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Filling in the gaps: a road map to establish a model system to study developmental programmed cell death

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Abstract

Only a handful of model systems for studying programmed cell death (PCD) exist. The model *Arabidopsis thaliana* has generated a plethora of knowledge, but it is essential to introduce new models to broaden our understanding of the commonalities of PCD. This review focuses on *Aponogeton madagascariensis* (the lace plant) as a choice model to study PCD in vivo. PCD plays a key role in plant development and defence. Thus, identifying key regulators across plants is a priority in the field. The formation of perforations in lace plant leaves in areas called areoles is a striking example of PCD. Cells undergoing PCD within areoles can be easily identified from a loss of their anthocyanin pigmentation. In contrast, cells adjacent to veins, non-PCD cells, retain anthocyanins, creating a gradient of cell death. The spatiotemporal pattern of perforation formation, a gradient of cell death within areoles, and the availability of axenic cultures provide an excellent in vivo system to study mechanisms of developmental PCD. The priorities to further develop this model involve sequencing the genome, establishing transformation protocols, and identifying anthocyanin species to determine their medicinal properties. We discuss practical methodologies and challenges associated with developing the lace plant as a model to study PCD.

Key words: lace plant, novel model organism, anthocyanin, leaf development, perforation formation, gradient of cell death

Programmed cell death models

Programmed cell death (PCD) is a ubiquitous cellular process for the controlled demise of the cell either to achieve a developmental step or in response to stress (Kuriyama and Fukuda 2002; Van Doorn 2005; Williams and Dickman 2008; Van Hautegem et al. 2015; Burke et al. 2020). PCD exists in unicellular microbes to eukaryotes. The intracellular dynamics and chronological order of cell death or apoptosis have been well characterized in animals, but less characterized and studied across plant species. The study of plant PCD study has advanced past Arabidopsis as a core model system to many dicots, monocots (Lee and Chen 2002; Valdivia et al. 2013), gymnosperms (Filonova et al. 2002), green algae (Barreto et al. 2022), and more (Dauphinee and Gunawardena 2015), providing a broader array of plants for comparative studies or the study of unique developmental processes. Still, the field is uncovering the roles of specific enzymes, signals, and transcription factors that help regulate plant-specific PCD processes (Olvera-Carrillo et al. 2015; Daneva et al. 2016; Burke et al. 2020). How the cell death field categorizes different forms of plant PCD under development or stress is still an ongoing debate (Galluzzi et al. 2018). Here, we describe the prominent plant model systems, molecular regulation of PCD, and different classifications of plant PCD. Additionally, we highlight some ongoing work to resolve the big questions about plant PCD and how the emerging experimental model system of the lace plant (*A. madagascariensis*) can become a favourable PCD model in the future.

As reviewed previously by Dauphinee and Gunawardena (2015), *Drosophila melanogaster* and mice models have been used to better understand animal PCD mechanisms by identifying cells dying by apoptosis during salivary gland and limb bud development, respectively (Baechrecke 2003). Furthermore, using the *Caenorhabditis elegans* model helped unravel the genetic mechanism and commonalities within the animal kingdom, leveraging the identification that 131 out of 1090 cells undergo destined PCD from development to maturity (Hengartner and Bryant 2000). Additionally, *C. elegans* is an ideal organism to visualize cell death under differential interference contrast microscopy; cell death in *C. elegans* displays morphological changes such as chromatin condensation, cell shrinkage, nuclear refractivity, and phagocytosis of the cell corpse (Conradt et al. 2016). It is difficult to envision that a single model organism as such can represent the diversity of processes and systems in plants, let alone angiosperms. Therefore, comparative approaches must be used to broaden our development of "models" in various taxa to characterize unique developmental and physiological features to

better understand how these may have evolved. Unlike the *C. elegans* model, there is a lack of well-characterized and easily accessible PCD cells across plant taxa to study plant PCD.

What makes a model species for studying plant PCD?

Model plants have research value based on the expected knowledge of biological processes gained in that species and how they may apply to other species (Borrill 2020). Small size and genome size, short growth period, fast generation time (intrinsic properties), and genetic amenability characterize a model plant. Another tier of model status can be reached by having research groups contribute to an ever-growing communal annotated genomic database or genetic strain repositories (communal properties) (Chang et al. 2016; Provart et al. 2016; Borrill 2020). As described in Sun et al. (2021) and Kress et al. (2022), there are ~450 000 species of Viridiplantae (green plants and green algae) but fewer than 300 "chromosome-scale" genome assemblies representing ~812 species found in the International Sequencing Database Consortium (INDSC; Arita et al. 2021). Of the Viridiplantae, there are ~350 000 angiosperm species (543 sequenced genomes identified by the INDSC), 1000 gymnosperm species (11 sequenced), 13 000 seedless vascular plant species (5 sequenced), and 20 000 bryophyte species (8 sequenced) and 22 000 (249 sequenced) species of green algae (Kress et al. 2022). Advancement in plant biology and plant PCD research has been made using the model Arabidopsis (Holland and Jez 2018). Arabidopsis has exponentially advanced our understanding of plant processes such as stress, defence, development, signalling, and evolution. At the same time, the rapid use of next-generation sequencing (NGS) for studying gene function has widened the scope for new emerging plant models and their respective research communities (Unamba et al. 2015).

Picea abies (Norway spruce) is an economically important conifer species and is one of a few gymnosperm PCD model species. Its large embryos offer an opportunity to observe the developmental PCD of the embryonic suspensor and its well-characterized cell death timeline using live cell imaging (Filonova et al. 2000; Reza et al. 2018). In addition, it is theorized that the female gametophyte produces a PCD-initiating signal to trigger PCD in the embryonic suspensor (Filonova et al. 2002; Zhivotovsky 2002). Norway spruce embryos provide a valuable model for studying embryo development because of their large and accessible size, sequenced genome, and the characterization of specific developmental stages through somatic embryogenesis (Reza et al. 2018). However, the limitation of the Norway spruce model is that its PCD characteristics and molecular dynamics can only be studied in vitro (Zhivotovsky 2002).

Model monocots for PCD

Arabidopsis (a eudicot) is limited in its ability to investigate monocot-specific processes (Brkljacic et al. 2011). Monocots differ from eudicots in cell wall hemicellulose composition, including a lower proportion of pectin. In addition, they have a seed aleurone layer, a different meristem structure, and fibrous root architecture (Brkljacic et al. 2011). Traditionally, monocot model plants have been studied to make biologically parallel discoveries compared to eudicots for important grasses like *Oryza sativa* (rice), *Triticum aestivum* (wheat), and *Zea mays* (maize). These agriculturally important models possess well-characterized PCD processes like endosperm development and aerenchyma formation (Young et al. 1997; Young and Gallie 1999; Gunawardena et al. 2001*a*, *b*; Dauphinee and Gunawardena 2015). However, only a few monocot genomes have been sequenced beyond these species.

Hurdles of studying PCD in plant models

Building on our ability to manipulate plant PCD is of growing interest to tackle declining plant health due to climate change and post-harvest loss (Kacprzyk et al. 2021). Consequently, there is growing momentum in further expanding our understanding of PCD and autophagy in plant stress responses and development (Thanthrige et al. 2021). Cereals and other monocot seeds like purple false brome, maize, wheat, and rice make unique models for studying the consumption of endosperm during embryogenesis (López-Fernández and Maldonado 2015). In addition, maize and rice serve as models for studying PCD during aerenchyma formation. *Zinnia elegans* and Arabidopsis mesophyll cells can also be studied for their ability to differentiate into tracheary elements during xylogenesis (Escamex et al. 2016; Iakimova and Woltering 2017).

Despite successes in elucidating these processes, a problematic hurdle is that plant PCD can occur in a small population of cells surrounded by healthy cells, making it difficult to access or probe within a complex developing tissue (Reape et al. 2008; Kacprzyk et al. 2011). Cell suspension cultures have overcome this hurdle (Kacprzyk et al. 2011; Malerba and Cerana 2021). Cell cultures are also more suitable for live microscopy because morphological changes are easier to observe in isolated cells, and the cells are more amenable to staining. Plant cell cultures of Arabidopsis, tobacco, and *Z. elegans* have been successfully used to study PCD processes (Van Doorn et al. 2011; Babula et al. 2012; Iakimova and Woltering 2017). The lack of natural cell differentiation is the trade-off in using cell cultures for studying PCD. Cell cultures represent a simplified and controllable system to analyze intracellular features to study the progression of plant PCD, but different types of PCD can be observed in cultures using similar stress inducers (Malerba and Cerana 2021; Sychta et al. 2021). Whether a conserved core mechanism exists in whole plants or cell cultures, there has been little research done in vivo to understand the changes that take place in organelles during developmental plant PCD (Lord and Gunawardena 2011; Lord et al. 2011; Wertman et al. 2012).

Single-cell RNA sequencing (scRNA-Seq) has been used successfully to profile individual single plant cell types in Arabidopsis roots and the stems of the nonmodel aquatic monocot duckweed (Denyer et al. 2019; Abramson et al. 2021). scRNA-Seq can quickly profile many cell types in developing plant tissues. However, some cell types, like quiescent centres in roots, have proven challenging to parse out (Denyer et al. 2019). Although profiling transcriptomes is only one omics

Table 1. Timeline of significant events in PCD classification for animals to plants, including a summary of advances.

Reference	Contribution to PCD classification
Kerr et al. (1972)	Defines a set of morphological characteristics as apoptosis
Fukuda (2000)	Suggests a classification system for plant PCD consisting of apoptosis-like cell death, cell death during leaf senescence, and cell death involving the vacuole
Kroemer et al. (2005)	Proposes three distinct categories of animal cell death based on morphological characteristics: apoptosis, autophagy, and necrosis
van Doorn and Woltering (2005)	Contextualizes plant PCD within established metazoan definitions. No example of plant PCD conforms to apoptosis. Some examples may be categorized under autophagy, nonlysosomal cell death linked to necrosis.
van Doorn et al. (2011)	Proposes a classification system for plant PCD as vacuolar cell death and necrosis
van Doorn (2011)	Proposes revision for previously suggested classification of plant PCD as autolytic and nonautolytic cell death
Reape and McCabe (2013)	Argues that protoplast retraction may be an active and fundamental part of plant PCD, and a key hallmark in distinguishing between apoptosis-like PCD and necrosis
Galluzi et al. (2018)	Proposes updated classification for animal PCD based primarily on molecular characteristics

approach to help finely unravel core PCD regulators, it shows promise to distinguish PCD genes critical to plant PCD mediation, even in nonmodel organisms (Alfieri et al. 2022). Finding a suitable plant model that provides easily accessible differentiating cells undergoing PCD would give the field a valuable system to resolve conserved pathways in plant PCD.

Classifications, comparisons, and gaps in PCD

Classifications of animal and plant PCD

PCD classification began over half a century ago with a definition for apoptosis initially proposed to describe morphological changes during controlled cell death (Kerr et al. 1972). These include cell shrinkage, chromatin condensation, membrane blebbing, nuclear fragmentation, and plasma membrane retraction (Kerr et al. 1972; Kroemer et al. 2005; Elmore 2007). These characteristics differ from those seen in uncontrolled cell death, termed necrosis, a passive process usually triggered by extreme or irreversible stress where cellular volume increases, organelles swell, the plasma membrane ruptures, and cellular bodies spill outside the cell (Kerr et al. 1972; Kroemer et al. 2005; Dauphinee et al. 2014). The term autophagy was also originally introduced in the classification scheme to describe cell death involving the absence of chromatin condensation and an increase in the shuttling of materials for degradation by double-membrane vesicles (autophagosomes) (Kroemer et al. 2009). More recently, molecular definitions have been the focus of refining the classification scheme of PCD in animals (Galluzzi et al. 2018).

Though much progress has been (and continues to be) made in classifying animal PCD, this system does not accurately encompass the morphological or molecular characteristics of plant PCD. Fukuda (2000) proposed a classification system for plant PCD based on cytological characteristics: apoptosis-like cell death, cell death during leaf senescence, and cell death involving the vacuole. Attempts were then made to contextualize plant PCD within the established metazoan definition. However, Van Doorn and Woltering (2005) claimed that apoptosis was an inaccurate classification since no plant cell showcases true apoptosis due to the cell wall preventing phagocytosis. Subsequently, the classification of plant PCD independent from animal definitions was proposed as vacuolar cell death and necrosis (van Doorn et al. 2011). Vacuolar cell death is carried out through a combination of autophagy-like processes and the release of hydrolases from the collapse of the vacuole resulting in the removal of cell contents (see Table 1 for a summary of perspectives on the classification of PCD). In contrast, necrosis consists of the premature collapse of the plasma membrane and the absence of vacuolar cell death features (van Doorn et al. 2011). Shortly after, van Doorn (2011) revised previous classifications and proposed two new classifications: autolytic and nonautolytic cell death. In addition, van Doorn (2011) suggested that autolytic cell death was loosely comparable to autophagic cell death in animal cells, while nonautolytic can be linked to necrosis. Following this, Reape and McCabe (2013) argued that distinguishing between cells that have undergone protoplast retraction and those that have not is an essential hallmark between apoptosis-like cell death and necrosis. Specifically, they argue that protoplast retraction may be an active part of the PCD process as opposed to a consequence of the stress generated by it (Reape and McCabe 2013). Kacprzyk et al. (2017) found that disrupting calcium flux signalling, ATP synthesis, and mitochondrial permeability inhibited protoplast retraction, suggesting it is part of the active biological process of apoptosis-like PCD. Protoplast retraction should, therefore, be a feature considered in future cell death classification schemes as its implications render the system put forth by van Doorn et al. (2011) unable to accurately capture the differentiation between categories of PCD involving protoplast retraction and those that do not.

Debate remains on the appropriate classification of plant PCD processes, and a system is yet to be widely adopted. However, it is agreed that more morphological, biochemical, and molecular investigation using model and nonmodel plant PCD processes are needed to sort “atypical” types of plant cell death (Bozhkov and Lam 2011; van Doorn 2011; van Doorn et al. 2011).

Comparisons of animal and plant PCD

Previous definitions of PCD have been morphological. However, progress is being made to uncover molecular commonalities between types of plant PCD (Daneva et al. 2016) and develop molecular definitions of animal PCD (Galluzzi et al. 2018).

Plant and animal PCD differ in some morphological and molecular hallmarks. Plant-specific organelles such as chloroplasts, cell walls, and large vacuoles play roles in the morphological and molecular underpinnings of plant PCD and contribute to its divergence from animal PCD. Chloroplasts are involved in energy production and, coupled with the cell wall, can produce reactive oxygen species (ROS) ultimately triggering PCD. Vacuoles clear cellular contents through autophagy and release hydrolytic contents (Yamada et al. 2005; Hatsugai et al. 2006; Rantong and Gunawardena 2018). Plant cells also possess a rigid cell wall that prevents cell fragmentation and lack key animal apoptotic components like Bcl-2 family proteins and true caspases (Minina et al. 2021). Additionally, plant-specific growth regulators are often involved in PCD; these include ethylene (Yakimova et al. 2006; Lombardi et al. 2012), auxin (Kacprzyk et al. 2022); jasmonic, abscisic, and gibberellic acid (Van Durme and Nowack 2016).

In animals, apoptotic pathways have been well characterized and, in most cases, result from either mitochondrial membrane permeabilization, activation of caspases, or both (Savitskaya and Onishchenko 2015). The common involvement of caspases has made them synonymous with apoptosis, though they are not strictly necessary for true apoptosis (Orrenius et al. 2003). Even though there is no evidence that plant genomes have conserved some central apoptotic regulators such as caspases, some commonalities between plant and animal PCD remain. For instance, metacaspases and various protease families in plants exhibit caspase-like activity (Lam and Zhang 2012; Fagundes et al. 2015; Balakireva and Zamyatnin 2019; Minina et al. 2020). Furthermore, animal apoptosis and plant PCD may have evolved from a common ancestor to cater to their cell modalities, morphologies, and contents (Minina et al. 2021). Additionally, there is evidence of pro- and anti-apoptotic animal proteins exhibiting functionality in plant models, as well as the lace plant itself (Lord and Gunawardena 2012*a*, 2012*b*).

Comparisons can also be made between instances of plant PCD. In Arabidopsis, distinct transcription factors were found between developmental and environmental PCD processes (Olvera-Carrillo et al. 2015). However, many were shared between developmental PCD processes, indicating potentially conserved pathways in developmental PCD. Jiang et al. (2021) reviewed recent progress in uncovering molecular regulation of plant developmental PCD events. Plant PCD processes involving male and female reproductive tissues, such as tapetum cell deletion and nucellus degradation are regulated by the crucial timing of transcription factors. For example, tapetum cell deletion is promoted by a few basic helix-loop-helix (bHLH) transcription factors. In rice, a specific tapetum degeneration retardation factor (OsTDR) activates a requisite cysteine protease gene that induces tapetal PCD (Xie et al. 2020; Jiang et al. 2021). From embryogenesis to leaf senescence, PCD can occur throughout a plant's life cycle revealing several molecular and morphological characteristics that may indicate that multiple overlapping PCD pathways evolved and exist in one plant species (Kacprzyk et al. 2011).

Issues and gaps in plant developmental PCD research

As outlined and discussed by Buono et al. (2019), there is still a lack of coherent understanding of the exact mechanisms regulating even the most well-studied model systems. Over the last few decades, many PCD pathways essential for growth and reproduction have been described in plants (Van Durme and Nowack 2016; Buono et al. 2019; Cubría-Radío and Nowack 2019; Xie et al. 2022). Pieces of the signalling cascade have been characterized and manipulated to build partial but not complete hypothetical mechanisms and models. However, there are very few examples of comprehensive biochemical pathways to explain the control of developmental PCD processes.

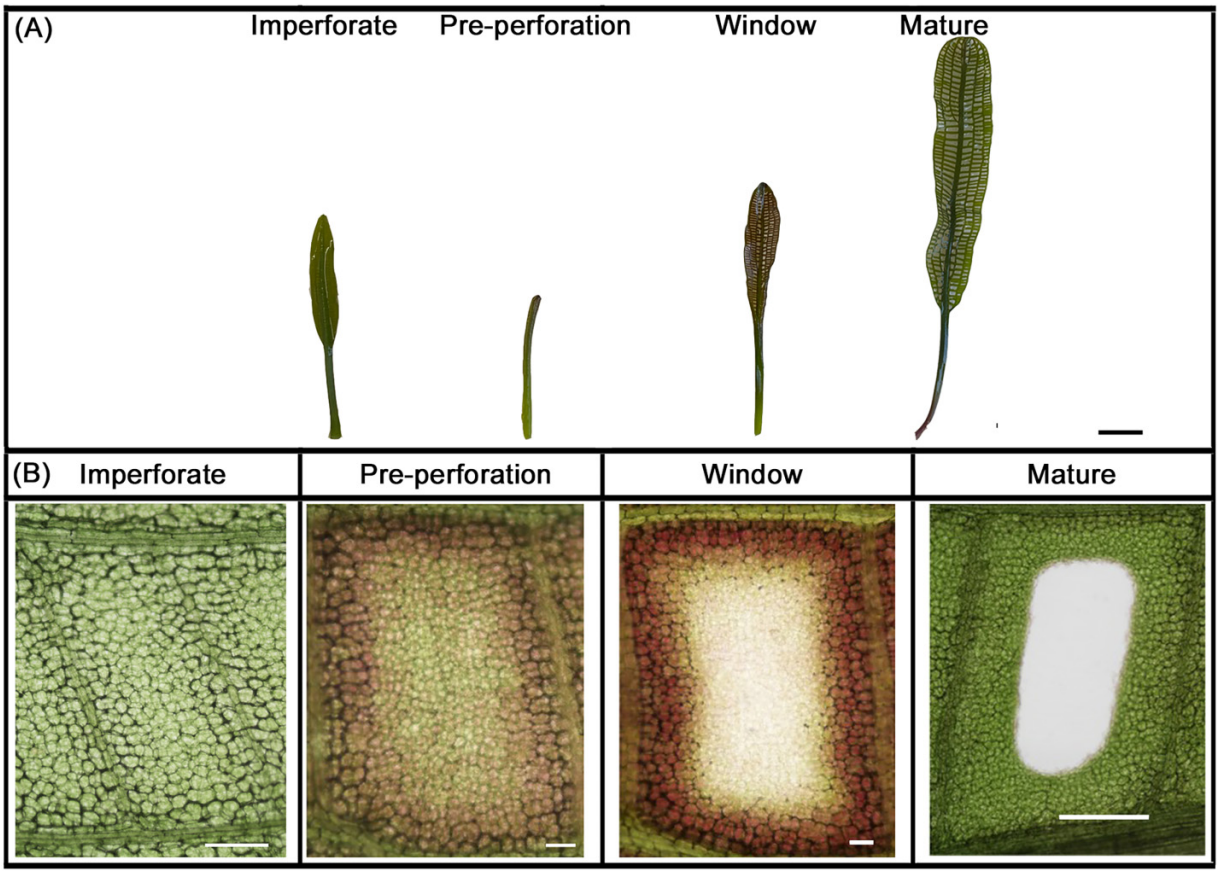
Though studying and labelling plant PCD process categories is challenging (Van Durme and Nowack 2016), the lack of agreed-upon plant PCD nomenclature makes discussing and comparing processes difficult. Cell degradation with PCD characteristics does not guarantee plant cell death execution, as can be seen in phloem sieve elements (Furuta et al. 2014; Van Durme and Nowack 2016). There are also examples of cell reduction without PCD, such as fusion events of nonreceptive synergid cells in Arabidopsis (Maruyama et al. 2015; Van Durme and Nowack 2016). Comparative analyses of PCD regulation across various plant cell types such as in Arabidopsis are a viable strategy for resolving the mechanisms of plant PCD, such as in Arabidopsis by Olvera-Carrillo et al. (2015). It is predicted that novel in vivo plant systems for studying PCD will further advance the field's understanding of plant PCD regulation (Kacprzyk et al. 2011). Therefore, we reiterate the importance of broadening the use of multiple plant models and nonmodels for future comparative studies.

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

The lace plant: what we know about this emerging model

Aponogeton madagascariensis is one of the 57 species of Aponogetonaceae but the only one that forms perforations

Fig. 1. Lace plant leaf developmental stages. Individual leaf stages harvested from whole lace plants (A) and micrographs of areoles (B) show the progress of PCD. Imperforate leaves are the first three to four leaves to emerge from the corm, and they do not accumulate anthocyanin or form perforations. Successive leaves, called adult leaves, emerge as pre-perforation stage furled leaves. As leaves unfurl, they enter the window stage, where anthocyanin is visible only in areas near veins. Next, the centrally located cells (PCD cells) enter PCD to initiate the formation of a hole. In the mature stage, PCD has ceased, and a hole is visible within the areoles. The four to five cell layers axial to the veins (NPCD cells) survive. Scale bars: (A) 2 cm and (B) 70 μm.



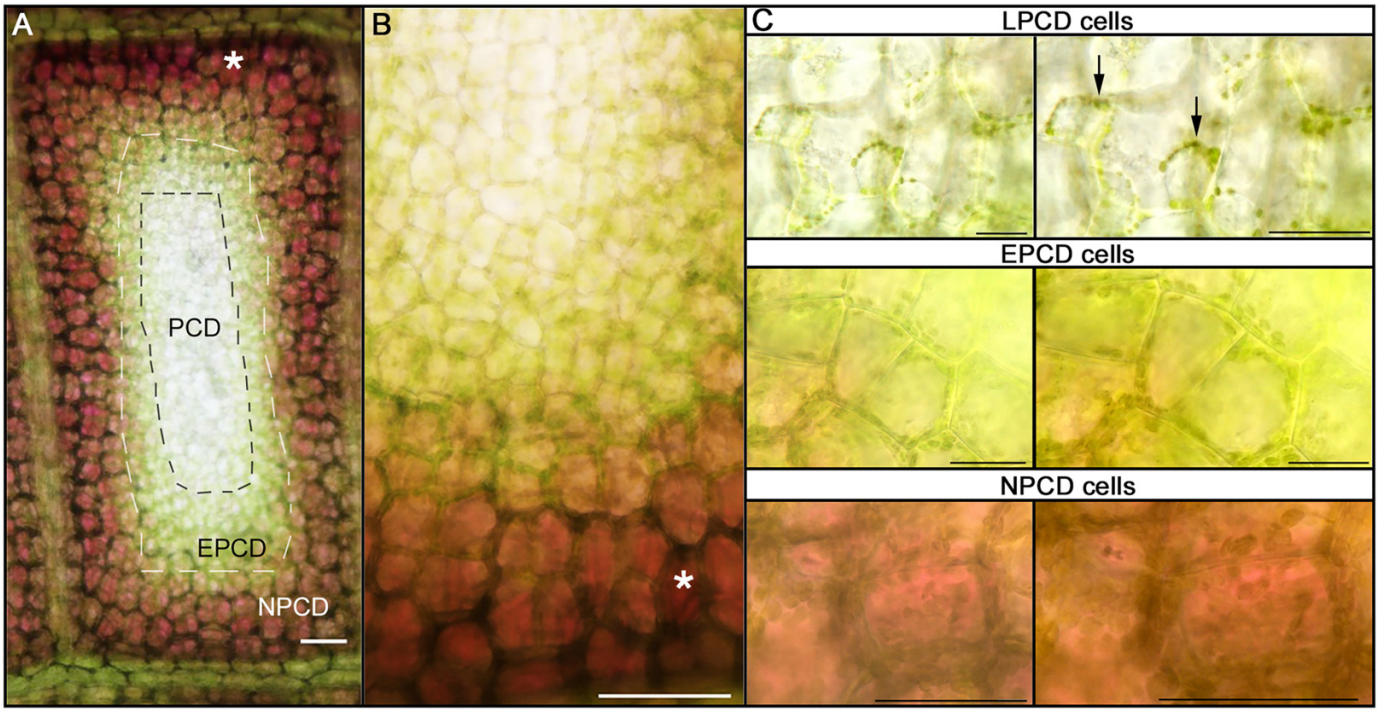
during leaf development (van Bruggen 1985, 1998). Although a few species with perforated leaves in Araceae also belong to the Alismatales, it is unknown if perforation formation has a common evolutionary origin (Gunawardena 2008). Therefore, the evolutionary advantage of leaf perforations in aquatic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

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

Predictability of perforation formation

The predictable spatiotemporal nature of PCD within lattice-like veins of mature lace plant leaves presents a unique opportunity to develop a model system to study PCD (Gunawardena et al. 2004). The first three to four leaves that emerge from the corm do not produce perforations and are observed to senesce relatively quickly after maturity. The leaves that follow (known as adult leaves) are enriched with a red pigment from the antioxidant anthocyanin and form perforations. The developmental stages of leaves are subdivided into pre-perforation, window, perforation formation, perforation expansion, and mature (Fig. 1). Newly emerged leaves are in the pre-perforation stage; they are furled, and the mesophyll cells of the lamina are full of anthocyanin. The initiation of PCD in window stage leaves is visible as a loss of anthocyanin pigmentation. In the perforation formation stage, the deletion of cells begins to progress outwards from the center of the areole, a region framed by longitudinal and transverse veins. During perforation expansion, the hole formed by PCD continues to expand until it reaches four to five cells from the veins. At maturity, the perforation is complete, and PCD has ceased (Fig. 1). Mesophyll cells at the perforation border transdifferentiate into epidermal cells protected

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



from water loss or infection by a suberin layer (Gunawardena 2007).

Live cell imaging of lace plant PCD

Aquatic lace plant leaves are ideal for live cell imaging due to their near-translucent nature, which has been useful for characterizing the chronological subcellular events that take place during PCD (Fig. 2; Lord et al. 2013; Dauphinee et al. 2017, 2019; Lord and Gunawardena 2013). Wertman et al. (2012) previously detailed the chronological order of lace plant developmental PCD using a combination of conventional light microscopy, transmission electron microscopy, and laser scanning confocal microscopy. Cells central to the areole undergo the first sign of PCD differentiation with the loss of anthocyanin pigment, but the cellular signalling that controls this change is unknown. This change in pigment is also observed in the senescence of petals in Arabidopsis, theorized to be a result of changes in selective permeability or pH of the vacuole (van Doorn 2004; Wertman et al. 2012). Next, in early-PCD cells (EPCD cells, Fig. 2C, Video S1) comes the loss of chlorophyll along with a decrease in chloroplast size and number (Wright et al. 2009; Wertman et al. 2012), also observed in Arabidopsis leaf senescence (Lim et al. 2007). Actin microfilaments re-organized from thin and organized to thicker and disorganized in arrangement before degradation, a standard feature found in early plant PCD cells, including Norway spruce suspensor deletion (Filonova et al. 2000; Smertenko and Franklin-Tong 2011). This feature is believed to occur to prime microfilaments for being targeted by upstream caspase-like proteases (Wertman et al. 2012). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive PCD cell nuclei indicate deoxyribonucleic acid fragmentation is detectable in lace plant PCD cells during actin degradation, followed by tonoplast changes (Wertman et al. 2012). Tonoplast rupture is common in plant PCD processes like tracheary element differentiation (Iakimova and Woltering 2017), suspensor deletion (Reza et al. 2018), and aerenchyma formation (Ni et al. 2014). Once the tonoplast ruptures, vacuolar aggregates cease their Brownian movement (Wertman et al. 2012), the nucleus condenses, the mitochondrial membrane potential is lost, and the plasma membrane retracts (~20 min after tonoplast rupture). Early- and late-PCD cells (LPCD, Fig. 2C, Video S2) have aggregates in their vacuole. There is evidence that chloroplasts are brought to the vacuole by autophagy (Wright et al. 2009; Dauphinee et al. 2019). In addition, an interesting observation in early and LPCD cells is the formation of perinuclear chloroplast aggregations that are common during developmental PCD (Wright et al. 2009; Lord et al. 2013; Dauphinee et al. 2014). The exact process is also observed in tobacco protoplasts and leaf aerenchyma formation in *Typha angustifolia* (Wright et al. 2009; Lord et al. 2011; Wertman et al. 2012;Ni et al. 2014). However, it is unknown if this morphological

Fig. 3. Whole plant growth under axenic conditions in 150 mL magenta boxes. New corm embedded in solid 1% agar MS media in magenta boxes (Day 0). Leaves emerge in a heteroblastic series beginning with imperforate leaves (I), followed by adult leaves emerging as furled pre-perforation leaves (P), which will develop into window leaves (W) where PCD is active, and anthocyanin gradients are visible. By Day 30, at least one of each developmental leaf stage is present in the culture. At least three perforated mature leaves (M) are visible, indicating that the whole plant is ready for pharmacological experimentation. Scale bars: 2 cm.

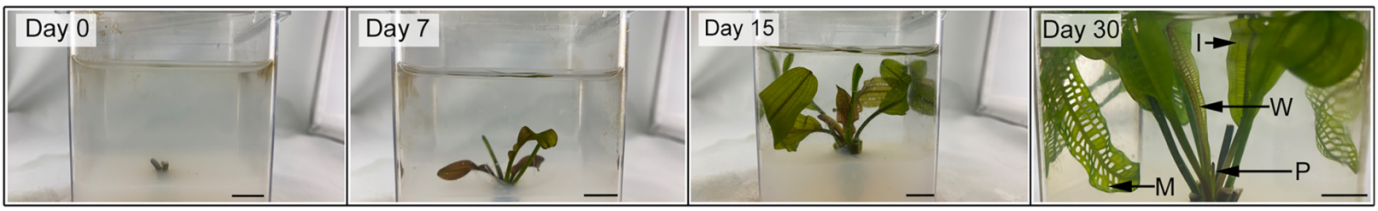


Fig. 4. Tracking newly furled leaf growth in 1 L glass culture jars. Furled adult or pre-perforation leaves (*black arrows*) emerge from the corm successively after three to four imperforate leaves have already emerged (Day 1). The laminar tissue of pre-perforation leaves has a red pigmentation from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

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

Cells proximal to the vasculature retain their anthocyanin (in the mesophyll layer) and chlorophyll pigmentation, maintaining homeostasis throughout the formation of perforations (non-PCD cells (NPCD), Fig. 2C, Video S3). NPCD cells maintain function during perforation formation providing a suitable control group to test and observe intracellular changes in EPCD and LPCD cells. This natural gradient of PCD is accessible in a single field of view, a distinct advantage over other plant PCD systems.

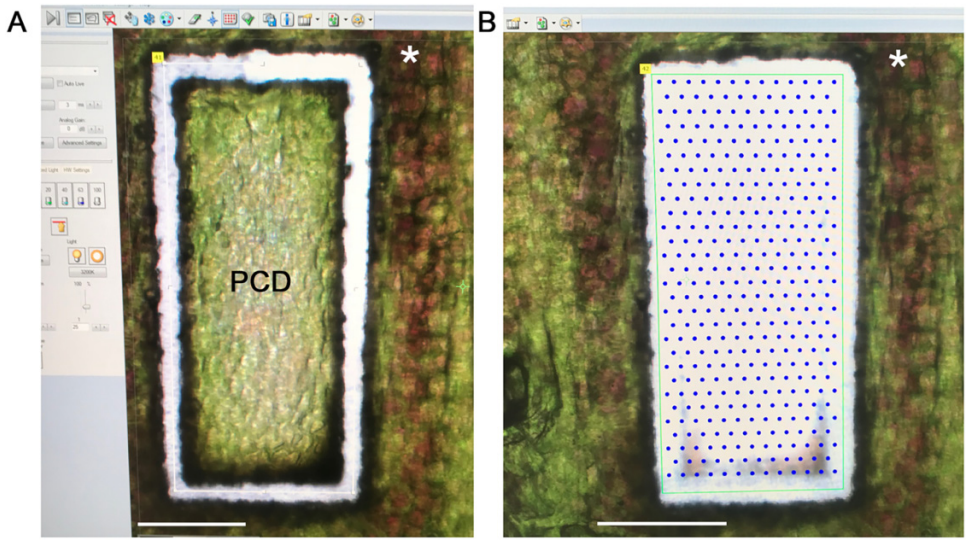
Sterile culture system

Although lace plants can be maintained in aquariums, they are propagated in sterile, controlled environments to avoid environmental disturbances and maintain a consistent leaf morphology and PCD pattern (Dauphinee and Gunawardena 2015). This transfer to a controlled sterile environment represents a trade-off where lace plant leaves are generally smaller than aquarium plants. Lace plants are propagated as cleaned corms under established protocols in G47 magenta box containers (Fig. 3) or 1 L glass jar vessels (Fig. 4). Cultures are supplemented with Murashige and Skoog (MS) media containing 1% agar and 3% sucrose (Fig. 3) and grown at 24 °C under 12 h light:12 h dark cycle without additional hormones to promote growth. Pharmacological whole-plant treatments in axenic cultures make for consistent conditions for optimal (i) protein and RNA extractions and (ii) monitoring leaf growth and morphological changes (Fig. 3). Whole plants can, therefore, be propagated and subjected to different types of treatment for weeks without infection, signs of necrosis, or accumulation of biproducts. In comparison, embryos of Norway spruce used to study suspensor deletion PCD need to be stimulated for development with growth regulators, a limitation the lace plant system does not experience (Högberg et al. 1998). In addition, the constant recycling of removing overgrown shoots and cleaning lace plant mother corms before transplanting to a new culture magenta box provides highly repeatable experiments using plants with little genetic variation.

Summary of molecular lace plant PCD findings to date

Twenty years of lace plant research have provided the plant PCD community with a series of observations of events that characterize lace plant leaf remodelling on a morphological, biochemical, and molecular level. The inhibition of ethylene biosynthesis produces leaves with fewer perforations (Dauphinee et al. 2012; Rantong et al. 2015). Downstream of ethylene, caspase-1-like activities triggered by the release of a mitochondrial signal have been postulated but not identified (Lord et al. 2013). The inhibition and promotion of lace plant heat shock protein 70 (Hsp70) in early developing leaves also affect anthocyanin levels, caspase-like protease activity, and the formation of perforations. However, the exact mechanism of its connection to PCD has not been elucidated (Rowarth et al. 2020). The morphological and cellular changes

Fig. 5. Laser capture-microdissection and catapult of leaf tissue. The user interface of the Zeiss Laser capture microdissector shows fresh window stage leaf tissue under 4× magnification. Laser cuts around PCD cells (PCD), separating them from healthy NPCD tissue (*) (A). The dotted region selects for desired tissue to be catapulted by air pulse into a microcentrifuge tube (B). Scale bars: 150 μm.



during lace plant leaf development are well categorized. However, the genetic control underpinning lace plant PCD remains elusive (Rantong and Gunawardena 2015; Rantong et al. 2016), partly due to a lack of genetic information for the Aponogetonaceae family. However, advancements in comparative RNA sequencing (RNA-Seq) analysis between PCD and NPCD-like cells in other plants have helped profile key differentially expressed genes (DEGs) that resemble PCD regulators (Rowarth et al. 2021).

To date, lace plant experiments have taken advantage of sterile culture systems, long-term live cell imaging, protoplast extractions, successful Western blotting protocols, and RNA extractions from whole leaves or cell populations using laser capture microdissection (Fig. 5, Video S4; Rowarth et al. 2020, 2021). Currently, the lace plant model system is limited from reaching its full potential due to the lack of genomic data, mutants, and a robust protocol for genetic transformation. For example, *Agrobacterium tumefaciens* strain GV2260 has been used to transform eudicots and monocots and, in optimal environments, was ~25% successful in transforming lace plant shoot apical meristem (SAM) explants (Gunawardena lab (unpublished data, 2017)). On the other hand, callus tissue transformation produced a limited number of regenerated leaves, making it a less viable process than SAM transformation.

Model status of the lace plant

New avenues to explore in lace plant PCD knowledge

Even with the established protocols for propagation and live cell imaging, the lace plant system still needs improved protocols to expand genomic and molecular information. For example, elucidating essential genes that regulate lace plant PCD was challenging due to little prior molecular work, sequencing data, or valid transformational protocols. Recently, progress was made in tackling this issue through a de novo RNA-seq project of the lace plant leaf transcriptome (Rowarth et al. 2021). In addition, optimizing RNA and protein extraction and detection workflows have enabled lace plant research to expand into the characterization of proteins.

Building the lace plant to model status

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

Transcriptome analyses of lace plant leaves, NPCD and PCD cells using RNA-Seq

Based on our comparative analysis, NPCD and PCD cells differentiate by balancing plant hormone and transcription factor activities that both promote and inhibit the PCD pathway in lace plants. Alternative splicing (AS) of mRNA variants occurs with 20%–30% of genes in Arabidopsis and rice, demonstrating a critical role of AS in gene expression (Campbell et al. 2006; Wang and Brendel 2006; Gassmann 2008; Baralle and Giudice 2017) and promotion of plant development and disease resistance (Reddy et al. 2013). Furthermore, AS events linked to Hsp81-2, 1-aminocyclopropane-1-carboxylic acid synthase, and WRKY33 have been demonstrated to mediate stress-induced PCD in *Vitis amurensis* (Xu et al. 2014) and in *Gossypium davidsonii* under salt stress (Zhu et al. 2018).

Table 2. Individual genomic information of select members of Aponogetonaceae compared to the lace plant.

Aponogetonaceae species	Ploidy level	Genome size (Gb/2 C)	2N chromosomes	Reference
Aponogeton madagascariensis (lace plant)	10X	5.083	78	Šmarda et al. (2014)
Aponogeton undulatus			70–74	Šmarda et al. (2014)
Aponogeton natans			80	Šmarda et al. (2014)
Aponogeton crispus			32	Šmarda et al. (2014)
Aponogeton longiplumulosus	10X	5.357	78	Šmarda et al. (2014)

Note: Most data were adapted from Šmarda et al. (2014).

These genes are also differentially expressed between NPCD and PCD cells of the lace plant. The results support the notion that quantifying AS profiles coupled with an liquid chromatography/mass spectrometry (LC/MS) study across the leaf and cell type transcriptomes would prove helpful in identifying mRNA compositions that may mediate lace plant PCD.

Our de novo RNA-Seq analysis has generated ~22 000 nonredundant protein-coding genes, but this transcriptome's completeness is unknown. To update these gene annotations, there must either be an improved assembly or an improved gene prediction protocol. One possibility includes single-molecule, long-read genome sequencing using PacBio Iso-Seq and Nanopore RNA-Seq to identify new genes and isoforms to revise previously sequenced isoforms (Wang et al. 2016; Van Bel et al. 2019). Complementary sequencing methods will increase the completeness of the lace plant genome (Van Bel et al. 2019).

Once a reference genome is completed, all protein-coding genes from this lace plant transcriptome project will be uploaded to NCBI (SRA accession IDs: SRR10524134-SR10524151 and BIOPROJECTID: PRJNA591467). Most of the protein-coding genes described in Rowarth et al. (2021) are closely aligned with Arabidopsis, *Oryza sativa*, and *Zea mays* homologs. Of the upregulated PCD-related genes across developmental lace plant leaf stages, homologs for Bcl-2-associated athanogene (Bag) family proteins, autophagy-related genes (Atgs), metacaspases, and vacuolar processing enzymes were detected, and a large majority of these families possessed high sequence similarity to Arabidopsis homologs. Transient expression of these PCD-related lace plant homolog genes by transformation into Arabidopsis, Norway spruce, or other transformable monocots will help resolve if they play a role in inducing a form of cell death in other developmental PCD models (Reza et al. 2018). In addition, our findings helped to identify conserved genes between aquatic monocots, gymnosperms, and other angiosperms but will also connect the role of these genes in the regulation of developmental PCD.

scRNA-Seq will further advance our work to characterize cell types within the NPCD and PCD cell populations. For example, many scRNA-Seq studies have resolved root, meristem, or inflorescences to characterize specific cell type trajectories (Denyer et al. 2019; Abramson et al. 2021). In addition, using scRNA-Seq in the lace plant system may provide an opportunity to characterize spatial variations in gene expression patterns during cell differentiation from healthy cells to PCD cells on a developmental time scale similar to what was achieved in duckweeds.

An optimized protocol for protein quantification

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

Protein subcellular localization using confocal microscopy has been useful within the NPCD/PCD cell gradient to characterize proteins involved in PCD. In addition, monoclonal antibodies have been used successfully to probe for several proteins (Dauphinee et al. 2019; Rowarth et al. 2020) within fixed cells. However, confirming quantification levels between PCD and NPCD cells may prove challenging, given that PCD cells are more susceptible to the permeation of stains and antibodies than NPCD cells.

Lace plant genome and genetics

More information on the lace plant genome must be obtained before it can be established as a bona fide model. The lace plant's sequenced "reference genome" will provide a complete reference library for future comparisons with other plant species. The *Aponogeton* group of genomes still falls under the "dark clades" of green plant life that possess little to no genomic information (Kress et al. 2022). At the same time, the gold standard approach to building an optimal reference genome is changing, and future technologies will allow chromosome and structural sequence information (Ballouz et al. 2019; Kress et al. 2022). Here we will briefly give an overview of the current understanding of the lace plant and *Aponogeton* genome information.

Little is known about the genomes of the Aponogetonaceae family. However, Šmarda et al. (2014) estimated ploidy level, genome size, and chromosome counts in a few *Aponogeton* species (Table 2; Šmarda et al. 2014). Additionally, the inconsistency of methods used to extract genomic material

Table 3. Individual genomic information of selected model and nonmodel plant groups with varying genome complexities.

Species (common name)	Ploidy	Genome size (Mb/2 C)	Chromosomes	References
Angiosperm eudicot				
Arabidopsis thaliana	2X	135	10	*
Vitis vinifera (grape)	2X	487	38	*
Brassica napus (rapeseed)	4X	1130	38	Chalhoub et al. (2014)
Angiosperm, monocot, terrestrial				
Brachypodium distachyon (purple false brome)	2X	272	10	Garvin (2007); Scholthof et al. (2018)
Hordeum vulgare (barley)	2X	5100	14	*
Miscanthus sinensis (Chinese silver grass)	2X	5500	38	Rayburn et al. (2009)
Oropetium thomaeum (resurrection plant)	2X	245	18	VanBuren et al. (2015)
Oryza sativa (rice)	2X	372	24	*
Paris japonica (Andromeda japonica)	8X	148 000	40	Pellicer et al. (2010)
Setaria viridis (green fox tail)	2X	395	18	Bennetzen et al. (2012)
Triticum aestivum (wheat)	6X	17 000	14	*
Zea mays (corn)	2X	2300	20	Schnable et al. (2009)
Gymnosperms				
Picea abies (Norway spruce)	2X	20 000	12	Nystedt et al. (2013)
Seedless vascular plants				
Azolla filiculoides (water fern)	2X	740	44	https://www.azollagenome.net
Ceratopteris richardii (C-fern)	2X	22 000	78	Sessa et al. (2014)
Selaginella moellendorffii (spike moss)	2X	106	20	Banks et al. (2011)
Bryophytes				
Anthoceros agresis (field hornwort)	2X	83	12	Szövényi et al. (2015)
Ceratodon purpureus (fire moss)	2X	340		Thornton et al. (2005)
Marchantia polymorpha (liverwort)		226		*
Physcomitrella patens (earth moss)	2X	473	54	*

Note: Most data are adapted from Chang et al. (2016). *, https://phytozome.jgi.doe.gov/pz/portal.html.

and karyotype from lace plant tissue for detection may be a source of error (Šmarda et al. 2012, 2014). The plastomes of five different *Aponogeton* species have also been analyzed and found to be suitable protein-coding genes for future intrageneric barcoding (Mwanzia et al. 2020). The Gunawardena lab is currently estimating the genome size of three different lace plant isolates using flow cytometry in collaboration with Drs. Sonja Yakovlev, Université Paris-Sud, Béatrice Satiat-Jeunemaitre, Institut de Biologie Intégrative, Michaël Bourge, and Nicolas Valentin, Imagerie-Gif, Plateforme de Cytométrie, France.

The genomes of approximately 788 plant species in reference or draft form (613) have been published (Bolger et al. 2017; Schmidt et al. 2017; Sun et al. 2021; Van Bel et al. 2021). However, other research groups have sequenced bigger genomes like Norway spruce, *Brassica napus* L. (rapeseed), and *Paris japonica* (Table 3). The manipulation of genomes and advances in sequencing technologies allow the accession of complex polyploid genomes. The combined use of short-read, long-read, single cell-read, chromosome conformation capture scaffolding (Hi-C) and Strand-Seq (Sun et al. 2021) increases the quality of polyploid assemblies. Examples of improved polyploid genomes include *Chenopodium quinoa* (Jarvis et al. 2017; Jiao et al. 2017), *Gossypium arboretum* (Huang et al. 2020), and *Arachis hypogaea* (Chen et al. 2019). An integrated strategy of multiple sequencing approaches and flow-sorted individual chromosomes is paving the way for future high-quality chromosome-level assemblies for the lace plant.

As reviewed by Kress et al. (2022), there is a need for a gold-standard sequencing and annotation protocol for these "dark clade" reference genomes like *Aponogeton*. With a large and complex genome, the first plan of attack is to assess gene space using short-read sequencing (Kress et al. 2022). The chromosome-level genome assemblies will need advanced technological resources for plant groups with large genomes. As sequencing, assembly, and annotation costs fall, and long-read sequencing improves, we are confident that future chromosome-level genome assembly will be feasible for plants with a large genome (Kress et al. 2022).

Gene editing

We must establish a gene editing method for the lace plant and create mutant lines to study gene function to reach comprehensive model status along with Arabidopsis (Holland and Jez 2018). The introduction of exogenous genes, the regeneration of transgenic cells, and the selection for and regeneration of transgenic plants have been successfully achieved in duckweeds (Yamamoto et al. 2001; Yang et al. 2020). Additionally, *Setaria viridis* can be transformed using a floral dip technique using a suspension culture of tobacco extract and

AGL1 *Agrobacterium tumefaciens* and acetosyringone (Martins et al. 2015). Oil palm (*Elaeis guineensis*) protoplasts can be transformed using polyethylene glycol mediation to generate nonchimeric callus with a high degree of success (Masani et al. 2014).

Agrobacterium-mediated transformation for monocot plants has had limited success due to their inability to counter wound damage by releasing phenolic compounds (Xi et al. 2018). However, the successful transformation of callus tissue harvested from a lace plant mother corm, confirmed by the detection of green fluorescent protein fluorescence in newly formed lace plant shoots, was achieved using 5% sucrose, 100 μmol/L acetosyringone, 10 μmol/L aminoethoxyurinal glycine, 15 μmol/L phloroglucinol pH 5.5 over 12 weeks with a ~25% success rate (Gunawardena Lab (unpublished data, 2017)).

Delivering double-stranded RNA into plant tissue using laser-assisted RNA delivery was successfully achieved with citrus leaves (Killiny et al. 2021). However, RNA interference methods can be flawed due to possible off-targeting effects and may not be the optimal mode of studying gene function (Senthil-Kumar and Mysore 2011; Kola et al. 2015). For a more stable transformation and a better understanding of genes in Aponogetonaceae, a CRISPR/Cas9 system for genome editing should be developed. CRISPR/Cas9-mediated targeted mutagenesis has been successfully optimized in the duckweed *Lemna aequinoctialis* via EHA105 *Agrobacterium* transformation with a rice ubiquitin promoter within the vector (Liu et al. 2019). Liu et al. (2019) discussed that the increased transformation efficiency in *Lemna aequinoctialis* over *Lemna minor* was due to an optimized sonication and vacuum filtration protocol. This method has also been successful in cowpea (*Vigna unguiculata*), chickpea (*Cicer arientinum*), and banana (*Musa* cv. AAB) (Indurker et al. 2010; Bakshi et al. 2011; Subramanyam et al. 2011), and could also be used to improve lace plant transformation.

Additionally, the CRISPR/Cas9 system can require labour-intensive trial and error runs to generate large constructs and multiple cassettes successfully (Wang et al. 2017). Nevertheless, advanced gene editing like CRISPR-Cas9 or 13 has many potential benefits for studying PCD control in the PCD/NPCD gradient of lace plant leaves, including (i) recovery or silencing of functional proteins, (ii) promotion of specific AS events, or (iii) tracking the shuttling of specific RNA inside cells with fluorescent detection. However, one of the limitations of using this biotechnology in studying plant PCD is the unknown biochemical effects on the Cas9/13 catalytic domain within eukaryotes and the occurrence of chimeric cells within mutant plants (Wang et al. 2017).

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

Limitations of growing lace plants

One limitation we experience with the lace plant system is establishing a protocol to work with the lace plant at a large scale, mainly via producing seeds. Numerous lab resources have been spent on inducing lace plant flowering and harvesting seeds for propagation and transformation. The sterile culture system for the lace plant in our lab was established using seedlings (Gunawardena et al., 2021). However, successful inflorescences are rare and unpredictable across propagated cultures as they are observed to abort soon after their initiation. There is ongoing work to treat aquarium-grown lace plants exogenously with gibberellin supplementation to induce more inflorescences. However, no optimal results have been obtained to date. A protocol to make lace plant seeds more readily available will help develop a community of researchers for the lace plant system.

Protocols for inducing the formation of inflorescences in other aquatic monocot systems have been successful. Protocols for maximizing flowering are described for several duckweed species and include providing additional photoperiod exposure in combination with salicylic acid or ethylenediamine-*N*, *N*′-bis(2-hydroxyphenylacetic acid) supplementation (Fourounjian et al. 2021). Longer photoperiod in combination with salicylic acid supplementation was optimal for inducing flowering in Arabidopsis and *Lemna gibba* (Fu et al. 2020).

Perspectives on PCD and applications of the lace plant PCD

The lace plant has been recognized as a unique emerging model for studying PCD in plant development (Gunawardena 2008; Kacprzyk et al. 2011, 2021; Dauphinee and Gunawardena 2015). However, the fact that this phenomenon is such a rare occurrence in the plant kingdom begs the question of whether or not the lace plant can be used as a PCD model compared to established plant models.

PCD is ubiquitous across plants, but the molecular machinery that regulates its response, timing, and execution can vary across models and PCD processes. Many genes and signalling networks are increasingly characterized across established plant models from transcriptional networks, nucleases, and protease cascades. The question that remains is finding (if any) about the subcellular genetic, morphological, proteomic, or biochemical changes are conserved across plant PCD processes and plant groups.

Using the unique cell death gradient to observe morphological variability in adjacent and accessible PCD and NPCD cells simultaneously in one field of view will contribute to the ongoing debates on cell death classifications (Lord and Gunawardena 2011; Wertman et al. 2012). Lace plant PCD may elucidate regulatory pathways better manipulated in

agriculturally important species processes like leaf senescence which share intracellular morphological changes similar to PCD cells during perforation formation (Wright et al. 2009; Wertman et al. 2012). The potential anticancerous properties of anthocyanins from window stage leaves may contribute to biomedical applications and our knowledge of how they can regulate PCD in other plant systems (Gunawardena et al. 2021). Previous cross-species experiments show that mammalian pro-death Bax and anti-death Bcl-XL affect PCD and tobacco stress resistance (Lacomme and Santa Cruz 1999; Qiao et al. 2002). These results suggest that commonly conserved modulators in the PCD process exist (or once did) between animals and plants. Testing functionally similar genes of interest between animal models, plant models and the lace plant system will likely lead to a broader, universal understanding of PCD regulation.

The Bag protein family is ubiquitous, with homologs existing in yeast, humans, metazoans, and plants, all containing an evolutionarily conserved BAG domain (Kabbage et al. 2017). Animal Bags are involved with Bcl-2, promote anti-apoptotic activity (Takayama et al. 1995; Kabbage et al. 2017), and act as co-chaperones to influence Hsp70 protein folding (Takayama et al. 1997; Lüders et al. 2000). Arabidopsis Bag protein homologs can be organized into those which possess a ubiquitin-like motif (UBL; Bag1–4) and those which possess a calmodulin-binding motif (CaM; Bag5–7) (Doukhanina et al. 2006). Plant Bags have been shown to mediate plant stress responses, PCD, and autophagy (Li et al. 2016*a*,*b*; Kabbage et al. 2017), but their direct function with plant Hsp70s is not elucidated (Kabbage et al. 2017). Given that several Bag homologs are differentially expressed during lace plant leaf development (Rowarth et al. 2021) and that Hsp70 plays a role in lace plant PCD (Rowarth et al. 2020), we are currently investigating the functionality of Bag protein activity in lace plant in vivo. The work will help build on our understanding of Bag proteins and their conserved nature across animal and plant PCD.

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

Another important area of plant PCD research is unravelling the role of autophagy. Autophagy has been documented to play a dual role in cell death by promoting or inhibiting PCD across different critical processes (Minina et al. 2014; Dauphinee et al. 2019). In lace plants, autophagy is active in NPCD and PCD cells, and autophagy-related genes (Atg8, Atg16, and Atg18) are differentially regulated at different stages of leaf development and under the influence of autophagy modulators (Mishra et al. 2017; Dauphinee et al. 2019; Rowarth et al. 2021). Furthermore, lace plant autophagy modulation using promoter rapamycin and inhibitor wortmannin has significantly affected anthocyanin accumulation and perforation formation (Rowarth et al. 2023). Within plant tissues, the role of autophagy is even more cryptic as it can influence PCD in a cell type- and time-dependent manner, as seen in root cap cells in Arabidopsis (Feng et al. 2022). The tractable model of differentiating cells within the lace plant gradient can be employed to characterize the role of autophagic flux during leaf development.

Summary and conclusions

The lace plant system has contributed to the elucidation of PCD processes shared across species, but its unique features can also help further elucidate PCD (Wright et al. 2009; Lord and Gunawardena 2011; Wertman et al. 2012; Dauphinee et al. 2014). Recently, there have been advances in sequencing draft genomes for plants with large complex genomes, such as Norway spruce and *Paris japonica* and transformation protocols for recalcitrant species like rapeseed (Li et al. 2021). Combining these new tools will facilitate the assembly of a draft genome for the lace plant and functional analyses of genes essential for leaf morphogenesis. The knowledge gained from this cellular gradient of PCD and anthocyanin pigmentation within developing leaves will leverage the lace plant as a more widespread model for studying plant PCD processes.

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

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

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Data availability

Data generated or analyzed during this study are provided in full within the published article and supplementary materials.

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The authors declare that there are no competing interests.

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Supplementary material

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