An ethylene-induced cDNA encoding a lipase expressed at the onset of senescence

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A cDNA clone encoding a lipase (lipolytic acyl hydrolase) expressed at the onset of petal senescence has been isolated by screening a cDNA expression library prepared from carnation flowers (*Dianthus caryophyllus*). The cDNA contains the lipase consensus sequence, ITFAGHSLGA, and encodes a 447-amino acid polypeptide with a calculated molecular mass of 50.2 kDa that appears to be a cytosolic protein. Over-expression of the clone in *Escherichia coli* yielded a protein of the expected molecular weight that proved capable of deesterifying fatty acids from *p*-nitrophenylpalmitate, tri-linolein, soybean phospholipid, and Tween in both *in vitro* and *in situ* assays of enzyme activity. The abundance of the lipase mRNA increases just as carnation flowers begin to senesce, and expression of the gene is also induced by treatment with ethylene. Southern blot analyses of carnation genomic DNA have indicated that the lipase is a single copy gene. The lipase gene is also expressed in carnation leaves and is up-regulated when the leaves are treated with ethylene. Deesterification of membrane lipids and ensuing loss of membrane structural integrity are well established early events of plant senescence, and the expression pattern of this lipase gene together with the lipolytic activity of its cognate protein indicate that it plays a fundamentally central role in mediating the onset of senescence.

Membrane deterioration is an early and characteristic feature of plant senescence engendering increased permeability, loss of ionic gradients, and decreased function of key membrane proteins such as ion pumps (1). One of the clearest manifestations of this is the onset of membrane leakiness measurable as increased conductivity of diffusates from intact tissue. This is detectable in carnation petals, for example, well before petal inrolling, the first morphological manifestation of senescence in this tissue, and also before the climacteric-like rise in ethylene production (2). The decline in membrane structural integrity at the onset of senescence appears to be largely attributable to accelerated metabolism of membrane lipids and ensuing change in the molecular organization of the bilayer. Indeed, loss of membrane phospholipid is one of the best documented indices of membrane lipid metabolism during senescence and has been demonstrated for senescing flower petals, leaves, cotyledons and ripening fruit (3, 4).

The selective depletion of phospholipid fatty acids from the membranes of senescing tissues results in an increase in the sterol:fatty acid ratio in the bilayer and a consequent decrease in bulk lipid fluidity. This has been demonstrated by fluorescence depolarization and electron spin resonance for microsomal membranes from senescing cotyledons, flowers, leaves, and ripening fruit (5–8) and for plasmalemma of ripening fruit and senescing flowers (5). The decrease in lipid fluidity is engendered by an enrichment of free sterols relative to fatty acids in the bilayer as fatty acids are cleaved from the membrane lipids and selectively removed, reflecting the fact that free sterols are known to restrict the mobility of phospholipid fatty acids (9). As well, in some senescing tissues, the decrease in bulk lipid fluidity appears to be caused in part by a selective depletion of polyunsaturated fatty acids from membranes and an ensuing increase in the saturated-to-unsaturated fatty acid ratio (6). There are also reports that the large changes in bulk membrane lipid fluidity accompanying senescence may alter the conformation of membrane proteins, rendering them prone to proteolysis (10, 11).

Recent data suggest that free fatty acids arising from the metabolism of membrane lipids may be removed from the bilayer by blebbing of lipid particles highly enriched in free fatty acids from the membrane surface into the cytosol (12–14). These lipid particles appear to be structurally analogous to oil bodies. Indeed, there is growing evidence that the free fatty acids released from senescing membranes are metabolized by glyoxylate cycle enzymes also induced at the onset of senescence (15). However, it is also clear that free fatty acids accumulate in senescing membranes and induce lipid-phase separations. The resulting mixture of liquid-crystalline and gel phase lipid domains in the bilayer contributes to the leakiness of senescing membranes because of packing imperfections at the phase boundaries (16).

Deesterification of membrane lipids at the onset of senescence and the resultant release of free fatty acids can be attributed to lipolytic acyl hydrolase, an enzyme that has broad substrate specificity and can deacylate phospholipids directly (17). Rose petals, for example, exhibit an increase in lipolytic acyl hydrolase at the onset of senescence that is accompanied by loss of membrane function (3). In the present study, we report the isolation and characterization of a cDNA clone from carnation petals that encodes a senescence-induced lipase exhibiting lipolytic acyl hydrolase activity. The carnation lipase contains the 10-amino acid consensus sequence characterizing animal, bacterial, and fungal lipases.

PLANT BIOLOGY

Materials and Methods

Plant Material and Subcellular Fractionation. Rooted carnation (*Dianthus caryophyllus* L. cv., Improved White Sim) cuttings were grown as described by Hudak and Thompson (18). Flowers at four stages of development (19) were cut and used directly. The four stages are as follows: I, petals cream-colored, not fully expanded from tight bud, outer petals about 30° from vertical; II, flower open with outer petals about 60° from vertical, no ethylene production; III, flower fully open, petals white and about 90° from vertical, small amounts of ethylene produced; IV, petals turned from white to cream-colored, inrolling of petal tips, loss of turgidity, flowers beginning to close, maximum ethylene production (19). Cut flowers at stage I of development were also placed in deionized water and were allowed to senesce under post-harvest conditions at room temperature. In some experiments, flowers at stage II of development were treated with 0.5 ppm of ethylene for 15 h in a sealed chamber to induce premature senescence (2).

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF026480).

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Lipase polyclonal antibodies were obtained by immunizing rabbits with lipid particles purified from carnation petal cytosol isolated just before the onset of visible flower senescence (stage III of development). These particles exhibit lipase (lipolytic acyl hydrolase) activity, and they were purified from the petal cytosol by flotation centrifugation (18).

RNA Isolation and Construction and Screening of a cDNA Library. Total RNA for Northern blot analysis was isolated from carnation petals and leaves as described by Puissant and Houdebine (20). For construction of a cDNA library, poly(A)$^+$ RNA for cDNA synthesis was purified from total RNA of petals at stage III of development by using a PolyA tract mRNA Isolation System (Promega). A cDNA library was prepared by using the ZAP express cDNA Synthesis Kit (Stratagene). This library and a cDNA library produced from petals of carnation flowers at stage IV of development (kindly provided by R. Woodson, Purdue University) were both screened for lipase clones.

Initially, $\approx 5 \times 10^5$ clones of the stage IV cDNA library were immuno-screened with lipid particle antiserum. Positive cDNA clones were recovered by the ExAssist Helper Phage/SOLR strain system and were recircularized in pBluescript SK(−) phagemid (Stratagene). This initial screen yielded a partial length lipase cDNA clone. A full-length lipase clone was obtained by screening the stage III petal cDNA library with a γ-^{32}P labeled 19-bp oligonucleotide probe (5′-ACCTACTAGGTTC-CGCGTC-3′) located in the ORF of the partial length lipase clone. Positive cDNA clones were excised from the phage and were recirculized into a pBK-CMV phagemid (Stratagene).

DNA Isolation, Sequencing, and Sequence Analysis. Plasmid DNA was isolated by alkaline lysis (21) and was sequenced at MOBIX (McMaster University, Hamilton, ON, Canada). The ORF of the carnation lipase cDNA was compiled and analyzed by using the BLAST Search (GenBank, Bethesda, MD), and sequence alignments were obtained by using the BCM Search Launcher (http://dot.imgen.bcm.tmc.edu:9331). Putative motifs in the amino acid sequence of the ORF were discerned by using LAMA Search in Block Maker of the BCM Search Launcher.

SDS/PAGE and Blotting Analyses. SDS/PAGE and Western blotting were carried out as described (22). Protein gels were stained with silver (22). For Northern blot analysis, total RNA was fractionated on 1.0% denatured formaldehyde agarose gels and was immobilized on a nylon membrane (ICN). To confirm equal loading, the fractionated RNA was stained with ethidium bromide and was visualized on the gel as well as on the nylon membrane after transfer by using an Image Master VDS (Amersham Pharmacia). Immobilized RNA was hybridized with lipase cDNA by incubating the nylon membranes overnight at 42°C with full-length cDNA labeled with α^{32}P-dCTP using a random primer kit (Boehringer Mannheim). The membranes were then washed once in 1 × standard saline citrate (SSC) containing 0.1% SDS at room temperature for 15 min and three times in 0.2 × SSC containing 0.1% SDS at 65°C for 15 min each. Hybridization was visualized by autoradiography after an overnight exposure at −70°C using Kodak x-ray film and two intensifying screens.

For Southern analysis, genomic DNA was isolated from petals of flowers at stage II of development (21) and was digested with the restriction endonucleases *Bam*HI, *Xba*I, *Xho*I, *Eco*RI, and *Hin*dIII. The digested products were fractionated on a 1.0% agarose gel, were immobilized on a nylon membrane, and were hybridized with lipase cDNA as for Northern blot analysis.

Expression and Purification of a Lipase Fusion Protein. To confirm that the cDNA isolated from carnation petals encodes lipase (lipolytic acyl hydrolase), the clone was over-expressed as a fusion protein in *Escherichia coli*, thereby enabling purification of expression product that could be used for assaying enzymatic activity. The lipase cDNA was subcloned into the fusion protein expression vector, pMalc (New England Biolabs), using *Eco*RI and *Xba*I cloning sites, and the resultant construct was expressed in *E. coli* BL-21(DE3), yielding a fusion protein consisting of the lipase linked through a proteolytic cleavage site, Factor Xa, to maltose-binding protein. The lipase fusion protein was purified from *E. coli* extracts by amylose column chromatography (21, 23). In some experiments, the fusion protein was denatured and treated with Factor Xa (1 μg/100 μg of fusion protein) as described by Sambrook *et al.* (21) to release the lipase.

Lipase Assays. Lipase (lipolytic acyl hydrolase) activity was measured by using both *in vitro* and *in situ* assays. For *in vitro* measurements, *p*-nitrophenylpalmitate and soybean phospholipid (40% phosphatidylcholine and 60% other phospholipids) were used as substrates, and the products of the reactions, *p*-nitrophenol and free fatty acids, respectively, were measured spectrophotometrically (24–26). Lipase activity was also measured *in vitro* by gas chromatography (GC) using a modification of the method described by Nixon and Chan (25) and Lin *et al.* (26). The reaction mixture contained 100 mM Tris·HCl (pH 8.0), 2.5 mM substrate (trilinolein, soybean phospholipid, or dilinoleylphosphatidylcholine), and enzyme protein (100 μg) in a final volume of 100 μl. The substrates were emulsified in 5% gum arabic before being added to the reaction mixture. To achieve this, the substrates were dissolved in chloroform, were added to the gum arabic solution, and were emulsified by sonication for 30 s. After emulsification, the chloroform was evaporated by a stream of N_2. The reaction was carried out at 25°C for varying periods of time up to 2 h. The reaction mixture was then lipid-extracted, and the free fatty acids were purified by TLC and were derivitized and quantified by GC (HP 5890 series II Gas Chromatography) (13). In some experiments, 100 μg of Factor Xa were added to the reaction mixture to progressively release the lipase from its association with maltose-binding protein during the reaction. Protein was assayed as described by Bradford (27) by using BSA as a substrate.

Lipolytic acyl hydrolase activity was measured *in situ* as described by Furukawa *et al.* (28) and modified by Tsuboi *et al.* (29). In brief, single colonies of *E. coli* BL-21 (DE3) harboring lipase fusion protein or maltose-binding protein alone were cultured in basal salt medium supplemented with Tween 40 or Tween 60 as the only source of carbon.

Results

Isolation of the Lipase cDNA Clone. A partial length (1,149 bp) cDNA clone containing the lipase gene family consensus sequence, ITFAGHSLGA, was isolated by screening a carnation petal cDNA expression library prepared from stage IV flowers with antibodies raised against carnation petal cytosolic lipid particles. The full-length lipase cDNA was obtained by screening another petal cDNA library prepared from stage III flowers with a 19-bp oligonucleotide corresponding to a central region of the gene sequence (Fig. 1). The full length lipase clone has a 1,341-bp ORF, a 47-bp 5′ noncoding region, and a 149-bp 3′ noncoding region (Fig. 1). The ORF encodes a 447-aa protein (GenBank accession no. AF026480) that contains the lipase consensus sequence as well as putative motifs for an amidation site, a protein kinase C phosphorylation site, a cAMP phosphorylation site, a casein kinase II phosphorylation site, and an *N*-glycosylation site (Fig. 1).

The carnation lipase sequence exhibits 63.8% similarity and 36.3% identity with an *Arabidopsis thaliana* lipase-like protein (GenBank accession no. AL021710), 56.1% similarity and 34% identity with an *A. thaliana* lipase isolog (accession no. U93215), 52.2% similarity and 27.5% identity with a cDNA clone from

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gcacgagccattccaaaactccttacaccactcaaaactattccaacATGGCTG
                                                    M  A
CAGAAGCCCAACCTTTAGGCCTCTCAAAGCCCGGCCCAACATGGCCCGAACTCC  108
A  E  A  Q  P  L  G  L  S  K  P  G  P  T  W  P  E  L
TCGGGTCCAACGCTTGGGCCGGGCTACTAAACCCGCTCAACGATGAGCTCCGTG
L  G  S  N  A  W  A  G  L  L  N  P  L  N  D  E  L  R
AGCTCCTCCTACGCTGCGGGGACTTCTGCCAGGTGACATACGACACCTTCATAA  216
E  L  L  L  R  C  G  D  F  C  Q  V  T  Y  D  T  F  I
ACGACCAGAACTCGTCCTACTGCGGCAGCAGCCGCTACGGGAAGGCGGACCTAC
N  D  Q  N  S  S  Y  C  G  S  S  R  Y  G  K  A  D  L
TTCATAAGACCGCCTTCCCGGGGGGCGCAGACCGGTTTGACGTGGTGGCGTACT  324
L  H  K  T  A  F  P  G  G  A  D  R  F  D  V  V  A  Y
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L  Y  A  T  A  K  V  S  V  P  E  A  F  L  L  K  S  R
CGAGGGAGAAGTGGGATAGGGAATCGAATTGGATTGGGTATGTCGTGGTGTCGA  432
S  R  E  K  W  D  R  E  S  N  W  I  G  Y  V  V  V  S
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N  D  E  T  S  R  V  A  G  R  R  E  V  Y  V  V  W  R
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G  T  C  R  D  Y  E  W  V  D  V  L  G  A  Q  L  E  S
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A  H  P  L  L  R  T  Q  Q  T  T  H  V  E  K  V  E  N
AGGAAAAGAAGAGCATTCATAAATCAAGTTGGTACGACTGTTTCAATATCAACC  648
E  E  K  K  S  I  H  K  S  S  W  Y  D  C  F  N  I  N
TACTAGGTTCCGCGTCCAAAGACAAAGGAAAAGGAAGCGACGACGACGATGATG
L  L  G  S  A  S  K  D  K  G  K  G  S  D  D  D  D  D
ACGACCCCAAAGTGATGCAAGGTTGGATGACAATATACACATCGGAGGATCCCA  756
D  D  P  K  V  M  Q  G  W  M  T  I  Y  T  S  E  D  P
AATCACCCTTCACAAAACTAAGTGCAAGAACACAACTTCAGACCAAACTCAAAC
K  S  P  F  T  K  L  S  A  R  T  Q  L  Q  T  K  L  K
AACTAATGACAAAATACAAAGACGAAACCCTAAGCATAACATTCGCCGGTCACA  864
Q  L  M  T  K  Y  K  D  E  T  L  S  I  T  F  A  G  H
GCCTAGGCGCGACACTATCAGTCGTGAGCGCCTTCGACATAGTGGAGAATCTCA
S  L  G  A  T  L  S  V  V  S  A  F  D  I  V  E  N  L
CGACCGAGATCCCAGTCACGGCCGTGGTCTTCGGGTGCCCAAAAGTAGGCAACA  972
T  T  E  I  P  V  T  A  V  V  F  G  C  P  K  V  G  N
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R  N  V  I  D  L  I  P  L  Y  P  V  K  L  M  G  Y  V
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N  I  G  I  E  L  E  I  D  S  R  K  S  T  F  L  K  D
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S  K  N  P  S  D  W  H  N  L  Q  A  I  L  H  V  V  S
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G  W  H  G  V  K  G  E  F  K  V  V  N  K  R  S  V  A
TGGTTAATAAGTCATGTGATTTTCTTAAGGAAGAATGTTTGGTTCCTCCAGCTT 1296
L  V  N  K  S  C  D  F  L  K  E  E  C  L  V  P  P  A
GGTGGGTTGTGCAGAACAAAGGGATGGTTTTGAATAAGGATGGTGAGTGGGTTT
W  W  V  V  Q  N  K  G  M  V  L  N  K  D  G  E  W  V
TGGCTCCTCCTGAGGAAGATCCTACTCCTGAATTTGATtgataatatttcatca 1404
L  A  P  P  E  E  D  P  T  P  E  F  D
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gttacttatttatatgtttattatatttgaaatgtgttttaagttacataaaat 1512
tgcaattagttttaaaaaaaaaaa                              1536
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Fig. 1. Nucleotide and inferred amino acid sequences of the senescence-induced lipase cDNA from carnation petals (GenBank accession no. AF026480). Nucleotide sequence: 5′ and 3′ noncoding cDNA sequence, lowercase letters; 19-bp oligonucleotide used for cDNA library screening, solid-line boxed region. Amino acid sequence: lipase consensus motif, solid-line boxed region; putative amidation site, single solid underline; putative protein kinase C phosphorylation site, broken-line boxed region; putative cAMP phosphorylation site, broken single underline; putative casein kinase II phosphorylation site, double solid underline; *N*-glycosylation site, double-line boxed region.

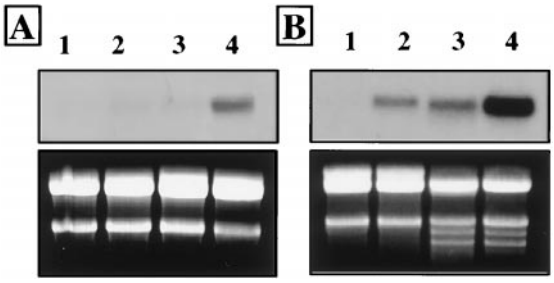

Fig. 2. Northern blot analysis of total RNA isolated from carnation flowers at different stages of post-harvest development and carnation leaves. Each lane contained 10 μg of RNA. Upper panels, RNA blots probed with carnation lipase cDNA; lower panels, agarose gels of fractionated RNA. (*A*) Lanes 1–4, flowers at stages I–IV of post-harvest development, respectively. (*B*), lanes: 1, stage II flowers; 2, stage II flowers treated with ethylene; 3, a mixture of senescing and nonsenescing leaves from stems bearing flowers at stage II of development; 4, leaves from stems bearing flowers at stage II of development that were treated with ethylene.

Ipomoea nil (accession no. U55867), 47.8% similarity and 27% identity with another *A. thaliana* lipase isolog (accession no. AC002388), and 38.4% similarity and 21.4% identity with an *A. thaliana* lipase homolog (accession no. Z97342). The substrate-binding consensus sequence for the lipase gene family is [LIV]-X-[LIVAFY]-[LIAMVST]-G-[HYWV]-S-X-G-[GSTAC], and the conserved serine is thought to be an active site residue (PROSITE). The carnation petal lipase has this conserved motif in common with animal, bacterial, and fungal lipases, including human lipoprotein lipase (GenBank accession no. 126314), mouse lipoprotein lipase (accession no. 417255), canine pancreatic lipase (accession no. 126316), *Staphylococcus aureus* lipase (accession no. 126333), and *Thermomyces lanuginosus* lipase (accession no. 2997733).

Expression of the Carnation Lipase During Flower Development. Changes in the expression of the carnation petal lipase were assessed by probing Northern blots of total RNA preparations from flowers at various stages of post-harvest development with lipase cDNA. Total RNA was isolated from flowers at stages I, II, III, and IV of development, which are morphologically and physiologically distinguishable (2). RNA was also isolated from stage II flowers that had been treated with 0.5 ppm ethylene for 15 h, a treatment known to induce premature senescence (2). A 1.53-kb mRNA, which corresponds in size to the lipase cDNA (Fig. 1), was clearly discernible in the Northern blots (Fig. 2*A*). The Northern blot analysis also indicated that, during post-harvest development of cut flowers, the lipase gene is strongly induced between stages III and IV of flower development, coincident with the onset of petal senescence (Fig. 2*A*, lanes 3 and 4). The same pattern of expression was observed when the flowers were allowed to open and senesce while still attached to the plant (data not shown). The lipase gene was also strongly expressed when senescence of young stage II flowers was induced prematurely by treatment with ethylene (Fig. 2*B*, lanes 1 and 2). The gene is expressed as well in the leaves of stems bearing flowers at stage II of development and was up-regulated when these stems were treated with ethylene (Fig. 2*B*, lanes 3 and 4). RNA for these blots was isolated from a mixture of senescing and nonsenescing leaves, and this presumably accounts for the fact that low levels of expression were detectable before ethylene treatment.

For Southern blot analysis, genomic DNA was digested with *Bam*HI and *Xho*I, which cut in the ORF of the lipase, and with three other enzymes, *Xba*I, *Eco*RI, and *Hin*dIII, which do not have cut sites in the ORF. When Southern blots of the digested

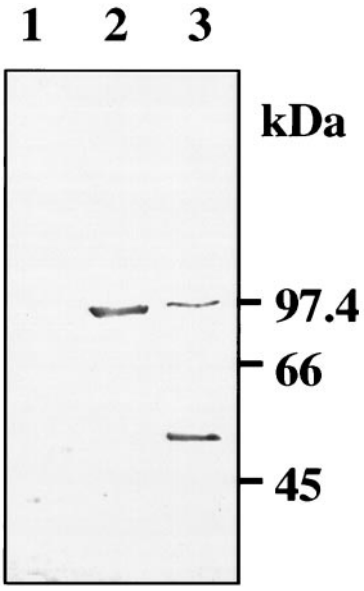

Fig. 3. Western blot analysis of purified lipase fusion protein from *E. coli*. The blot was probed with lipase antibody. Lanes: 1, maltose-binding protein control; 2, lipase fusion protein; 3, lipase fusion protein cleaved with Factor Xa. Molecular weight markers (kDa) are indicated.

DNA were probed with the carnation lipase cDNA, two DNA fragments were discernible for DNA treated with *Bam*HI or *Xho*I, which have cut sites in the ORF, whereas only a single band was detectable for DNA digested with the other restriction enzymes (data not shown). These observations indicate that the carnation petal lipase is a single-copy gene.

Expression of the Lipase cDNA. The carnation lipase cDNA was over-expressed as a fusion protein (lipase fused with maltose-binding protein) in *E. coli*. For this purpose, the lipase cDNA was linked through a proteolytic cleavage site to the cDNA for maltose-binding protein. The fusion product was purified by amylose column chromatography and was visualized as a 97.4-kDa protein on a Western blot probed with lipase antibody (Fig. 3, lane 2). Maltose-binding protein alone was not detectable (Fig. 3, lane 1). When the fusion protein was proteolytically cleaved, a 50.2-kDa polypeptide corresponding to the free lipase protein was clearly discernible in Western blots probed with the lipase antibody, and some residual uncut fusion protein was also detectable (Fig. 3, lane 3). As well, the size of the free lipase protein, 50.2 kDa, matches the size of the ORF of the lipase cDNA.

Lipolytic acyl hydrolase activity of the purified lipase fusion protein was assayed spectrophotometrically by using *p*-nitrophenylpalmitate and soybean phospholipid as exogenous substrates. For maltose-binding protein alone, which served as a control, there was no detectable lipase activity with phospholipid as a substrate (Table 1). When *p*-nitrophenylpalmitate was used as a substrate with maltose-binding protein alone, a small amount of *p*-nitrophenol, the expected product of a lipase reaction, was detectable reflecting background levels of *p*-nitrophenol in the commercial preparation of *p*-nitrophenylpalmitate (Table 1). However, in the presence of purified lipase fusion protein, strong lipase activity manifested as the release of free fatty acids from phospholipid and of *p*-nitrophenol from *p*-nitrophenylpalmitate was evident (Table 1).

In other experiments, the enzymatic activity of the lipase fusion protein was assayed by GC, a technique that enables quantitation and identification of free fatty acids released from

Table 1. Spectrophotometric measurements of the lipolytic acyl hydrolase activity of maltose-binding protein and lipase fusion protein expressed in *E. coli* and purified by amylose column chromatography

	Product	
Protein species	*p*-nitrophenol	Free fatty acid
Maltose-binding protein	0.71 ± 0.02	ND
Lipase fusion protein	12.02 ± 1.81	46.75 ± 1.24

Two substrates, *p*-nitrophenylpalmitate and soybean phospholipid, were used. Activities are expressed as nanomoles of product formed (*p*-nitrophenol from *p*-nitrophenylpalmitate and free fatty acid from soybean phospholipid)/milligrams of protein/minute. Means ± SE for $n = 3$ replications are shown. ND, not detectable.

the substrate. Trilinolein, soybean phospholipid, and dilinoleylphosphatidylcholine were used as substrates, and the deesterified fatty acids were purified by TLC before being analyzed by GC. In keeping with the spectrophotometric assay (Table 1), there was no detectable lipase activity for maltose-binding protein alone with either soybean phospholipid or dilinoleylphosphatidylcholine, indicating that these substrates are essentially free of deesterified fatty acids (Table 2). However, when the lipase fusion protein was used as a source of enzyme, palmitic, stearic, and linoleic acids were deesterified from the soybean phospholipid extract, and linoleic acid was deesterified from dilinoleylphosphatidylcholine (Table 2). In contrast to the phospholipid substrates, detectable levels of free linoleic acid were present in trilinolein, but the levels of free linoleic acid were significantly increased in the presence of lipase fusion protein, indicating that the lipase is capable of deesterifying fatty acids from triacylglycerol as well (Table 2). Different batches of purified lipase fusion protein exhibited different levels of lipase specific activity, presumably reflecting differences in folding of the protein during its formation in *E. coli*. When the protease, Factor Xa, was added to the enzyme reaction mixture to progressively release the lipase from its association with maltose-binding protein during the reaction, there was no increase in activity. However, fusions with maltose-binding protein are often not cleavable with Factor Xa without prior denaturation, a finding that is thought to reflect inaccessibility of the protease to the proteolytic cleavage site in the folded fusion protein (pMAL protein fusion and purification system instruction manual, New England BioLabs).

The lipolytic acyl hydrolase activity of the lipase fusion protein was also measured *in situ.* Single colonies of *E. coli* harboring the lipase fusion protein or maltose-binding protein alone as a control were cultured in basal salt medium containing Tween 40, an ester of palmitic acid, or Tween 60, an ester of stearic acid, as the only source of carbon. Thus, bacterial growth only occurs if the fatty acid esters are hydrolyzed. Only cells containing the lipase fusion protein grew in the presence of Tween 40 and Tween 60 (Fig. 4). The lag phase of ≈96 h is consistent with the fact that the Tween has to gain entry to the cells and be deesterified before growth occurs.

Discussion

Several lines of evidence indicate that deesterification of membrane fatty acids mediated by a lipolytic acyl hydrolase is an early and seminal feature of carnation petal senescence. Of particular significance in this context is the finding that there is a large increase in the sterol:phospholipid fatty acid ratio of microsomal membranes from petals coincident with the onset of petal inrolling, which is the first morphological manifestation of petal senescence (30). This has been shown to reflect a selective depletion of fatty acids from the membrane bilayers and to

Table 2. GC measurements of the lipolytic acyl hydrolase activity of maltose-binding protein and lipase fusion protein expressed in *E. coli* and purified by amylose column chromatography

Substrates		Products, μg/mg protein*	
		Maltose-binding protein	Lipase fusion protein
Tri-linolein†	Linoleic acid (18:2)	15.9 ± 0.75	33.4 ± 1.58
Soybean phospholipid‡	Palmitic acid (16:0)	ND§	4.80
	Stearic acid (18:0)	ND	9.68
	Linoleic acid (18:2)	ND	5.80
Dilinoleylphosphatidylcholine‡	Linoleic acid (18:2)	ND	20.0

*Reaction was allowed to proceed for 2 hr and was not continuously linear over this period.
†Means ± SE for $n = 3$ replications are shown.
‡Single experiment.
§ND, not detectable.

engender a large decrease in bulk membrane fluidity (6). Moreover, there is evidence for other senescing systems that this decrease in bulk lipid fluidity induces conformational changes in membrane proteins that render them prone to proteolysis (10, 11). There is also an increase in the free to esterified fatty acid ratio in the membranes of senescing carnation petals, reflecting enhanced fatty acid deesterification (6). This indicates that some of the fatty acids hydrolytically cleaved from phospholipid at the onset of senescence remain within the membrane bilayers, a contention that is further supported by the fact that domains of gel phase lipid enriched in free fatty acids are detectable in membranes of carnation petals at a very early stage of senescence (31). Thus, those free fatty acids that are not released from the membranes apparently phase-separate within the bilayer, and the resulting mixture of liquid-crystalline and gel phase domains is known to render membrane bilayers leaky (32). Indeed, membrane leakiness has been shown to be an early and pronounced manifestation of carnation petal senescence (2). Finally, there appears to be a selective deesterification of unsaturated fatty acids from the membranes of senescing carnation petals. This is evident from the finding that there is an increase in the saturated:unsaturated fatty acid ratio of senescing petal membranes (6). Moreover, there is also evidence that the hydrolytic release of free linoleic and linolenic acids from membrane phospholipid results in enhanced lipoxygenase activity and an accumulation of peroxidized lipids in bilayers, leading to further destabilization of membrane structure (33).

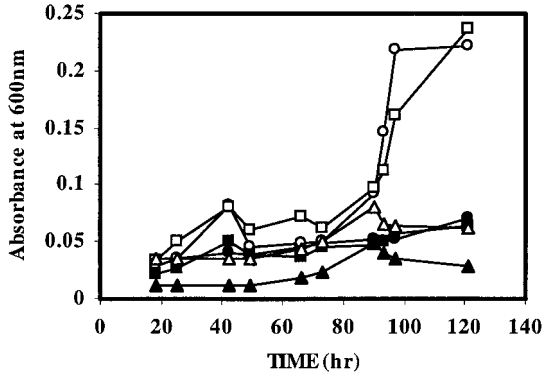

Fig. 4. *In situ* assay of lipolytic acyl hydrolase activity in *E. coli* cells harboring lipase fusion protein. Cells were cultured in basal salt medium supplemented with Tween 40 or Tween 60. ●, cells with maltose-binding protein cultured in medium containing Tween 40; ○), cells with lipase fusion protein cultured in medium containing Tween 40; ■, cells with maltose-binding protein cultured in medium containing Tween 60; □, cells with lipase fusion protein cultured in medium containing Tween 60; ▲, cells with maltose-binding protein cultured in the absence of Tween; △, cells with lipase fusion protein cultured in the absence of Tween. The assay was performed four times with comparable results.

These observations collectively indicate that the action of lipolytic acyl hydrolase on membranes is a pivotal feature of senescence, setting in motion a cascade of events that leads to loss of cell function. In the present study, we describe the isolation and characterization of a lipase gene encoding lipolytic acyl hydrolase in carnation petals that is strongly expressed coincident with the onset of petal inrolling, the first morphological manifestation of senescence. Although lipase genes encoding proteins that deesterify complex lipids have been cloned previously from plants, animals, and bacteria (e.g., GenBank accession nos. AL021710, AL126316, and AL126333), the carnation lipase is the first ethylene- and senescence-induced lipase gene to be identified. Indeed, the timing of the expression of this lipase gene during development of the carnation flower suggests that the hydrolytic activity of its cognate protein is initiating membrane deterioration that leads to senescence. Of particular significance is the finding that expression of the lipase gene is strongly induced by ethylene, for it has been demonstrated that manifestations of carnation petal senescence thought to be attributable to fatty acid deesterification, including membrane leakiness and consequent petal inrolling, a decline in phospholipid levels, and changes in membrane lipid fluidity and phase properties, are also induced by ethylene (2, 4, 30). The carnation lipase is capable of deesterifying fatty acids from a range of substrates, including phospholipid, triacylglycerol, and wax esters, and thus exhibits the defining feature of a lipolytic acyl hydrolase (17). It can also be inferred from the sequence of the cDNA ORF that the carnation lipase is a cytosolic protein. Specifically, the ORF does not have a recognizable signal sequence or transit peptide. It does, however, feature three putative phosphorylation sites, and in this sense resembles animal lipases that are normally soluble enzymes that in some instances have been shown to be regulated by phosphorylation (34). The finding that the carnation lipase is a cytosolic lipolytic acyl hydrolase means that it is presumably capable of hydrolyzing complex lipids in all cellular membranes with exposure to the cytosol as well as in cytosolic lipid particles, and is thus able to mediate the extensive hydrolysis of membrane lipids that characterizes senescence (4). It is also noteworthy that the carnation lipase contains the 10-aa consensus sequence, [LIV]-X-[LIVAFY]-[LIAMVST]-G-[HYWV]-S-X-G-[GSTAC], that has been shown to be a common feature of lipases cloned from animals (including humans), bacteria, and fungi.

Inasmuch as phospholipid deesterification and ensuing membrane leakiness appear to be common features of senescence for all plant tissues (1, 4, 16), it seems reasonable to infer that up-regulation of lipase gene expression characterizes the onset of senescence in plant tissues generally. The initial action of lipase on membrane phospholipids and the consequent release

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of free fatty acids will perturb the structure of the bilayer and destabilize membranes, leading to leakiness and loss of intracellular compartmentation. In addition, however, it is known that perturbed phospholipid bilayers are better substrates for lipase action than their unperturbed counterparts (35), and thus it seems reasonable to propose that lipase action on membranes becomes autocatalytic as senescence advances. That is, the initial perturbation of membrane bilayers mediated by lipase action during the early stages of senescence, including, for example, lateral phase separation of free fatty acids within the bilayer, renders the bilayer more prone to lipase attack, an eventuality that leads to complete dissolution of membranes and mobilization of their fatty acid equivalents. Indeed, recent evidence that glyoxylate cycle enzymes are induced in senescing leaves indicates that deesterified membrane fatty acids in senescing plant tissues may be metabolized into phloem-mobile sugars (15).

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