogenous ethylene plays a role in developmentally regulated PCD in the lace plant. The results show that suppressed ethylene biosynthesis significantly reduces perforation formation without affecting normal leaf development and that this effect can be reversed by stimulating endogenous ethylene production. Ad-

ditionally, to our knowledge, this is the first study to support the involvement of climacteric-like ethylene production in the remodelling of leaves. Future work will investigate the downstream interactions of ethylene with other phytohormones and apply molecular techniques to determine the regulation of genes associated with ethylene and lace plant PCD.

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