IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES DURING LACE PLANT LEAF DEVELOPMENT

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Premise of research.   The lace plant is an excellent and unique model for studying developmentally regulated programmed cell death (PCD) in plants. Perforations form in highly predictable and easily accessible and distinguishable areas in lace plant leaves. However, little is known about the genes involved in regulation of this PCD or leaf development. In this study, for the first time, a general gene expression profile for lace plant leaf development was investigated.

Methodology.   A cDNA–amplified fragment length polymorphism involving 64 primer combinations was used for a half-genome analysis of 4666 transcripts. Two hundred and thirty differentially expressed transcript-derived fragments (TDFs) were sequenced. A partial expressed sequence tag (EST) database for window-stage (in which PCD is occurring) leaves was also established. Through a reverse transcription polymerase chain reaction, the possible role of ubiquitin in lace plant PCD was investigated.

Pivotal results.   Seventy-nine TDFs were successfully annotated. The isolated TDFs and ESTs encoded genes involved in processes such as photosynthesis, biosynthesis pathways, gene regulation, stress responses, defense against pathogens, and PCD, among others. Indirect evidence through ubiquitin transcript levels suggests involvement of proteasome machinery in lace plant development and PCD. This study provides a foundation for selective studies on regulation of lace plant leaf development and PCD.

Keywords: cDNA–amplified fragment length polymorphism, expressed sequence tags, lace plant, leaf development, perforation formation, programmed cell death.

Introduction

Programmed Cell Death

Programmed cell death (PCD) is essential for the response to stress and for adaptation, survival, and development in eukaryotes. PCD is genetically regulated and common in all forms of multicellular organisms (Lam 2004; Gadjev et al. 2008). PCD is involved throughout plant life—from death of the embryonic suspensor to death of the entire plant (Thomas and Franklin-Tong 2004; Gadjev et al. 2008). Plant PCD can be divided into two broad categories: environmentally induced and developmentally regulated (Gunawardena et al. 2004; Gunawardena 2008; reviewed in Rantong and Gunawardena 2015). Environmentally induced PCD is employed in response to salt stress, drought (Hameed et al. 2013), pathogens (Coll et al. 2014), ultraviolet light (Nawkar et al. 2013), heat (Lord and

Gunawardena 2011), and other external environmental cues. Developmentally regulated PCD is caused by intrinsic developmental cues and is involved in processes such as self-incompatibility in pollen (Thomas and Franklin-Tong 2004), embryonic suspensor deletion (Giuliani et al. 2002; Rogers 2005), aerenchyma formation (Gunawardena et al. 2001; Evans 2003), root cap shedding (Fendrych et al. 2014), leaf senescence (Lee and Chen 2002), and leaf remodeling in the lace plant (Gunawardena et al. 2004) and *Monstera* (Gunawardena et al. 2005).

The Lace Plant

The lace plant (fig. 1*A*) is an aquatic monocot endemic to Madagascar. It is a member of the family Aponogetonaceae. It is unique because it is one of the few known plant species that employ PCD to form perforations during normal leaf development. The only other known plants that utilize PCD for perforation formation are the *Monstera* species in the family Araceae (Gunawardena et al. 2005). In both of these plant species, perforations form through developmentally regulated PCD (Gunawardena and Dengler 2006). However, unlike in *Monstera*, perforation formation in the lace plant is highly predictable. The perforations form in highly predictable areas, between longitudinal and transverse veins (fig. 1*B*–1*E*). Perforation sites are

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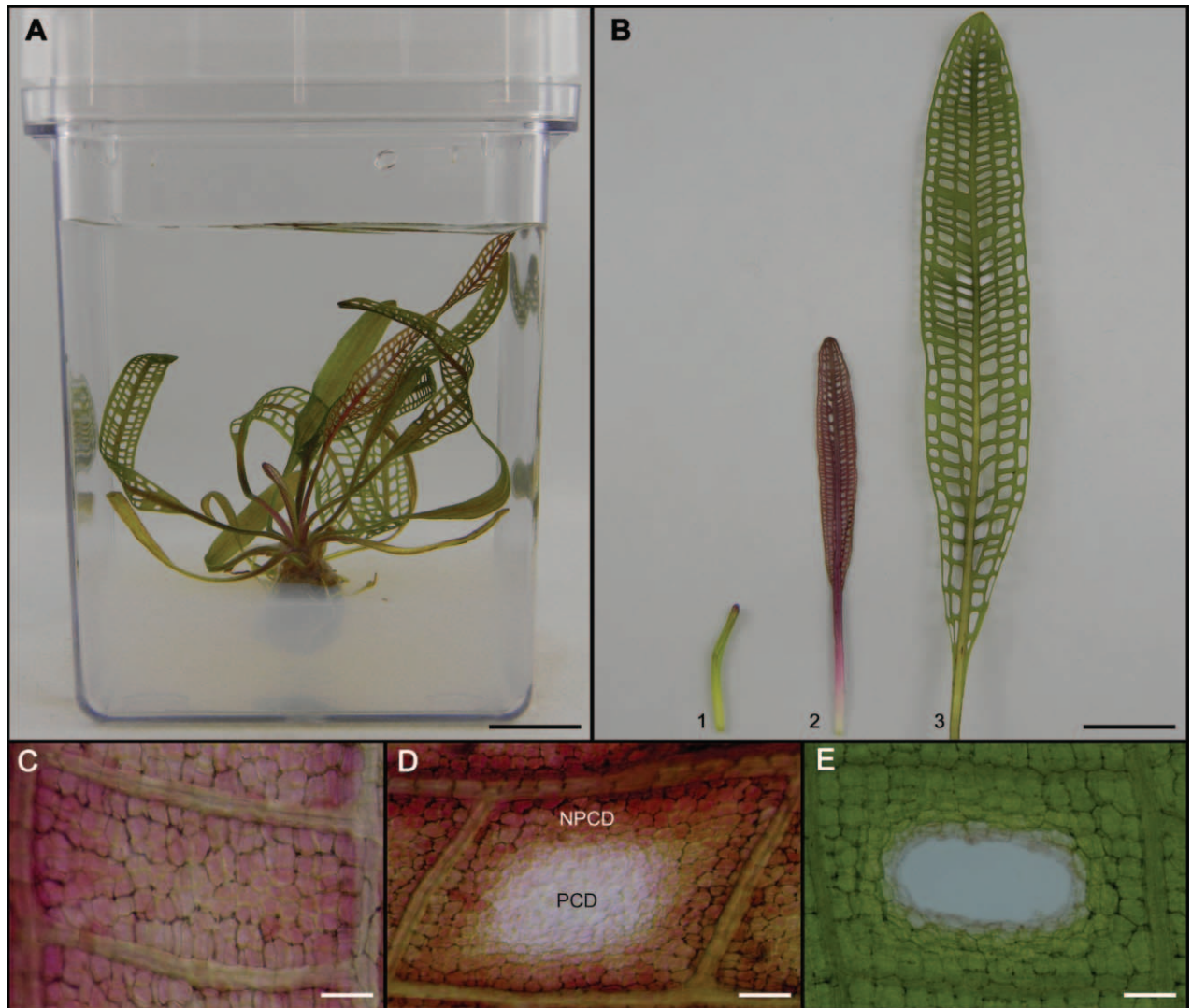[2] Author for correspondence; e-mail: arunika.gunawardena@dal.ca.

Fig. 1   Propagation of the lace plant and leaf development. *A*, Whole lace plant. The lace plants used in experiments in this study were grown in Magenta boxes under sterile conditions. The leaves emerge from a corm, and perforations form through developmentally regulated programmed cell death (PCD) to result in lace-like mature leaves. *B*, Three lace plant leaf developmental stages with different stages of perforation formation. In the preperforation stage (1), leaves have just emerged from the corm, and there are no obvious signs of perforation formation or PCD. Window-stage leaves (2) are pink due to anthocyanin, and PCD is occurring at the perforation site. In mature-stage leaves (3), perforations are complete, and there is no PCD occurring. *C*, An areole in a preperforation-stage leaf; all the cells look pink, and there is no sign of PCD or perforation formation. *D*, Within an areole in a window-stage leaf, cells at the center of the areole (PCD) lose their pigmentation and undergo PCD. The rest of the cells (NPCD), about four or five cell layers away from the vascular tissue, remain intact and alive. *E*, In mature-stage leaf areoles, PCD has stopped four or five cell layers away from vasculature, and all the cells are green. Scale bars: $A = 1.25$ cm, $B = 1$ cm, $C = 150$ μm, $D = 360$ μm, $E = 500$ μm.

easily visible, and perforations form during a specific stage of leaf development (window stage; fig. 1*B*, 1*D*). Lace plant leaves are also almost transparent, a favorable characteristic for microscopy. Plants can be easily propagated through sterile cultures in Magenta boxes (fig. 1*A*) to provide microbe-free experimental tissue. These characteristics make the lace plant a good model for studying developmentally regulated PCD in plants (Dauphinee and Gunawardena 2015).

The process of perforation formation and the morphological aspects of PCD in the lace plant have been well studied (Gunawardena et al. 2004, 2006, 2007; Gunawardena and Dengler 2006; Gunawardena 2008; Wright et al. 2009; Elliott and

Gunawardena 2010; Wertman et al. 2012; Lord et al. 2013; Dauphinee et al. 2014). Gunawardena et al. (2004) divided and characterized the formation of perforations in lace plant leaves into five stages based on morphological characteristics. The preperforation stage (stage 1) occurs immediately following leaf emergence from the corm as it begins to unfurl. At this stage, the vascular tissue is well developed, displaying a grid-like pattern over the surface of the leaf; no signs of PCD are present at this point (fig. 1*C*). The window stage (stage 2) begins when leaves display the loss of pigments, such as chlorophyll and anthocyanin, in distinct areas (perforation sites) between transverse and longitudinal veins. These areas become almost transparent in

comparison to the cells not undergoing PCD (four or five cell layers from the vascular tissue), which remain intact and retain their pigments. In the perforation-formation stage (stage 3), cells at the center of the perforation site begin to separate from adjacent cells, and a small hole forms through the leaf blade. During perforation expansion (stage 4), the perforations become larger as the leaf expands, and more cells die at the perforation site. In a mature leaf (stage 5), perforations are fully developed, and cells at the perforation border have transdifferentiated to become epidermal cells (Gunawardena et al. 2004). For this research, three stages of lace plant leaf development were used. Stage 1 was the preperforation stage, in which leaves did not display any obvious signs of PCD (fig. 1B, 1E). Stage 2 was the window stage, where PCD is actively taking place at the perforation site (fig. 1B, 1D). Stage 3 was the mature stage, where perforations were complete and no PCD was taking place (fig. 1B, 1E).

Morphological cellular changes that occur in cells undergoing PCD in the lace plant have been well documented. These changes occur in a time period of about 48 h and include a decrease in the size of chloroplasts and starch grains, fluctuations in the number of transvacuolar strands, aggregation of chloroplasts around the nucleus, formation of conglomerates of organelles within the vacuole, swelling of the vacuole, a decrease in the size of the nucleus, changes in the actin filaments' architecture, loss of mitochondrial membrane potential, cessation of mitochondrial streaming, tonoplast rupture, and collapse of the plasma membrane (Wright et al. 2009; Wertman et al. 2012; Lord et al. 2013). All of the above-mentioned cellular changes take place in the cells undergoing PCD (PCD cells; fig. 1D) during perforation formation. Cells four or five layers from vascular tissue, which do not undergo PCD at this stage of leaf development (NPCD cells; fig. 1D), retain normal morphological cellular characteristics.

Even though the cellular changes that occur during PCD in lace plant leaf development are well studied, little is known about the genes involved in regulation of PCD during perforation formation (Rantong et al. 2015). The genomic sequence of the lace plant is also unknown; therefore, cDNA–amplified fragment length polymorphism (cDNA-AFLP) and expressed sequence tag (EST) database approaches were employed in this study. The cDNA-AFLP approach was used to provide a gene expression profile and elucidate genes that show differential expression during different stages of leaf development. It is a useful tool for future molecular studies, which will be focused more on studying genes involved in lace plant PCD. In addition, an EST database was created for window-stage leaves, during which PCD is occurring, to identify some of the genes expressed during this leaf developmental stage. The transcript expression pattern of ubiquitin throughout lace plant leaf development was also studied through a quantitative polymerase chain reaction (qPCR) to provide insights into the involvement of the proteasome-ubiquitin protein degradation pathway in lace plant PCD and leaf development.

Material and Methods

Plant Propagation

The lace plants were grown as described by Gunawardena et al. (2006)—in sterile conditions within Magenta boxes. They were grown under daylight-simulating fluorescent bulbs (Daylight Deluxe, F40T12/DX; Philips, Markham, Ontario), which provided approximately 125 μmol m^{-2} s^{-1} of 12L : 12D cycles. Leaves in the preperforation, window, mature, and senescence (only for qPCR reactions) stages were selected from these plants and used for RNA extraction.

RNA Extraction

Approximately 200 mg of leaf tissue was used for each RNA extraction. The midrib was removed from the leaf tissue. RNA extraction was carried out according to the TRI reagent (Sigma-Aldrich, Oakville, Ontario) method, with some modifications. The modifications included elimination of air-drying the RNA pellet, using double the recommended volume of the TRI reagent, and maintaining the samples at a low temperature throughout RNA extraction.

cDNA-AFLP Technique

A cDNA-AFLP was carried out and processed essentially as described by Vuylsteke et al. (2007). First, 64 AFLP primer combinations with three selective nucleotides (BT1, BT2, BT3, and BT4, each in combination with Mse11, Mse12, Mse13, Mse14, Mse21, Mse22, Mse23, Mse24, Mse31, Mse32, Mse33, Mse34, Mse41, Mse42, Mse43, and Mse44) were used for selective amplification. The obtained fragments were separated on polyacrylamide gels. Gels were dried on blotting paper to enable the isolation of fragments from gels. For each primer combination, 50–100 transcript fragments were detectable, varying in length from 100 to 1000 bp. AFLP-QuantarPRO software (Keygene, Wageningen, Netherlands) was used to quantify band intensities in different lanes; oblique lanes were corrected, and unique transcript fragments were quantified, resulting in individual intensities for each time point per transcript fragment. Data were further corrected by doing a total lane correction. The residuals from these data normalizations were subjected to a one-factor ANOVA using the TIGR MultiExperiment Viewer of the TM4 software suite (Saeed et al. 2003). Genes with P values of <0.001 were retained for further analysis. The expression values of selected fragments were median centered across each gene and again log2 transformed. The resulting data sets were subjected to hierarchical average linkage clustering (with a Euclidian distance metric) with the MultiExperiment Viewer of TM4 (Saeed et al. 2003). For each identified subcluster, the median-centered expression value for each replica was graphically presented (log2 values). After data analysis, differentially expressed fragments were cut from gel and placed in $T_{10}E_{0.1}$ buffer for elution. These fragments were PCR amplified and directly sequenced.

Construction of cDNA Library and EST Sequencing

Window-stage leaves were used for the cDNA library, and total RNA from these leaves was extracted as described above. Spectrophotometry (ratio A260/280) and gel electrophoresis were used to verify the quality of the RNA. First-strand cDNA synthesis was carried out using the M-MuLV reverse transcriptase (200 U/μL; New England Biolabs, Pickering, Ontario), dT primer 5'-T18MN-3' (Integrated DNA Technology, Coralville,

IA), dNTP mix (New England Biolabs), 5X First Strand Buffer (Invitrogen, Burlington, Ontario), RNase inhibitor (40 U/μL; New England Biolabs), and 0.1 M DTT (Invitrogen). Double-stranded cDNA was synthesized using DNA polymerase 1 (10 U/μL; Fermentas, Burlington, Ontario), dNTP mix and random hexamers (NNNNNN; Integrated DNA Technology), T4 DNA polymerase (5000 U/mL; Fermentas), and 0.5 M EDTA (pH 8; Fermentas). The blunt-ended, double-stranded DNA was then purified by phenol/chloroform extraction. The dsDNA was ligated into pUC19 DNA/SmaI Digested and Dephosphorylated (Fermentas) using T4 DNA Ligase (5 U/μL; Fermentas). Once transformed into *Escherichia coli* DH5 α (Invitrogen) competent cells, clones were manually picked, and plasmids were extracted using the GenElute plasmid miniprep kit (Sigma-Aldrich). The plasmids with inserts were sent to Macrogen (Rockville, MD) for sequencing. Sequences were edited using the BioEdit Sequence Alignment Editor (Carlsbad, Ottawa, Ontario) to trim low-quality and vector portions. Sequence annotation was performed by comparing insert sequences with nonredundant protein (blastx) and nucleotide (blastn) sequence databases at the National Center for Biotechnology Information (NCBI).

qPCR

For transcript level analysis, in addition to the three stages of leaf development described above, the senescence stage was also used. The Protoscript M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs) was used for cDNA synthesis, according to manufacturer's instructions. The QuantiTect SYBR Green PCR Kit (Qiagen, Mississauga, Ontario) was used for qPCR in 20-μL reactions using a Rotor-Gene 2000 (Qiagen) and following the manufacturer's instructions. Ubiquitin primers used in qPCR were 5′-GCCCTCACTGGAAAGACCATTACCC-3′ and 5′-TCCCG-CAAAAATCAGCCTCTGC-3′. Actin was used as a reference gene, and actin qPCR primers used were 5′-TACGACAGGTAT-CGTGCTTG-3′ and 5′-CAAGCACGATACCTGTCGTA-3′. The PCR product was verified through ligation into a pGEM-T vector, cloned, and sequenced. qPCR conditions used were initial denaturing at 95°C for 15 min, 40 cycles of denaturing at 94°C for 20 s, primer annealing at 60°C for 20 s, and elongation at 72°C for 30 s. The melting curve was observed to determine purity of the PCR product. mRNA absolute copy numbers were determined through standard curves, which were generated as explained by Bustin et al. (2005). Ubiquitin absolute mRNA copy numbers were divided by actin mRNA copy numbers to generate mean normalized expression values.

Statistical Analysis

GraphPad Prism, version 5.00 (San Diego, CA), was used to analyze qPCR data. To determine significant differences in transcript levels, a general linear model of variance was used. After determining significance in the overall relationship, a Tukey post hoc test was used to compare means of individual treatments. Data were determined to be statistically significant if $P < 0.05$. Data were collected from 16 independent RNA samples (four RNA samples of each leaf developmental stage). Tissue from at least three different leaves was used for each RNA sample.

Results

Sequence Analysis of Transcript-Derived Fragments

To assess differential gene expression throughout lace plant leaf development, three leaf developmental stages of lace plant perforation formation were selected. These are the preperforation, window, and mature stages (fig. 1B). Through cDNA-AFLP analysis, transcript levels of 4666 transcripts were monitored throughout the three stages of leaf development. Figure 2 shows a section of a typical cDNA-AFLP gel that was run during this analysis. Most of the monitored transcripts displayed similar levels of expression, but those that showed differential expression were selected for further analysis. ANOVA analysis ($P < 0.001$) identified a total of 186 differentially expressed transcripts, of which 168 were isolated from the gels. Visual analysis resulted in an additional 62 fragments that displayed differential expression between the leaf developmental stages. A total of 230 fragments were isolated, reamplified, and sequenced.
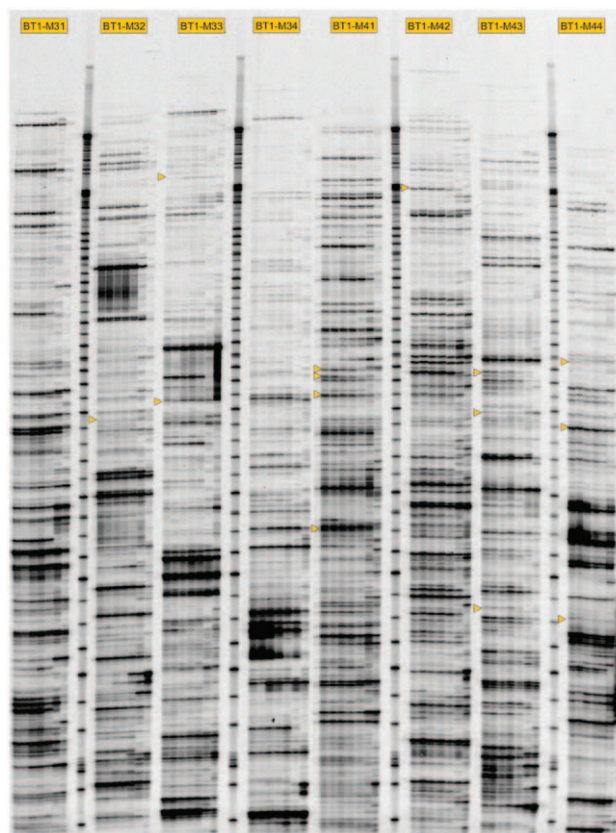

Fig. 2 cDNA–amplified fragment length polymorphism (cDNA-AFLP) gel displaying differentially expressed transcript-derived fragments (TDFs). One of the cDNA-AFLP gels displays the pattern TDFs amplified by BT1-Mse31, BT1-Mse32, BT1-Mse33, BT1-Mse34, BT1-Mse41, BT1-Mse42, BT1-Mse43, and BT1-Mse44. Lanes represent different samples from preperforation (lanes 1 and 2), window (lanes 3 and 4), mature (lanes 5 and 6), and senescent (lane 7) stages. Differentially expressed TDFs are highlighted by arrowheads. Note that the senescence stage had only one successfully analyzed sample (lane 7); therefore, due to lack of sufficient replicates, this leaf developmental stage was omitted from data analysis.

The median-centered expression values for these fragments were calculated and used to produce a hierarchical clustering (fig. 3). Out of the 230 sequenced transcript-derived fragments (TDFs), 77 were successfully functionally annotated through alignments in the NCBI databases (table 1). Some of the TDFs were representatives of the same or similar genes. For example, TDF45, TDF91, TDF117, TDF160, and TDF231 encoded LRR receptor–like serine/threonine-protein kinases; TDF9 and TDF10 encoded polyubiquitin; TDF44, TDF229, and TDF230 encoded chlorophyll a/b binding proteins; and TDF34 and TDF35 encoded a cold-regulated 413 plasma membrane protein 2–like gene. Some of the TDFs (TDF19, TDF102, and TDF232) were significantly similar to uncharacterized proteins of unknown functions.

Functional Annotation and Expression Analysis

Hierarchical average linkage clustering grouped genes with similar expression patterns into 24 clusters (figs. 3, 4). Of the 24 clusters, TDFs in four (clusters 4, 6, 9, and 15) displayed increased expression from the preperforation stage to the window stage. The other TDF clusters showed a decrease in expression (clusters 2, 3, 13, 14, 16, 20, and 22), while the rest displayed no difference in expression between the two developmental stages. Gene clusters that showed increased expression from window stage to mature stage were clusters 1, 5, 7, 8, 9, 10, 15, 16, and 18. TDFs in clusters 11, 14, 20, 21, 22, and 23 showed decreased expression levels from window stage to mature stage, while the rest of the subclusters did not show any significant differences. TDFs in clusters 12 and 24 showed higher expression in mature stage than in preperforation stage but did not show any significant difference in expression between mature and window stages. Only one gene cluster (cluster 2) displayed low TDF expression levels in the window stage, in comparison with both preperforation and mature stages. Clusters 6, 18, and 20 consisted of the highest number (16, 17, and 15, respectively) of TDFs, making up 20.9% of the total number of TDFs showing differential expression. Clusters 2 and 24 had the lowest number of TDFs, each composed of three and two TDFs, respectively. The rest of the gene clusters consisted of between six and 13 TDFs. Clusters 6, 18, and 20 consisted of TDFs encoding protein kinases (TDF45, TDF160, and TDF231), membrane proteins (TDF196 and TDF48), ribosomal proteins (TDF189, TDF186, and TDF182), proteins involved in biosynthetic pathways (TDF49, TDF54, and TDF185), ATP binding (TDF45), phosphatases (TDF42), components involved in ubiquitination (TDF50, TDF57, and TDF165), transcriptional regulators (TDF171), and chloroplastic proteins (TDF44, TDF46, and TDF183), among others. The smallest cluster was cluster 24, which was made up of two TDFs (TDF229 and TDF230). Both of these TDFs encode chloroplastic proteins.

Lace Plant Window-Stage Leaf EST Database

Window-stage leaves were chosen for construction of an EST database since they represent one of the most important stages of perforation formation. PCD was occurring in these leaves; therefore, some PCD-associated genes were likely to be isolated. Also, these leaves contain both PCD and NPCD cells; therefore, genes both expressed and downregulated during PCD

are represented in these leaves. Most of the genes associated with PCD should be expressed in the window-stage leaves. A total of 147 ESTs were successfully cloned and sequenced, 62% of
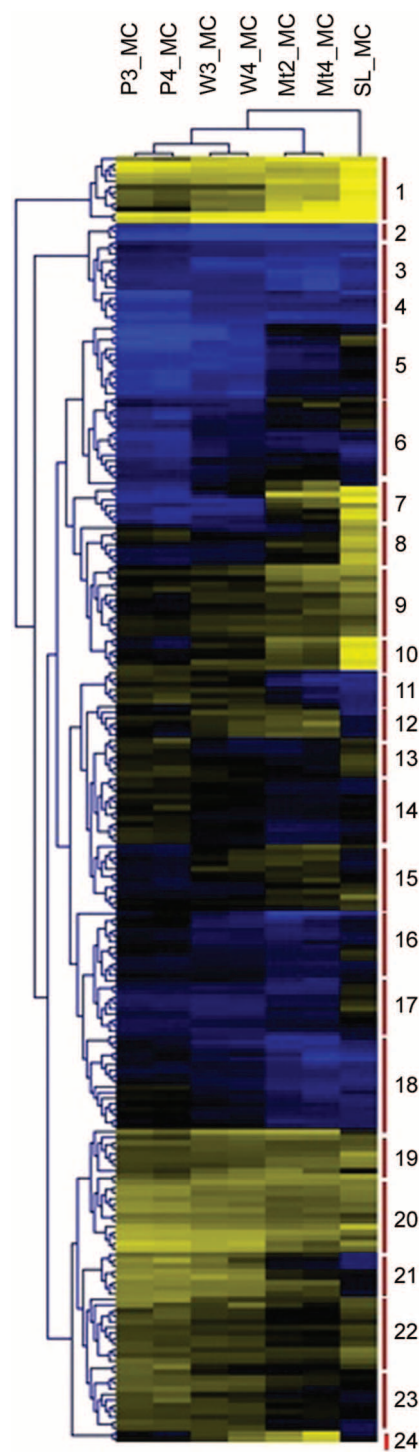

Fig. 3 Hierarchical clustering of differentially expressed transcript-derived fragments (TDFs). Hierarchical clustering of the median-centered normalized expression values of genes differentially expressed during the stages of lace plant leaf development is shown. Yellow indicates higher transcript levels, and blue indicates lower transcript levels.

Table 1

Annotated Transcript-Derived Fragments (TDFs) Showing Differential Expression during the Three Stages of Lace Plant Leaf Development

TDF	Primer combination	Length (bp)	Annotation	E-value	Accession no.
TDF4	bt3-m41	385	RING/FYVE/PHD zinc finger superfamily protein	5.00E-16	JZ845395
TDF9	bt4-m32	268	Ubiquitin	6.00E-36	JZ845396
TDF10	bt4-m32	256	Ubiquitin	5.00E-33	JZ845397
TDF19	bt1-m33	321	Lzipper-MIP1, SleB, and DUF547 domain-like protein	4.00E-27	JZ845400
TDF20	bt2-m14	603	60S ribosomal protein L19	4.00E-45	JZ845401
TDF22	bt3-m11	157	Haloacid dehalogenase-like hydrolase domain-containing protein 3	8.00E-11	JZ845398
TDF23	bt3-m13	225	Glucan endo-1,3-beta-glucosidase 3 precursor	7.00E-26	JZ845399
TDF26	bt4-m23	433	Histidinol dehydrogenase	5.00E-32	JZ845402
TDF30	bt1-m21	219	Uridine kinase-like protein	3.00E-24	JZ845403
TDF43	bt2-m24	501	YELLOW-LEAF-SPECIFIC GENE 9 (YLS9)-like	7.00E-21	JZ845404
TDF42	bt2-m42	346	Probable protein phosphatase 2C 74	.046	JZ845405
TDF36	bt3-m22	150	Thiamine thiazole synthase	2.00E-23	JZ845406
TDF34	bt3-m32	329	Cold-regulated 413 plasma membrane protein 2-like	4.00E-35	JZ845407
TDF35	bt3-m32	326	Cold-regulated 413 plasma membrane protein 2-like	3.00E-44	JZ845408
TDF56	bt3-m13	227	ATP-binding cassette transporter	7.00E-16	JZ845409
TDF45	bt3-m13	368	LRR receptor-like serine/threonine-protein kinase	.19	JZ845410
TDF50	bt3-m14	404	F-box/kelch-repeat protein SKIP11-like	5.00E-62	JZ845411
TDF46	bt3-m21	467	Protein CURVATURE THYLAKOID 1B	5.00E-41	JZ845412
TDF57	bt3-m43	203	Hepatocyte growth factor-regulated tyrosine kinase substrate-like	2.00E-05	JZ845413
TDF49	bt3-m43	659	Isopentenyl pyrophosphate isomerase	3.00E-86	JZ845414
TDF44	bt4-m11	498	Light-harvesting complex II chlorophyll-*a/b* binding protein	4.00E-12	JZ845415
TDF54	bt1-m11	292	Cinnamyl alcohol dehydrogenase	3.00E-16	JZ845416
TDF48	bt3-m34	410	Mitochondrial chaperone BCS1-B	1.00E-60	JZ845417
TDF60	bt2-m42	193	Peptidase M20/M25/M40 family-like protein	.8	JZ845418
TDF77	bt2-m13	127	Nucleotidyltransferase/DNA polymerase	2.0	JZ845419
TDF81	bt2-m32	499	Calcium-binding protein CML24-like	.87	JZ845420
TDF76	bt2-m43	330	40S ribosomal protein S21	6.00E-24	JZ845421
TDF74	bt3-m34	476	Glycosyl hydrolase	2.00E-52	JZ845422
TDF90	bt1-m33	212	Allene oxide cyclase 4	2.00E-20	JZ845423
TDF91	bt2-m13	100	LRR receptor-like serine/threonine-protein kinase	1.00E-04	JZ845424
TDF98	bt1-m21	439	High-mobility group B protein 6-like	6.00E-23	JZ845425
TDF93	bt1-m42	433	F-box-like/WD repeat-containing protein TBL1XR1	5.00E-41	JZ845426
TDF96	bt2-m22	271	High-mobility group B protein 7-like	2.00E-26	JZ845427
TDF97	bt2-m32	332	Ubiquitin-conjugating enzyme E2 20-like	3.00E-64	JZ845428
TDF103	bt2-m21	161	Synechocystis YCF37-like protein	.21	JZ845429
TDF104	bt3-m14	389	Homogentisate phytyltransferase 1, chloroplastic	1.00E-22	JZ845431
TDF102	bt3-m14	436	Pentatricopeptide repeat (PPR) superfamily protein	8.00E-16	JZ845431
TDF101	bt3-m14	348	Protein CURVATURE THYLAKOID 1B, chloroplastic	6.00E-23	JZ845432
TDF100	bt1m44	331	Geranylgeranyl dehydrogenase	3.00E-62	JZ845433
TDF105	bt4-m12	76	50S ribosomal protein L15	.014	JZ845434
TDF117	bt4-m11	147	Leucine-rich repeat receptor-like serine/threonine-protein kinase BAM3	4.00E-14	JZ845435
TDF118	bt4-m13	200	SUN domain-containing protein 1-like	1.00E-11	JZ845436
TDF126	bt2-m11	260	Peptidyl-prolyl cis-trans isomerase CYP26-2, chloroplastic	3.00E-26	JZ845437
TDF130	bt2-m31	183	Pentatricopeptide repeat-containing protein, chloroplastic	7.2	JZ845438
TDF124	bt3-m14	304	Integral membrane protein	3.00E-22	JZ845439
TDF141	bt2-m12	307	Fructose-bisphosphate aldolase	1.00E-49	JZ845440
TDF145	bt3-m21	190	Fatty acyl-CoA reductase 4	3.00E-25	JZ845441
TDF143	bt4-m34	439	Gamma-terpinene synthase	1.00E-32	JZ845442
TDF138	bt1-m12	75	DNA/RNA-binding protein KIN17-like	9.00E-05	JZ845443
TDF156	bt2-m21	198	Cytochrome P450	3.0	JZ845444
TDF155	bt2-m33	148	Pentatricopeptide repeat-containing protein At4g33170-like	3.2	JZ845445
TDF165	bt1-m43	285	Ribosome biogenesis protein wdr12	2.00E-05	JZ845446
TDF172	bt2-m24	148	N-lysine methyltransferase-like protein	.008	JZ845447
TDF231	bt3-m11	365	LRR receptor-like serine/threonine-protein kinase ERECTA	2.00E-06	JZ845449
TDF160	bt3-m11	399	LRR receptor-like serine/threonine-protein kinase ERECTA	3.00E-11	JZ845450
TDF163	bt3-m14	241	Auxin-responsive aux/iaa gene family member	1.00E-16	JZ845451
TDF171	bt3-m31	273	Homeobox-leucine zipper protein HOX32 isoform X2	2.00E-05	JZ845452
TDF175	bt3-m41	392	30S ribosomal protein S5, chloroplastic-like	1.00E-37	JZ845453
TDF196	bt2-m32	164	Alpha-tubulin	7.00E-07	JZ845455
TDF191	bt3-m12	168	Relaxin-3 receptor	7.0	JZ845456
TDF186	bt3-m41	200	60S ribosomal protein L32-1-like	7.00E-32	JZ845457

Table 1 (*Continued*)

TDF	Primer combination	Length (bp)	Annotation	E-value	Accession no.
TDF185	bt3-m42	151	CDP-diacylglycerol–inositol 3-phosphatidyltransferase 2	1.00E-08	JZ845458
TDF183	bt4-m11	82	Translocase of chloroplast 34	5.00E-04	JZ845459
TDF182	bt4-m41	300	50S ribosomal protein L17, chloroplastic-like	1.00E-23	JZ845460
TDF200	bt1-m44	195	50S ribosomal protein L3-2, chloroplastic	3.00E-16	JZ845461
TDF204	bt2-m31	277	FRIGIDA-like protein 3	.56	JZ845462
TDF197	bt3-m12	108	Transcriptional regulator	1.6	JZ845463
TDF203	bt3-m44	201	Remorin	7.00E-04	JZ845464
TDF201	bt3-m44	238	Proliferating cell nuclear antigen	1.00E-21	JZ845465
TDF206	bt1-m12	116	WUSCHEL-related homeobox 8-like protein	3.00E-04	JZ845466
TDF209	bt3-m14	210	Beta-glucosidase	1.00E-09	JZ845467
TDF214	bt4-m22	72	Galacturonosyltransferase	1.2	JZ845468
TDF219	bt1-m23	184	Protein LHCP TRANSLOCATION DEFECT-like	1.00E-24	JZ845469
TDF232	bt1-m41	217	XP_001849016-like protein	.22	JZ845470
TDF228	bt3-m13	113	Thionin	8.95E-03	JZ845471
TDF222	bt3-m21	242	Serine/arginine-rich splicing factor RS2Z32-like	.53	JZ845472
TDF227	bt4-m21	289	U5 small nuclear ribonucleoprotein 40 kDa protein	3.00E-60	JZ845473
TDF229	bt4-m31	509	Light harvesting chlorophyll *a/b* binding protein	6.00E-71	JZ845474
TDF230	bt4-m44	535	Chlorophyll *a/b* binding protein 6, chloroplastic-like	4.00E-73	JZ845475
TDF173	bt2-m44	160	Defensin-like protein	3.1	JZ845448
TDF189	bt2-m44	111	Phospho-N-acetylmuramoyl-pentapeptide-transferase-like	3.00E-09	JZ845454

which were successfully annotated as identified (table 2) known proteins or uncharacterized proteins. The rest of the ESTs did not reveal homology within the GenBank database, using default parameters. Some ESTs, such as the putative senescence-associated protein (table 2), were isolated multiple times, suggesting that they are abundantly expressed in window-stage leaves.

Studying Ubiquitin Transcript Levels through qPCR

Several proteins involved in the ubiquitin-dependent protein degradation mechanism that involves the proteasome were isolated through both cDNA-AFLP (TDF9 and TDF10) and the EST database (EST3, EST121, and EST306). To determine the involvement of this mechanism in lace plant PCD and leaf development, ubiquitin transcript levels were investigated throughout leaf development through qPCR. In addition to the three leaf development stages discussed above, senescent-stage leaves were also included in transcript level analysis, since PCD is occurring in window- and senescent-stage leaves. Actin was used as a reference gene, and its transcript levels were constant throughout leaf development (fig. 5A). Ubiquitin transcript levels were significantly higher in senescence-stage leaves than in all the other developmental stages ($P < 0.05$). Window-stage leaves had significantly higher transcript levels than preperforation- and mature-stage leaves ($P < 0.05$). There were no significant differences in ubiquitin transcript levels between preperforation- and mature-stage leaves (fig. 5).

Discussion

Due to a lack of the known genome sequence, it has been challenging to study the genetic regulation of PCD and leaf development in the lace plant. The data accumulated through both cDNA-AFLP and the EST database provide a foundation to study the genetic regulation of PCD in the lace plant and other processes of interest. TDFs isolated through cDNA-AFLP encoded genes involved in processes such as translation, photosynthesis, gene regulation, stress responses, defense against pathogens, biosynthesis pathways, and PCD, among others. Table 3 lists the subclusters, their annotated TDFs, and the cellular processes the TDFs are involved in. Only one or none of the TDFs in subclusters 2, 7, and 8 was successfully annotated; therefore, these subclusters were not included in the table.

The TDFs potentially involved in PCD include polyubiquitin, ubiquitin-conjugating enzyme, YLS9, and LRR receptor–like serine/threonine-protein kinases (Bachmair et al. 1990; Woffenden et al. 1998; Heyndrickx et al. 2012; reviewed in Rantong and Gunawardena 2015). Ubiquitin is currently the most structurally conserved protein isolated. Its amino acid sequence is the same in all higher plants (Callis et al. 1995). Ubiquitin is involved in protein degradation in conjunction with the 26S proteasome during several processes, including PCD (Bachmair et al. 1990; Woffenden et al. 1998). During senescence, the ubiquitin-dependent proteasome system is responsible for the tremendous protein turnover (Vierstra 1996). Transcript level analysis suggests that ubiquitin might be involved in protein turnover that occurs during perforation formation and senescence in the lace plant (fig. 5). The increased ubiquitin transcript levels during the leaf developmental stages where PCD is occurring (window and senescence) support the potential involvement of ubiquitin and the proteasome protein degradation mechanism in lace plant PCD. The ubiquitin-conjugating enzyme (TDF97) is also involved in the ubiquitin/26S proteasome system. It is part of a sequence of enzymes involved in the pathway that attaches ubiquitin molecules to target proteins (Vierstra 2009). YLS9 is involved in processes such as hypersensitive response, leaf senescence, and regulation of PCD (Zheng et al. 2004; Heyndrickx et al. 2012). The specific molecular mechanism through which YLS9 is involved in these processes is unknown. LRR receptor–like serine/threonine-protein kinases are involved in signalling during response to pathogen attack,
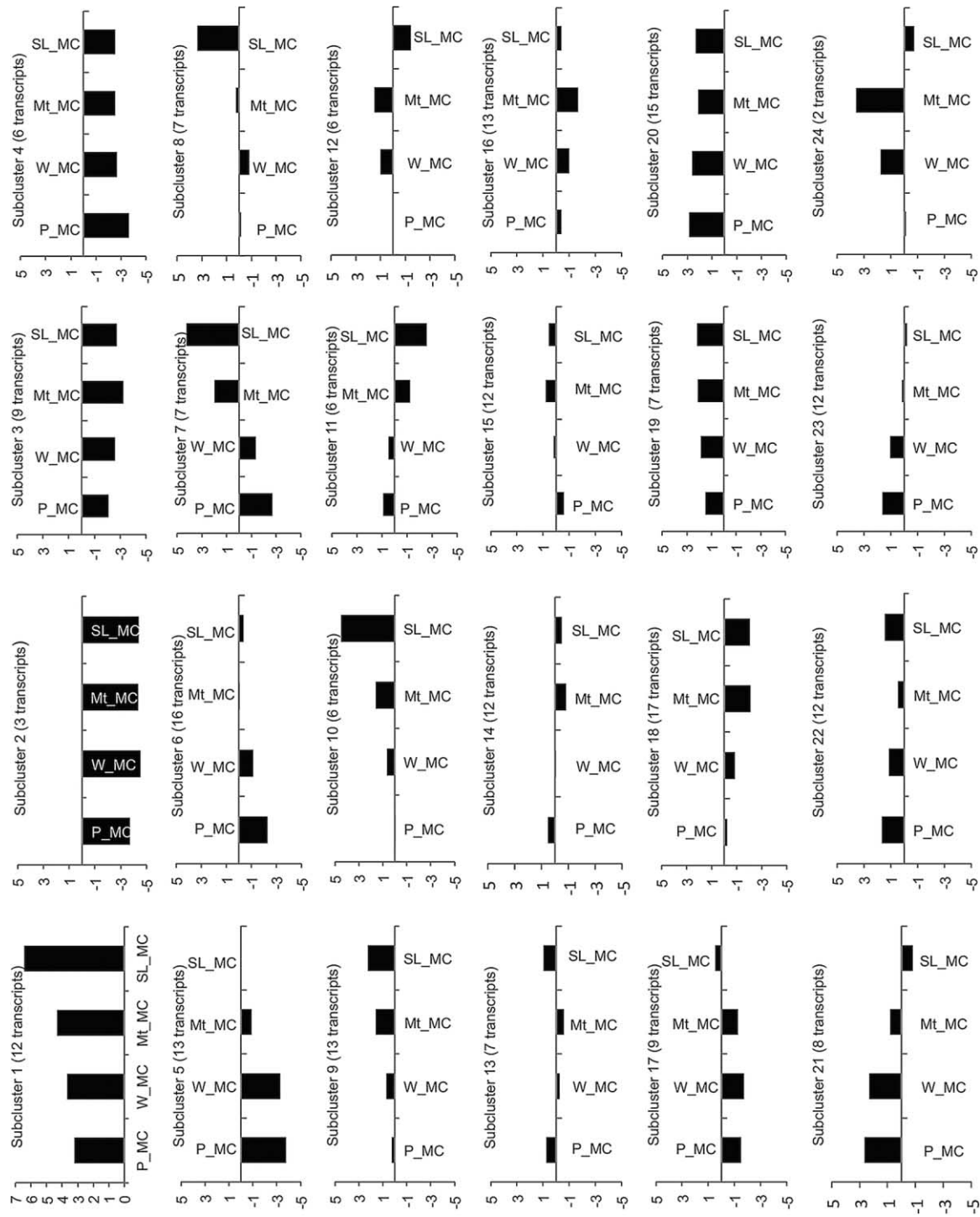
Fig. 4 Illustration of the cluster analysis through histograms. The transcript patterns of the 230 fragments in the different leaf developmental stages were clustered into 24 groups. The different leaf stages were window (W), preperforation (P), mature (Mt), and senescence (SL).

Table 2

Successfully Annotated Expressed Sequence Tags (ESTs) from Lace Plant Window-Stage Leaves

EST	Length (bp)	Annotation	E-value	Accession no.
1	261	Cytochrome P450 monooxygenase	3.00E-60	…
3	428	20S proteasome beta subunit PBG1	1.00E-33	JZ897089
23	698	GTP-binding protein SAR1A	3.00E-72	JZ897090
49	486	23S ribosomal RNA	2.00E-28	…
56	351	50S ribosomal protein L22	9.00E-50	JZ897091
505	744	Chloroplast photosystem II 10 kDa protein	3.00E-37	JZ897092
508	504	Tankyrase 1	1.00E-27	JZ897093
510	876	Cytochrome P450 monooxygenase	1.00E-38	…
512	116	Chloroplast hypothetical protein	2.00E-04	…
517	315	40S ribosomal protein S17	2.00E-10	JZ897094
522	150	60S ribosomal protein L34	1.00E-25	JZ897095
531	443	Photosystem I reaction center subunit psaK	2.00E-40	JZ897096
536	591	40S ribosomal protein S3a	5.00E-135	JZ897097
539	992	rRNA intron-encoded homing endonuclease	4.00E-61	…
549	382	Calmodulin-binding protein 1	9.00E-09	JZ897098
551	367	Retrotransposon protein	1.00E-08	…
552	445	Mitochondrial import receptor subunit TOM20	4.00E-06	JZ897099
439	583	GTP-binding protein SAR1-like	1.00E-65	JZ897100
77	158	WRKY24-like	3.00E-04	JZ897101
96	237	WRKY transcription factor	6.00E-07	JZ897102
100	253	WRKY-like	8.00E-02	JZ897103
384	962	Oxygen-evolving enhancer protein 1	2.00E-170	JZ897122
511	284	26S large subunit ribosomal RNA gene	1.00E-140	…
386	250	18S small subunit ribosomal RNA gene	1.00E-121	…
395	135	23S ribosomal RNA gene	4.00E-43	…
155	418	Cellulose synthase-like protein G3	9.00E-31	JZ897105
491	260	Putative senescence-associated protein	8.00E-39	…
326	463	Cell wall-associated hydrolase	1.00E-53	…
403	171	NADH dehydrogenase subunit 3	2.00E-06	JZ897114
89	138	UDP-glycosyltransferase	5.00E-07	JZ897115
13	492	Thionin	3.00E-07	JZ897116
172	197	Serine/threonine-protein kinase Nek2-like	8.00E-03	JZ897112
306	454	EIN3-binding F-box protein 1-like	7.00E-06	JZ897106
151	713	Leishmanolysin	4.00E-126	JZ897107
166	794	Methylsterol monooxygenase 2-2-like	4.00E-141	JZ897108
113	493	Chlorophyll *a/b* binding protein	2.00E-51	JZ897104
114	864	RNA-directed DNA polymerase (reverse transcriptase); ribonuclease H	9.00E-73	JZ897109
115	244	Fructose-bisphosphate aldolase, class I	8.00E-34	JZ897110
116	168	Transformation/transcription domain-associated protein	9.00E-17	JZ897111
118	564	Putative retroelement	1.00E-39	JZ897113
119	962	Oxygen-evolving enhancer protein 1	2.00E-170	JZ897117
120	295	50S ribosomal protein L21	7.00E-25	JZ897118
121	345	Ubiquitin-specific protease 14	1.00E-31	JZ897119
122	610	Cyclin-B2-2-like	3.00E-60	JZ897120
123	603	f13j11 PRLI-interacting factor G	2.00E-65	JZ897121

response to abiotic stresses, and PCD (Song et al. 1995; Oh et al. 2010; Park et al. 2014).

In addition to the TDFs involved in PCD, the EST database contains some of the genes known to be involved in different forms of PCD in other plant species. These include the 20S proteasome beta subunit (EST3), EIN3-binding F-box protein 1 (EST306), a calmodulin-binding protein (EST549), a senescence-associated protein (EST491), WRKY transcription factors (EST77, EST96, and EST100), and cytochrome P450 monooxygenase (EST1 and EST510; also isolated through cDNA-AFLP). Other TDFs and ESTs isolated through both cDNA-AFLP and the EST database include thionin and chlorophyll *a/b* binding proteins.

The 20S proteasome β subunit is involved in the ubiquitin-proteasome-dependent regulated degradation of proteins (Baumeister et al. 1998; Kim et al. 2006). It is a part of the 26S proteasome, which recognizes and degrades ubiquitinated proteins. The 26S proteasome is comprised of a 20S core particle with 19S regulatory particles attached to it. The 20S proteasome consists of 28 subunits that are encoded by distinct but related genes (Baumeister et al. 1998). In eukaryotes, the subunits are divided into archaeal α or β subunits; a β subunit was isolated in this study. Several functions of the 20S proteasome have been described in eukaryotes, the most significant being its involvement in ubiquitin-dependent proteolysis (Coux et al. 1996; Hoch-
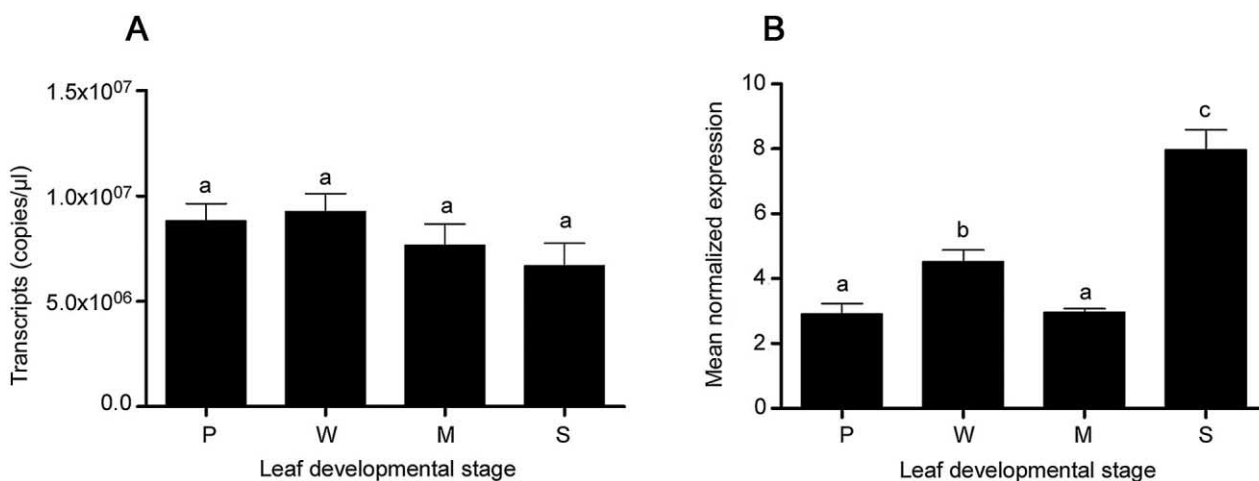
A

B

Fig. 5   Actin and ubiquitin transcript levels during different stages of leaf development. *A*, Actin transcript levels. Actin was used as a reference gene, and it had similar transcript levels throughout leaf development. *B*, Normalized ubiquitin transcript levels during the different stages of leaf development. There was no significant difference in ubiquitin transcript levels in preperforation- and mature-stage leaves. Window- and senescence-stage leaves had significantly higher transcript levels than preperforation- and mature-stage leaves. Senescence-stage leaves had more ubiquitin transcript levels than window-stage leaves. Bars represent SE ($n \geq 8$). Means with the same letters are not significantly different ($P > 0.05$). M = mature stage, P = preperforation stage, S = senescence stage, W = window stage.

strasser 1996; Vierstra 1996). It would be useful to determine its involvement in lace plant PCD and perforation formation as well as the role of the 26S proteasome as a whole. This would provide insights into whether protein turnover and nutrient recycling occur during perforation formation in the lace plant. EIN3-binding F-box protein 1 is a ubiquitin protein ligase involved in ethylene-regulated PCD in plants (Gagne et al. 2004). It is a constituent of a protein complex (SCF^EBF1/2) that directs the targeting of EIN3 for degradation through a ubiquitin-dependent pathway involving the proteasome (Guo and Ecker 2003; Gagne et al. 2004). EIN3 is a transcription factor required for induction of gene expression regulated by ethylene, including during ethylene-dependent PCD (Chao et al. 1997; Alonso et al. 2003). PCD in the lace plant is ethylene dependent (Dauphinee et al. 2012; Rantong et al. 2015); therefore, it is likely that EIN3-binding F-box protein 1 plays an important role in the regulation of lace plant PCD. Through orchestrating the degradation EIN3, EIN3-binding F-box protein 1 is also important for enhancing plant growth (Gagne et al. 2004).

Calmodulin-binding proteins such as AtBAG6 in *Arabidopsis* are known to be involved in PCD (Kang et al. 2006). AtBAG6 has a BCL-2-associated athanogene (BAG) domain, and when it is overexpressed, it can induce PCD. Calmodulin-binding protein 1 (EST 549) was isolated from the lace plant; however, it is unknown whether it is involved in lace plant PCD. Nevertheless, it is known that calcium signaling is essential in PCD during perforation formation in the lace plant (Elliott and Gunawardena 2010). Calmodulin-binding proteins are involved in a wide range of other processes, such as transcriptional regulation, phosphorylation, and metabolism (Snedden and Fromm 2001; Hoeflich and Ikura 2002).

Other clones of interest are the WRKY transcription factors. WRKY transcription factors are ubiquitous in plants and pos-

sess highly conserved characteristic amino acid motifs, which contain the WRKYGQK sequences followed by Cys(2)-His(2) or Cys(2)-His-Cys zinc-binding motifs (Zhou et al. 2011). There are 74 members of this gene family in *Arabidopsis* and 109 in rice (Eulgem and Somssich 2007; Ross et al. 2007). They are believed to be involved in regulation of several physiological processes in plants, such as immunity (Pandey and Somssich 2009), embryogenesis (Lagacé and Matton 2004), hormonal signaling (Zhang et al. 2004; Xie et al. 2005), trichome and seed coat development (Johnson et al. 2002), regulation of biosynthesis pathways (Xu et al. 2004), and senescence (Lin and Wu 2004; Buchanan-Wollaston et al. 2005). WRKY transcription factors play a role in plant immunity by regulating the expression of defense response genes (Eulgem et al. 2000; Heise et al. 2002). Plant immunity and senescence involve PCD; therefore, the WRKY transcription factors likely play a role in PCD. It would be a significant step to determine the role of these transcription factors in lace plant perforation formation and determine whether they regulate the expression of any PCD-associated genes during perforation formation.

Cytochrome P450s are generally known as key enzymes involved in synthesizing a large variety of secondary plant metabolites such as phytoalexins, lignin, and flavonoids (Butt and Lamb 1981; reviewed by Schuler and Werck-Reichhart 2003). Buchanan-Wollaston (1997) reported the expression of three cytochrome P450 genes in senescencing *Brassica napus* leaves but not in leaves not undergoing senescence. Xu et al. (2006) also showed that in *Petunia inflata*, cytochrome P450 was upregulated in the tonoplast during petal senescence. This suggests that they are somehow involved in leaf senescence, but their specific role in senescence is not yet elucidated. In cotton, cytochrome P450s have been shown to regulate systemic cell death (Sun et al. 2014). They are also involved in jasmonate metabolism,

Table 3

Subclusters and Cellular Processes That the Successfully Annotated Transcript-Derived Fragments (TDFs) Are Involved In

Subcluster no.	TDFs	Cellular processes
1	TDF4, TDF9, and TDF10	Transcription, transportation of mRNA, translation, organization of the cytoskeleton, protein folding, remodeling of chromatin, and posttranslational modification of proteins in several ways including protein activity, localization, interaction, and proteasome-dependent degradation
3	TDF20, TDF22, and TDF23	Hydrolytic activity, plant defense against pathogens, and a ribosomal protein involved in translation
4	TDF26 and TDF30	Histidine biosynthesis, redox reactions, response to ultraviolet light, RNA splicing, biosynthesis of methionine, and phosphorylation, among other processes
5	TDF42, TDF43, TDF36, TDF34, and TDF35	Hypersensitive response, programmed cell death, protein dephosphorylation thiazole biosynthesis, and response to stresses such as drought and cold
9	TDF81, TDF76, and TDF74	Calcium binding, component of the ribosomes, and biomass degradation
10	TDF90 and TDF91	Jasmonate biosynthesis and recognition of pathogens
11	TDF93, TDF97, TDF96, and TDF98	DNA binding, protein ubiquitination, and response to drought and salt stress
12	TDF100, TDF101, and TDF104	Cell differentiation and several biosynthetic processes including chlorophyll metabolism and photosynthesis
14	TDF117 and TDF118	A protein with serine/threonine kinase activity involved in ATP binding and a component of nuclear envelope bridging complexes
15	TDF124, TDF126, and TDF130	Protein folding, a protein that binds to organellar transcripts in chloroplasts and mitochondria, and an integral membrane protein
16	TDF141, TDF145, TDF138, and TDF143	Glucose catabolic process, lipid metabolism, a monoterpene synthase, DNA/RNA-binding, arrangement of microtubules and associated proteins in the cytoskeleton, response to salt, wounding, and oxidative stresses
17	TDF155	Terminal oxidase, also thought to be involved during leaf senescence
21	TDF197, TDF200, TDF201, TDF203, and TDF204	rRNA binding, cell differentiation, transcriptional regulation, DNA binding, regulation of the cell cycle, and DNA replication
22	TDF206, TDF209, and TDF214	Carbohydrate metabolism, a transcriptional regulation, and pectin biosynthesis
23	TDF219, TDF222, TDF227, and TDF228	Ribosomal biogenesis, plant defense against pathogens, RNA binding and splicing, and importing and transport of light-harvesting complex proteins

plant defense, and wounding response. As one of the isolated and identified clones, it would be interesting to determine whether the isolated cytochrome P450 monooxygenase is involved in perforation formation by comparing its expression patterns between dying and control cells.

Another EST worthy of further investigation is the putative senescence-associated protein. This clone was isolated 38 times, suggesting that it is a highly expressed protein in window-stage lace plant leaves. The specific name of the gene encoding this protein could not be determined. The homologs in other species are listed only as putative senescence-associated proteins. Senescence is a form of PCD; therefore, this protein is likely somehow involved in PCD. With its apparently high expression in window-stage leaves, it may be involved in the developmentally regulated PCD that leads to perforation formation in lace plant leaves. It would be helpful to discover the exact functional annotation of this protein and also to determine its involvement in lace plant perforation formation.

Some of the genes commonly known to be involved in plant PCD, such as caspase-like enzymes and genes involved in hormonal regulation of PCD (except EIN3-binding F-box protein 1), were not identified within the EST database. This is mainly because the technique does not specifically target these genes, is random, and is biased toward highly expressed genes. As a result,

most of the ESTs were from generally highly expressed proteins such as the ribosomal and chloroplast proteins. These proteins are not directly related to PCD; however, they will be useful in future research, such as phylogenetic and developmental studies.

The involvement of more PCD-related genes in lace plant PCD can now be studied through direct methods such as real-time PCR, which requires prior knowledge of gene sequences. Through qPCR, we provided evidence that supports the involvement of ubiquitin in lace plant PCD, occurring during perforation formation and leaf senescence (fig. 5). Ubiquitination has been shown to be involved in other plant PCD examples such as leaf senescence (Pinedo et al. 1996; Wang et al. 2014) and hypersensitive response (Marino et al. 2012). During daylily leaf senescence, ubiquitin and ubiquitin conjugates have been shown to increase (Courtney et al. 1994).

To focus more on PCD, separation of dying cells and nondying cells within window-stage leaves before analysis is required. This has recently been achieved through laser-capture microscopy and allows for PCD-specific analysis of gene function (Rantong et al. 2015). The molecular data generated in this study can also be used to study the role of specific genes in other plant developmental processes outside of PCD. This study also makes the lace plant the first species within the Aponogetonaceae family with a considerable amount of gene sequences

elucidated. Molecular data available on all the other species within this family are limited to the chloroplastic maturase K (matK) and nuclear DNA (nrITS) loci used in phylogenetic analysis of the Aponogetonaceae family (Les et al. 2005; Chen et al. 2015). The lack of substantial genome sequences from closely related species makes it challenging to successfully annotate new sequences. The large number of unidentified proteins in both the cDNA-AFLP and EST approaches can be accredited to the lack of sequence data and molecular studies in close relatives of the lace plant.

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