Cloning of Phospholipase D α and Lipoxygenase Genes CmPLD α 1 and CmLOX1 and Their Expression in Fruit, Floral, and Vegetative Tissues of ‘Honey Brew’ Hybrid Honeydew Melon

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ABSTRACT. Increases in phospholipase D [PLD (EC 3.1.4.4)] and lipoxygenase [LOX (EC 1.13.11.12)] activities are thought to play a critical role in senescence of mesocarp tissues in netted and nonnetted muskmelon (*Cucumis melo* L.) fruits. We have cloned and characterized two full-length cDNAs, CmPLD α 1 and CmLOX1, encoding PLD α and LOX proteins in honeydew melon (*C. melo* Inodorus Group cv. Honey Brew). Relative levels of expression of the corresponding genes were determined by semi-quantitative RT-PCR in developing and mature fruit mesocarp tissues [20–60 d after pollination (DAP)], as well as in roots, leaves, and stems from 4-week-old and flowers from 6- to 7-week-old plants. The coding regions of CmPLD α 1 and CmLOX1 cDNAs are, respectively, 2427 and 2634 nucleotides long, encoding proteins 808 and 877 amino acids in length. CmPLD α 1 is very similar to PLD α genes from castor bean (*Ricinus communis* L.), cowpea (*Vigna unguiculata* L.), strawberry (*Fragaria xananassa* Duch.) and tomato (*Lycopersicon esculentum* Mill.) (77% nucleotide identity), and is the first PLD gene cloned from a cucurbit species. CmLOX1 has 94% nucleotide identity to a cucumber (*Cucumis sativus* L.) LOX gene expressed in roots and 80% identity to cucumber cotyledon lipid body LOX. In general, transcript of CmPLD α 1 was much more abundant than that of CmLOX1, but relative levels of transcript in the various organs and tissues were similar for the two genes. Expression was highest in roots, flowers, and fruit mesocarp tissues. CmPLD α 1 expression in fruit was essentially constitutive throughout development, although maximum levels occurred at 50 and 55 DAP, respectively, in middle and hypodermal mesocarp. CmLOX1 expression was generally higher in middle than in hypodermal mesocarp with maximum transcript levels occurring at 55 and 50 DAP, respectively. Overall, the patterns of expression of CmPLD α 1 and CmLOX1 are consistent with a model in which their encoded enzymes act in tandem to promote or accelerate senescence in fruit mesocarp tissues.

Much research on the role of membrane lipid metabolism in fruit ripening, senescence, and postharvest deterioration of quality has focused on the cascade of enzymes involved in phospholipid (PL) hydrolysis and fatty acid peroxidation. An irreversible increase in PL catabolism is thought to predispose plant cells to membrane dysfunction and eventual cell death (Paliyath and Droillard, 1992; Thompson, 1988). The proposed senescence cascade begins with removal of PL polar head groups by phospholipase D (EC 3.1.4.4), yielding phosphatidic acid (PA), a non-bilayer-forming PL. Next, PA is dephosphorylated to diacylglycerol (DAG) by a specific PA phosphatase. Lipolytic acyl hydrolase then cleaves DAG, yielding free fatty acids (Brown et al., 1987; Paliyath and Thompson, 1987). These disrupt membrane structure, and linoleic and linolenic acid serve as substrates for lipoxygenase

(EC 1.13.11.12) (Fobel et al., 1987). LOX catalyzes formation of highly reactive hydroperoxides (Vick and Zimmerman, 1987) and may also generate superoxide radicals (Lynch and Thompson, 1984). Fatty acid hydroperoxides produced by LOX can perturb the membrane bilayer directly, or break down to yield toxic volatiles and free radicals that attack additional membrane components (Thompson et al., 1987).

With the advent of genomics and proteomics, PLD and LOX are now known to be members of two complex plant gene families that have important roles in growth and development, and in responses to biotic and abiotic stress (Feussner and Wasternack, 2002; Laxalt and Munnik, 2002; Porta and Rocha-Sosa, 2002; Siedow, 1991; Wang, 2000, 2002). During the last decade, five distinct types of plant PLDs have been cloned and characterized. Designated as α , β , γ , δ , and ζ , they differ in structure, cofactor requirements, substrate specificity, subcellular and tissue localization, and physiological function (Qin and Wang, 2002; Wang, 2000, 2002). PLD α is the most abundant and widely distributed PLD class, and is most likely the key PLD involved in general catabolism of membrane PL (Wang, 2000). One series of studies indicated that PLD α is probably involved in membrane restructuring and proliferation in young, rapidly growing tissues (Wang, 2000). However, it has also been shown that ethylene and abscisic acid (ABA) induce expression and activation of PLD α ,

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and promote senescence in detached leaves (Fan et al., 1997). Although oxidative stress and reactive oxygen species (ROS) appear to be an integral part of postharvest deterioration of fruit quality (Hodges, 2003), studies of various types of stress-induced senescence involving ethylene suggest that lipid hydrolysis by PLD α and other enzymes can be an earlier, more important event (Lee et al., 1998; Page et al., 2001; Pavelic et al., 2000). Moreover, there is evidence that, via production of PA, PLD α can stimulate ROS (superoxide) production in response to stress (Sang et al., 2001).

The LOX gene family in plants also encodes distinct types of enzymes with different patterns of expression, localization, and functions. Demonstrated physiological roles of plant LOXs include production of jasmonic acid and other signaling and defense volatiles in response to wounding or insect feeding, mobilization of storage lipids during seed germination (in cucumber), and serving as storage proteins in vegetative tissues (Feussner and Wasternack, 2002; Porta and Rocha-Sosa, 2002). The proposed involvement of LOX in deterioration of cell membranes during plant senescence has been less thoroughly explored, but there is evidence that specific LOX isoforms are key components of senescence in flowers, including carnation (*Dianthus caryophyllus* L.) (Fobel et al., 1987), daylily (*Heemerocallis* L.) (Panavas and Rubinstein, 1998), and rose (*Rosa* L.) (Fukuchi-Mizutani et al., 2000). As well, of the five LOX isogenes identified in tomato, one (TomloxB) is thought to contribute to ripening and senescence of tomato fruit (Griffiths et al., 1999; Kausch and Handa, 1997).

Loss of plasma membrane integrity, associated with a marked increase in electrolyte leakage in mesocarp tissues, has been identified as a key parameter in senescence of netted and nonnetted muskmelon fruit (Lacan and Baccou, 1996, 1998; Lester and Stein, 1993). Ripening, softening, and senescence of nonnetted honeydew and netted muskmelon (*C. melo* Reticulatus Group) fruits proceed from the seed cavity outward (Lester, 1988). The middle mesocarp (MM) constitutes the bulk of the edible tissue and is prone to over-softening, whereas during postharvest storage, integrity of the hypodermal mesocarp (HM) becomes critical with respect to water loss and quality maintenance (Lester, 1998, 2000). Rates of lipid peroxidation and generation of free radicals were much greater in a rapidly senescing than in a slowly senescing variety of nonnetted muskmelon (Lacan and Baccou, 1998), and increased LOX activity in HM tissue occurred with the onset of senescence in netted and nonnetted muskmelons (Lester, 1990, 1998). Substantial loss of membrane PL and increases in PA and the sterol:PL ratio are also typically associated with ripening and senescence of muskmelon mesocarp tissues (Lacan and Baccou, 1996, 1998; Lester 1990, 1998; Lester and Stein, 1993; Lester and Whitaker, 1996), suggesting a role of PLD in PL catabolism. We have begun efforts to clone PLD and LOX genes from honeydew melon and to characterize their expression and the activities of their encoded enzymes in relation to development, ripening, and senescence of melon fruit. Here we report cloning of the full-length cDNAs CmPLD α 1 and CmLOX1 (GenBank accession numbers DQ267933 and DQ267934, respectively). Semi-quantitative RT-PCR was used to determine the relative levels of expression of the corresponding genes in developing fruit mesocarp tissues, as well as in roots, leaves, stems, and flowers.

Materials and Methods

PLANT MATERIAL AND TISSUE SAMPLING. 'Honey Brew' hybrid honeydew muskmelon plants were grown in a greenhouse with

supplemental lighting at the USDA-ARS Subtropical Agricultural Research Center in Weslaco, Texas as described previously by Lester (2000). Flowers were hand pollinated and one fruit per plant was allowed to develop. Four developing and mature fruit were harvested at 20, 30, 40, and 50 d after pollination (DAP), and at abscission (full-slip, typically $\approx$ 55 DAP). Fifty DAP is considered commercial maturity (3/4-slip). One group of full-slip melons was held at 10 °C and 85% relative humidity for 5 d to simulate postharvest transport. Melon fruit were washed and processed shortly after harvest. Using a vegetable peeler, the epidermis was stripped away, followed by sequential excision of the HM and MM tissues, which were immediately frozen in liquid nitrogen. Vegetative tissues, including leaves, stems, and roots, were harvested simultaneously from a set of five plants at 4 weeks postgermination. After rinsing to remove any soil, leaves (one young, fully expanded leaf per plant) were dissected to yield separate lamina and petiole samples, and roots were dissected to yield main and lateral root samples. Twenty-five male and 25 female flowers were harvested when first fully opened from a second set of five plants (five male and female flowers from each plant) over the span of 6–7 weeks postgermination. All tissues were quickly frozen in liquid nitrogen. Forty-gram lots (fresh weight) of fruit mesocarp tissues and 5 to 10-g lots of floral and vegetative tissues were sealed in 50-mL plastic screw-cap centrifuge tubes. The tubes were packed in dry ice and shipped by overnight courier to the USDA-ARS Produce Quality and Safety Laboratory in Beltsville, Md., where they were stored at -80 °C until used for RNA extraction.

EXTRACTION AND PURIFICATION OF RNA. Total RNA was isolated as described by Pechous et al. (2005) with modification. Frozen tissue samples (5–10 g) were ground to a powder in liquid N₂ using a mortar and pestle. Small subsamples (0.2 g) of the homogenized, powdered tissue were transferred to 15-mL screw-cap centrifuge tubes, followed by immediate addition of 0.25 mL of plant aid solution and 2 mL of lysis buffer from the RNAqueous RNA extraction kit (Ambion, Austin, Texas). After vortexing and incubation for 10 min at 25 °C, the samples were processed according to the manufacturer's instructions. The crude RNA preparations were then treated with DNA-free DNase (Ambion) to degrade genomic DNA, extracted with 1 phenol : 1 chloroform (v/v), and total RNA was precipitated by addition of 1 volume isopropyl alcohol plus 0.1 volume of 3 M sodium acetate, pH 5.5. The precipitated RNA was pelleted by centrifugation, washed with cold 70% ethanol, and resuspended in diethyl pyrocarbonate-treated water. Quality of the extracted RNA was checked by agarose gel electrophoresis and RNA was quantified spectrophotometrically.

CLONING OF CmPLD α 1 AND CmLOX1 COMPLETE cDNAs. Total RNA isolated from leaf lamina tissue of *C. melo* 'Honey Brew' plants was used for the initial cloning of PLD and LOX cDNA fragments, including products of 3' and 5' RACE (rapid amplification of cDNA ends). As the initial step in cloning a *C. melo* PLD α gene, degenerate primers were designed based on two segments of the highly conserved second active site in plant PLD α s with the amino acid sequences DAYINA and NQYFLGS. Degeneracy was determined by sequence alignment of PLD α cDNAs from cabbage (*Brassica oleracea* L. var. *capitata* L.), tomato, castor bean, cowpea, and arabidopsis [*Arabidopsis thaliana* (L.) Heynh.] (GenBank accession numbers AF090444, AF154425, L33686, U92656, and U36381, respectively). The two primers, with the sequences 5'-A GAY GCT TAY ATT AAT GCT AT-3' and 5'-AAY CAA TAT TTC CTT GGA AGT T-3' (Y = C + T),

were used as the outer and inner primers, respectively, in a 3' RACE reaction. First strand cDNA was synthesized from 5 µg of leaf total RNA using oligo dT adaptor primers provided with the Invitrogen Life Technologies (Carlsbad, Calif.) 3' RACE System kit according to the manufacturer's instructions. 3' RACE was then performed using the two nested degenerate primers as the forward primers and a pair of nested primers provided with the kit (5'-CUA CUA CUA CUA GGC CAC GCG TCG ACT AGT AC-3' and 5'-GGC CAC GCG TCG ACT AGT AC-3') as the reverse primers. PCR was performed with the melting temperature 94 °C, annealing temperature 55 °C, and extension temperature 72 °C for 90 s run for 34 cycles. The amplified ≈1.3-kb product was gel purified using the QIAquick Gel Extraction Kit (QIAGEN, Valencia, Calif.) and cloned into pGEM-T Easy cloning vector (Promega Corp., Madison, Wis.) for sequencing.

After confirming that the 3' RACE product shared high sequence identity with known plant PLD α s, two gene-specific nested reverse primers based on the partial cDNA sequence were synthesized for use in a 5' RACE reaction (outer primer 5'-TT GCC AAG ACA GAA GAA TGT CA-3' and inner primer 5'-CT CCT CTG CCA ATC TAA TAT-3'). 5' RACE was performed following instructions in the FirstChoice RLM-RACE kit (Ambion) manual. Five µg of total RNA was first treated with calf intestine alkaline phosphatase and tobacco acid pyrophosphatase. A 45-bp RNA adaptor (5'-GCU GAU GGC GAU GAA UGA ACA CUG CGU UUG CUG GCU UUG AUG AAA-3') was then ligated to the RNA population using T4 RNA ligase. First strand cDNA was synthesized from the adaptor-ligated RNA using the random decamers provided in the kit. 5' RACE was carried out using the gene-specific nested reverse primers and a pair of nested forward primers provided with the kit (outer primer 5'-GCT GAT GGC GAT GAA TGA ACA CTG-3' and inner primer 5'-CGC GGA TCC GAA CAC TGC GTT TGC TGG CTT TGA TG-3'). PCR was performed, and the ≈2.0-kb product was gel purified and cloned into pGEM-T Easy cloning vector (Promega) for sequencing. Comparison and alignment of the nucleotide sequences of the 3' and 5' RACE products provided a full-length PLD α cDNA sequence. To confirm that the PLD α gene (*CmPLD α 1*) is expressed in *C. melo* fruit, random hexamer primed cDNA was generated from pooled fruit mesocarp tissue RNA (all six stages) and was used as template with the gene-specific primers 5'-ATG GAA GGT ACT TCG CTT CAC GGA-3' (forward) and 5'-TTA GGT AGT GAG GAT GGG AGG GAG-3' (reverse) to PCR-amplify the open reading frame of *CmPLD α 1*.

Cloning of a *C. melo* LOX gene began with reverse transcription of leaf RNA followed by gene-specific PCR amplification. cDNA was generated from 5 µg total RNA using the Thermoscript RT-PCR System (Invitrogen) with random hexamer primers following the manufacturer's guidelines. The initial PCR performed to amplify a *C. melo* LOX cDNA fragment utilized the forward primer 5'-GAA GAA TTT GCA AGA GAA ATG-3' and reverse primer 5'-AA CTT ATG GAT TGG ATG AAG CAC-3', which represent two identical segments of the *C. sativus* root LOX and cotyledon LOX cDNAs reported in GenBank under accession numbers U36339 and X92890 (see Table 2). PCR included the primer pairs at 300 nM, dNTPs at 200 µM, 1.5 mM MgCl $_2$, 2.5 units of *platinum Taq* DNA polymerase (Life Technologies, Rockville, Md.), and 2 µL of the RT reaction products as template. Thirty cycles of PCR were performed, with melting at 94 °C for 30 s, annealing at 52 °C for 30 s, and extension at 68 °C for 60 s, plus a 2-min initial melting step at 94 °C and a 5-min final extension at 68 °C. The cucumber gene-specific LOX primer pair yielded a

PCR product of ≈0.6 kb, which was gel purified and cloned into pGEM-T Easy cloning vector (Promega) for sequencing. Sequence analysis revealed high identity with plant LOX cDNAs reported in GenBank, particularly U36339 and X92890.

Based on the sequence of the initial RT-PCR product, gene specific nested primers were designed for use in a 3' RACE reaction (outer primer 5'-CCT CCC AAA AGC AAA CTT GA-3' and inner primer 5'-C GAG TTG AGT CTG CCA CAC C-3'). First strand cDNA was synthesized from 5 µg of total RNA as instructed in the Ambion FirstChoice RLM-RACE kit manual using the 3' RACE adaptor primer 5'-GCG AGC ACA GAA TTA ATA CGA CTC ACT ATA GGT12 VN-3' provided with the kit. 3' RACE was then performed using the two LOX gene-specific nested forward primers and a pair of nested reverse primers provided with the kit (outer primer 5'-GCG AGC ACA GAA TTA ATA CGA CT-3' and inner primer 5'-CGC GGA TCC GAATTAATA CGA CTC ACT ATA GG-3'). PCR was performed for 34 cycles with the melting temperature 94 °C, annealing temperature 55 °C, and extension temperature 72 °C for 90 s. The amplified ≈1.6-kb product was gel purified using the QIAquick Gel Extraction Kit (QIAGEN) and cloned into pGEM-T Easy cloning vector (Promega) for sequencing.

Two gene-specific nested reverse primers based on the partial LOX cDNA sequence obtained by 3' RACE were synthesized for use in a 5' RACE reaction (outer primer 5'-GC ATG AGT ATG CAA CCA ATG GCT-3' and inner primer 5'-G GTG TGG CAG ACT CAA CTC G-3'). 5' RACE was performed using the FirstChoice RLM-RACE kit (Ambion) as described for cloning of *CmPLD α 1*. The ≈1.7-kb PCR product was gel purified and cloned into pGEM-T Easy cloning vector (Promega) for sequencing. Comparison and alignment of the nucleotide sequences of the 3' and 5' RACE products provided a full-length LOX cDNA sequence. To confirm that the LOX gene (*CmLOX1*) is expressed in *C. melo* fruit, random hexamer primed cDNA was generated from pooled fruit mesocarp tissue RNA (all six stages) and was used as template with the gene-specific primers 5'-ATG TTT GGA ATT GGG AAG AAC-3' (forward) and 5'-TCA AAT AGA GAT ACT GCT TGG AAT-3' (reverse) to PCR-amplify the *CmLOX1* open reading frame.

SEMI-QUANTITATIVE RT-PCR. Two micrograms of total RNA extracted from the fruit, vegetative, or floral tissues were used to generate cDNA samples by random priming with Superscript III reverse transcriptase (Invitrogen). Semi-quantitative RT-PCR was performed using the QuantumRNA Universal 18S PCR kit (Ambion) with a 6.5:3.5 competitor to 18S primer pair ratio. Two microliters of the cDNA samples were used as the template in each of two replicate PCR reactions run for 24 cycles with 55 °C annealing temperature and an extension for 1 min at 68 °C. The *CmPLD α 1* forward primer 5'-A GAC GCT TAC ATT AAT GCT AT-3' (coding region nucleotides 1503–1523) and reverse primer 5'-TTA GGT AGT GAG GAT GGG AGG GAG-3' (nucleotides 2427–2404), the *CmLOX1* forward primer 5'-CCT CCC AAA AGC AAA CTT GA-3' (coding region nucleotides 1210–1229) and reverse primer 5'-G TGT TGC AAT CAC AAA TGG C-3' (nucleotides 1660–1641), and the 18S universal primers yielded PCR products 925, 451, and 315 bp long, respectively. Products of the multiplex PCRs of each sample using the set of *CmPLD α 1* or *CmLOX1* primers plus the set of 18S primers were run on a 1.2% agarose gel and visualized by staining with ethidium bromide (0.1 µg·mL $^{-1}$). The PCR products were quantified using a densitometry program in the Fluor-S imaging system (Bio-Rad Laboratories, Hercules, Calif.). Relative levels of the *CmPLD α 1* or *CmLOX1*

primer products were then normalized based on the density of the corresponding 18S products for each sample.

DNA SEQUENCING. Automated dideoxy sequencing was performed at the Iowa State University DNA Sequencing and Synthesis Facility. Reactions were set up using the Applied Biosystems (Foster City, Calif.) Prism BigDye Terminator cycle sequencing kit with AmpliTaq DNA polymerase (version 3.1; Roche Molecular Systems, Alameda, Calif.), and reactions were electrophoresed on an Applied Biosystems 3730 DNA analyzer. The programs BLASTN and BLASTP (Altschul et al., 1997) were used to search the National Center for Biotechnology Information GenBank nucleotide and protein sequence databases.

Results

CLONING AND CHARACTERIZATION OF *CmPLDα1* AND *CmLOX1* cDNAs. PCR amplification of partial cDNAs using pairs of degenerate or gene-specific primers based on highly conserved regions of reported plant PLDα and LOX cDNA sequences, followed by 3' and 5' RACE, enabled us to clone one complete PLDα cDNA (*CmPLDα1*) and one complete LOX cDNA (*CmLOX1*) from *C. melo* 'Honey Brew'. *CmPLDα1* is a total of 2877 nucleotides long, with a 2427-bp open reading frame (ORF) encoding a protein 808 amino acids (AAs) long. The *CmLOX1* cDNA is 2887 bp long, with a 2634-bp ORF encoding an 877-AA protein. National Center for Biotechnology Information database searches using the BLASTN and BLASTP programs (Altschul et al., 1997) showed that *CmPLDα1* is most similar to α-type PLDs from castor bean, cowpea, strawberry, and tomato (Table 1), sharing 77% and 82% to 84% identity, respectively, in the nucleotide and deduced protein sequences. Not surprisingly, BLAST searches showed that *CmLOX1* is most similar to a pair of type-1 (non-chloroplastic) 13-LOX genes from roots and cotyledons of cucumber (Table 2). In fact, *CmLOX1* appears to be a very close homolog of the cucumber root LOX1, with 94% nucleotide and 93% amino acid

identity, whereas *CmLOX1* differs substantially from a second cucumber cotyledon cDNA (GenBank accession AJ271161) that encodes a 9-LOX.

EXPRESSION OF *CmPLDα1* AND *CmLOX1* IN MESOCARP TISSUES DURING FRUIT DEVELOPMENT, RIPENING, AND SENESCENCE. Levels of *CmPLDα1* and *CmLOX1* transcripts in fruit middle and hypodermal mesocarp tissues (MM and HM, respectively) were determined by semi-quantitative RT-PCR at five growth stages encompassing fruit development and ripening (20–55 DAP), as well as after 5 d storage of ripe fruit (55 DAP) at 10 °C (Fig. 1). As depicted at the top of Fig. 1A, honeydew melon fruit enlarged from about 10 cm in diameter at 20 DAP to full size at 50 DAP, which is usually considered to be commercial harvest maturity, referred to as 3/4-slip. By ~55 DAP the ripe melons abscise from the peduncle (full-slip).

Since the same competitor to 18S primer pair ratio and the same number of PCR cycles were used in the quantitative RT-PCR analyses of *CmPLDα1* and *CmLOX1* transcript levels, it is clear from the agarose gel images in Fig. 1A that *CmPLDα1* transcript was generally much more abundant than that of *CmLOX1* in fruit mesocarp tissues. *CmPLDα1* expression was high and essentially constitutive throughout fruit development and storage, although maximum transcript levels in MM and HM tissues occurred at 50 and 55 DAP, respectively, and transcript was slightly more abundant in MM than in HM at most stages (Fig. 1B). In contrast, *CmLOX1* expression was generally low, increased with fruit maturation and ripening, and had a different pattern in MM and HM tissues (Fig. 1C). *CmLOX1* transcript declined over three-fold between 20 and 30 DAP in MM tissue, increased steadily to a maximum at 55 DAP, and then decreased about two-fold during the 5-d cool storage period. In HM tissue, *CmLOX1* transcript was quite low at all fruit stages with the exception of 50 DAP, when the level was at least three-fold higher than that at each of the other five stages.

Table 1. Nucleotide and encoded amino acid sequence identities of PLDα cDNAs from castor bean, cowpea, strawberry, and tomato to 'Honey Brew' honeydew melon *CmPLDα1* (GenBank accession numbers: cDNA, DQ267933; encoded protein, ABB82551). The plant organs from which the mRNAs were isolated are also shown.

Plant source	Organ mRNA	GenBank accession no.		Sequence identity (%)	
		cDNA	Protein	Nucleotide	Amino acid
Castor bean	leaf	L33686	AAB37305	1875 / 2427 (77)	675 / 808 (84)
Cowpea	leaf	U92656	AAB51392	1869 / 2430 (77)	675 / 809 (83)
Strawberry	fruit	AY758359	AAW83125	1879 / 2436 (77)	669 / 810 (83)
Tomato	root	AY013252	AAG45485	1864 / 2430 (77)	662 / 809 (82)

Table 2. Nucleotide and encoded amino acid sequence identities of three cucumber LOX cDNAs to 'Honey Brew' honeydew melon *CmLOX1* (GenBank accession numbers: cDNA, DQ267934; encoded protein, ABB82552). Product regiospecificities of the bacterially expressed *C. sativus* LOX enzymes are also shown if known.

Cucumber organ	GenBank accession no.		Sequence identity (%)		Product ^a 13S / 9S
	cDNA	Protein	Nucleotide ^b	Amino acids ^c	
Root	U36339	AAA79186	2477 (94)	815 (93)	54 / 46
Cotyledon	X92890	CAA63483	2111 (80)	670 (76)	84 / 16
Cotyledon	AJ271161	CAB83038	1639 (62)	504 (57)	n.d. ^w

^aRegiospecificity of recombinant LOX enzyme (% 13S- and 9S- hydroperoxy and hydroxy products).

^bOpen reading frames of U36339, X92890, and AJ271161 are 2634, 2637, and 2646 bp, respectively.

^cEncoded LOX proteins AAA79186, CAA63483, and CAB83038 are 877, 878, and 881 amino acids long, respectively.

^wNo data are available.

Fig. 1. Semi-quantitative RT-PCR results using random-primed cDNA produced from RT reactions including 2 μ g of total RNA from middle mesocarp (MM) and hypodermal mesocarp (HM) tissues of honeydew melons harvested 20, 30, 40, 50, and 55 d after pollination, plus 55-d fruit held 5 d postharvest at 10 $^{\circ}$ C (55 + 5d). Images of ethidium bromide-stained 1.2% agarose gels (A) show the relative intensity of the amplified 0.93-kb *CmPLD α 1* (PLD) and 0.45-kb *CmLOX1* (LOX) fragments, and the 0.32-kb 18S control cDNA fragment, generated using the QuantumRNA Universal 18S PCR kit (Ambion, Austin, Texas) with a 6.5:3.5 competitor to 18S primer pair ratio (Mol mkr = DNA molecular size markers). Bar graphs show the *CmPLD α 1*:18S (B) and *CmLOX1*:18S (C) gel band density ratios (Rel. intensity = relative intensity) obtained by densitometry measurements. Error bars indicate the SE for replicate PCR amplifications of the RT cDNA products run on two separate gels.

EXPRESSION OF *CmPLD α 1* AND *CmLOX1* IN VEGETATIVE AND FLORAL TISSUES. Semi-quantitative RT-PCR was also used to determine levels of *CmPLD α 1* and *CmLOX1* transcripts in vegetative and floral tissues from, respectively, 4- and 6- to 7-week-old 'Honey Brew' plants (Fig. 2). The tissues/organs analyzed included leaf laminae and petioles, main and lateral roots, stems, and both female and male flowers. As observed in fruit MM and HM tissues, the ethidium bromide-stained agarose gel images in Fig. 2A show that *CmPLD α 1* transcript was typically much more abundant than that of *CmLOX1* (with the exception of stems, in which the levels of both transcripts were quite low). The pattern of expression in the various tissues was similar for the two genes; transcripts of *CmPLD α 1* and *CmLOX1* were most abundant in main and lateral root tissues, and levels were also relatively high

Fig. 2. Semi-quantitative RT-PCR results using random-primed cDNA produced from RT reactions including 2 μ g of total RNA from 'Honey Brew' honeydew melon leaf (lamina), main and lateral root, stem, female and male flower, and petiole tissues. All tissues were harvested simultaneously from a set of five 4-week-old plants, with the exception of flowers, which were harvested as they opened from five 6- to 7-week-old plants. Images of ethidium bromide-stained 1.2% agarose gels (A) show the relative intensity of the amplified 0.93-kb *CmPLD α 1* (PLD) and 0.45-kb *CmLOX1* (LOX) fragments, and the 0.32-kb 18S control cDNA fragment, generated using the QuantumRNA Universal 18S PCR kit (Ambion, Austin, Texas) with a 6.5:3.5 competitor to 18S primer pair ratio (Mol mkr, DNA molecular size markers). Bar graphs show the *CmPLD α 1*:18S (B) and *CmLOX1*:18S (C) gel band density ratios (Rel. intensity = relative intensity) obtained by densitometry measurements. Error bars indicate the SE for replicate PCR amplifications of the RT cDNA products run on two separate gels.

in female and male flowers (Fig. 2 B and C). Expression of the two genes was roughly comparable in stem and petiole tissues, whereas in leaf laminae there was a disproportionately low, barely detectable level of *CmLOX1* transcript (Fig. 2 B and C).

Discussion

Extensive loss of membrane PL, increased electrolyte leakage, and accumulation of lipid peroxidation products are common features of senescence in plant tissues (Paliyath and Droillard,

1992; Thompson, 1988; Thompson et al., 1998). PLD and LOX catalyze the first and last steps of the senescence cascade which has been proposed to account for PL catabolism and loss of membrane function in senescing plant organs including flowers, leaves, cotyledons, and fruits (Paliyath and Droillard, 1992; Thompson et al., 1998). It has been demonstrated that concerted action of the four cascade enzymes (PLD, phosphatidate phosphatase, lipolytic acyl hydrolase, and LOX) in microsomal membranes from ripening tomato fruit pericarp tissue results in degradation of phosphatidylcholine and production of linoleic and linolenic acid hydroperoxides (Todd et al., 1990, 1992). Accumulated evidence indicates that senescence of mesocarp tissues in netted and non-netted muskmelon fruits is associated with increased leakage of ions across cell membranes (Lacan and Baccou, 1996; Lester, 1988) and decreased membrane H⁺- and Ca²⁺-ATPase activity (Lester, 1998; Lester and Stein, 1993) as a consequence of PL catabolism and lipid peroxidation (Lacan and Baccou, 1996, 1998; Lester and Whitaker, 1996). Moreover, increased activities of LOX and PLD have been linked with these changes in muskmelon fruit tissues (Lacan and Baccou, 1998; Lester, 1990, 2000).

To date, there have been few studies of PLD α and LOX gene expression and enzyme activities in relation to fruit development, ripening, and senescence. The most extensive data available are for tomato, which for many years has served as a model fruit for investigation of genetic and biochemical regulation of ripening. Three PLD α and five LOX isogenes have been reported in tomato (Chen et al., 2004; Laxalt et al., 2001; Pinhero et al., 2003; Whitaker et al., 2001). Of the PLD α s, *LePLD α 2* (AF154425) and *LePLD α 3* (AY013253) transcripts increase markedly during development and ripening of fruit pericarp tissue (Laxalt et al., 2001; Whitaker et al., 2001), whereas expression of *LePLD α 1* (AF201661; AY013252), which was cloned using root tissue RNA (Pinhero et al., 2003), appears to decrease after the mature green stage (Laxalt et al., 2001). Surprisingly, fruit from two transgenic lines of one tomato cultivar transformed with an antisense construct of *LePLD α 1* did exhibit somewhat reduced PLD α gene expression and enzyme activity as well as delayed senescence (Pinhero et al., 2003), perhaps as a result of partial suppression of all three PLD α isogenes. We found that *CmPLD α 1* transcript was abundant in fruit and flower tissues, as was shown for *LePLD α 2* and *LePLD α 3*, but unlike the two tomato genes, expression of *CmPLD α 1* was also quite high in roots. In addition, expression of *CmPLD α 1* was nearly constitutive in honeydew melon mesocarp tissues, with a modest increase in mature, ripening fruit (50–55 DAP), whereas *LePLD α 2* transcript increased up to 8-fold over the course of tomato fruit development and ripening (Whitaker et al., 2001). In the open reading frame, *CmPLD α 1* has 71% to 77% identity with the three tomato PLD α cDNAs, sharing the greatest identity with *LePLD α 1* (Table 1). The close similarity of *CmPLD α 1* with the PLD α cDNAs from castor bean, cowpea, and strawberry (Table 1) may be a matter of phylogenetic relatedness rather than specific gene/enzyme function. To our knowledge, *CmPLD α 1* is the first PLD gene reported for the Cucurbitaceae.

All five tomato LOX genes (Tomlox A–E) are expressed in tomato fruit, but the timing, magnitude, and inducibility of expression, as well as the localization and function of the LOX proteins, differ substantially (Chen et al., 2004; Griffiths et al., 1999). TomloxC and TomloxD are both chloroplast-targeted but have different roles; TomloxC is specifically required for aroma/flavor volatile production (Chen et al., 2004), whereas TomloxD is dedicated to wound-inducible production of jasmonic

acid (Heitz et al., 1997). Of the remaining three genes, TomloxB and TomloxE appear most likely to play a role in fruit ripening and senescence because they are up-regulated by ethylene, their expression increases dramatically in the late stages of ripening, and they are essentially fruit-specific (Chen et al., 2004; Griffiths et al., 1999; Kausch and Handa, 1997). In contrast, we found that expression of *CmLOX1* was generally low and transcript levels in young roots and flowers were comparable to or higher than those in fruit mesocarp tissues. On the other hand, the developmental pattern of *CmLOX1* expression in fruit mesocarp (i.e., a steady increase to a maximum at 55 DPA in MM and a transient $\approx$ 4-fold increase at 50 DPA in HM) suggests that this LOX could be involved in triggering the onset of ripening and senescence. It would not be surprising if *CmLOX1* has a role in muskmelon root physiology since it is such a close homolog of the cucumber *crlox-1* cDNA (U36339; Table 2) cloned from a 4-d-old root tissue cDNA library (Matsui et al., 1998). The protein encoded by *CmLOX1*, like the CRLOX-1 enzyme, does not include an N-terminal transit peptide sequence (Type-1 LOX) and has a critical Threonine-Histidine pair at AA 595–596 that predicts 13-LOX regiospecificity [i.e., its products should be predominantly 13-hydroxy and 13-hydroperoxy fatty acids (Hornung et al., 1999)]. However, bacterially-expressed CRLOX-1 yielded nearly equal proportions of 13S and 9S products from linoleic and linolenic acid (Table 2; Matsui et al., 1998), whereas plant 13-LOX enzymes typically yield >95% 13S products (Feussner and Wasternack, 2002). Another very unusual feature of CRLOX-1 is its ability to directly oxygenate polyunsaturated fatty-acyl moieties in phosphatidylcholine (Matsui et al., 1998). It seems quite likely that *CmLOX1* will exhibit the same enzymatic properties, considering the high percentage of sequence identity with CRLOX-1 (Table 2).

For those plant species such as tomato that have been studied in depth, multiple PLD and LOX genes and/or enzymes have been identified, typically two or more PLD α s and as many as 14 LOXs [in potato (*Solanum tuberosum* L.)]. In the present work, we were successful in cloning one cDNA of each. Cloning of a second PLD α is in progress, and it remains to be determined whether this *CmPLD α 2* and possibly other PLD α and LOX isogenes are expressed in fruit tissues. Nevertheless, the patterns of expression of *CmPLD α 1* and *CmLOX1* are consistent with a model in which their encoded enzymes act in tandem to promote or accelerate senescence in fruit mesocarp tissues. In light of the much higher and more constitutive expression of *CmPLD α 1* compared with *CmLOX1*, it is possible that the stage-specific, transient expression of *CmLOX1* serves as a trigger to initiate the degradation of cell membranes associated with melon fruit ripening and senescence. One scenario, considering the unusual enzymatic activity of closely related CRLOX-1, is that *CmLOX1* first attacks membrane PL, producing molecular species that include hydroxy and hydroperoxy fatty acids. These oxygenated PL species might then be preferentially hydrolyzed by PLD or PLA₂ (Banaś et al., 1992) to start the senescence cascade. It has also been reported that membrane-associated PLD in senescing plant cells shows a substrate preference for PL molecular species with linoleic and/or linolenic acid at both the *sn*1 and *sn*2 positions (Brown et al., 1987), thus, after the action of lipolytic acyl hydrolase, providing free fatty acid substrates for LOX. Finally, both PA and oxidized fatty acids, the respective products of PLD and LOX, are known to act as Ca²⁺ ionophores in membranes (Serhan et al., 1981). An increase in cytoplasmic Ca²⁺ above submicromolar levels activates and promotes membrane asso-

ciation of PLD α , and has numerous other deleterious effects on cellular metabolism (Paliyath and Droillard, 1992; Thompson, 1988; Thompson et al., 1998).

As stated above, prior research by Lester and coworkers as well as others has established that PLD and LOX enzymes likely play a key role in senescence of muskmelon mesocarp tissues. In particular, Lester (1990, 1998) showed that increased LOX activity is linked with loss of plasma membrane integrity during senescence of HM tissue. The present study laid the foundation for future testing of the hypothesized role of PLD and LOX at the molecular level. Work is currently in progress examining PLD α and LOX activities and protein levels in relation to CmPLD α 1 and CmLOX1 isogene-specific expression in 'Honey Brew' fruit MM and HM tissues. Our ultimate aim is to determine whether fruit-directed suppression or silencing of specific PLD α and LOX genes results in delayed senescence and extended shelf life of honeydew muskmelon fruit.

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