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Expression of Lipoxygenase in Wounded Tubers of *Solanum tuberosum* L.[1]

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A lipoxygenase cDNA clone from *Solanum tuberosum* L. was analyzed to study the role of lipoxygenases in potato development and wound response. Sequence analysis and comparison of the deduced amino acid sequence revealed high homology to other plant lipoxygenases. Expression of the cDNA sequences in *Escherichia coli* and subsequent analysis of bacterial protein extracts showed lipoxygenase activity using linoleic, linolenic, or arachidonic acid as substrates. Transcripts encoding the potato lipoxygenase were most abundant in tuber tissue, lower in roots, and hardly detectable in leaves, petioles, and stems. The induction of lipoxygenase expression in tubers by wounding was dependent on various parameters. Whereas lipoxygenase transcript levels increased in discs from stored tubers incubated under aerobic conditions, tubers taken from a growing plant did not accumulate lipoxygenase transcripts in response to wounding. Incubation of tuber discs in buffer did not lead to an increase in lipoxygenase RNA levels; however, methyl jasmonate stimulated lipoxygenase expression after 24 h in stored tubers. Proteinase inhibitor II mRNAs decreased in stored tubers as well as in discs from growing tubers.

Plant LOXs are members of a class of nonheme iron-containing dioxygenases that play a role in different aspects of plant growth and development (reviewed by Siedow, 1991). In addition to being involved in the synthesis of regulatory molecules such as JA and ABA (Vick and Zimmerman, 1987; Creelman et al., 1992), LOXs have been implicated to play a role in plant senescence and mobilization of lipid reserves during seed germination (Vick and Zimmerman, 1987; Hildebrand, 1988).

Another possible function of LOXs lies in the plant's defense response to pathogens. It has been demonstrated in several systems that LOX activity, or the abundance of LOX transcript, increased during the hypersensitive response elicited by pathogens (Bostock et al., 1992, and refs. therein). Furthermore, products of the LOX pathway exhibit antifungal (Ohta et al., 1990) or antibacterial activities (Croft et al., 1993).

LOXs catalyze the introduction of molecular oxygen into polyunsaturated fatty acids with *cis,cis*-1,4-pentadiene structures such as linoleic and linolenic acid, thereby creating a 1-hydroperoxy-*trans,cis*-2,4-pentadiene derivative. Several compounds with regulatory properties can arise from LOX products, depending on the substrates. In plants, LOXs are involved in the formation of JA, traumatic acid, or C_6 volatiles (reviewed by Anderson, 1989). Insertion of molecular oxygen into linolenic acid at the 13 position results in the formation of 13-hydroperoxylinolenic acid, which is dehydrated and modified to yield 12-oxo-phytodienoic acid and is subsequently reduced and shortened via β-oxidation to give JA (Vick and Zimmerman, 1987). JA and its methyl ester (MeJA) exhibit phytohormone-like activities and are involved in different processes in plant development, such as promotion of senescence and inhibition of cell growth (reviewed by Anderson, 1989). JA is also involved in the plant's response to wounding, because several wound-inducible genes, including those encoding the proteinase inhibitors I and II of potato (*Solanum tuberosum* L.) and tomato, have been shown to be inducible by JA (Farmer and Ryan, 1990; Farmer et al., 1992).

In potato, LOXs are of additional interest because of the identification of 3-oxo-2(5′-β-D-glucopyranosyloxy-2′-*cis*-pentenyl)-cyclopentane-1-acetic acid as the tuber-inducing activity in potato leaves (Yoshihara et al., 1989). The structural similarity of the aglycon of this compound to JA led to the observation that jasmonates can efficiently induce tuber formation in potato (Koda et al., 1991; Pelacho and Mingo-Castel, 1991).

High levels of LOX activity have been reported in potato tubers (Pinsky et al., 1971), and these can be increased by wounding (Lulai, 1988). Tuber LOX has been intensively studied because it is one of the few plant enzymes showing 5-LOX activity on arachidonic acid (Mulliez et al., 1987). The importance of 5-LOX in the creation of biologically active leukotrienes in animals and problems in obtaining sufficient quantities of stable enzyme from mammalian sources have shifted the attention to LOX from potato tubers. There are at least three LOX isoenzymes in potato tubers (Mulliez et al., 1987), the major form of which has been studied in detail to understand the reaction mechanism of 5-LOXs (Shimizu et al., 1984, 1990; Mulliez et al., 1987; Reddanna et al., 1990). Moreover, it was shown that potato tuber LOX is able to catalyze the hydroperoxidation of arachidonic acid as well as the subsequent conversion to leukotriene A_4 (Shimizu et al., 1984).

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Abbreviations: JA, jasmonic acid; LOX, lipoxygenase (linoleate:oxygen oxidoreductases; EC 1.13.11.12); MeJA, methyl jasmonate; PAL, phenylalanine ammonia-lyase; PI-II, proteinase inhibitor II.

Our interest in tuber-specific gene expression (Rosahl et al., 1986a, 1986b) caused us to analyze a potato LOX cDNA clone that we had previously isolated (Feltkamp et al., 1994). In addition to analyzing the induction of LOX at the transcript level in potato tubers by wounding, we studied the organ-specific expression pattern of LOX to obtain insight into the role played by LOX in different processes such as tuberization and wound response.

MATERIALS AND METHODS

Isolation and Characterization of a LOX cDNA Clone

The λ clone StLOX1 was obtained in a screen using degenerated oligonucleotides as described by Feltkamp et al. (1994). The 5′ part of StLOX1 was subsequently used to isolate StLOX1–5. *Not*I inserts of the two clones were subcloned into pUC and pET vectors (see below) using standard cloning techniques (Sambrook et al., 1989). Sequence analysis was performed using the dideoxy method (Sanger et al., 1977) with the Sequenase kit (United States Biochemical) after creation of deletion clones by exonuclease III digestion (Sambrook et al., 1989).

Genomic Southern Analysis

DNA was isolated from potato (*Solanum tuberosum* L. cv Desirée) plants according to the method of Murray and Thompson (1980) and digested with restriction enzymes. After gel electrophoresis and transfer to Hybond-N filters (Amersham, Braunschweig), filters were hybridized to a radioactively labeled 1.4-kb internal *Eco*RI fragment of the StLOX1 cDNA. Hybridization was performed in 5× SSPE, 5× Denhardt's, 0.1% SDS, 50% formamide, 100 μg/mL denatured salmon sperm DNA. Filters were washed at 65°C with 3× SSC, 0.5% SDS.

Expression of LOX Sequences in *Escherichia coli*

The pET expression vectors (Rosenberg et al., 1987) were used to express the LOX sequences in *E. coli*. The *Not*I insert of cDNA clone StLOX1 was cloned into the *Bam*HI site of pET3b after both ends had been filled in, yielding the clone pET-LOX1. To create a second fusion encoding different amino acids at the N terminus, the 1.6-kb insert of λ clone StLOX1–5 was cloned into the *Bam*HI site of pET3a as described above, resulting in pET-LOX5. *Xba*I restriction sites in the pET vector and at the 5′ end of the LOX cDNA clone were used to exchange the fragment encoding the N-terminal amino acids of pET-LOX1 for those of pET-LOX5. The resulting clone was called pET-LOX2. See below for amino acid sequences encoded by these clones.

The three constructs were transferred into BL21 (DE3) cells (Studier and Moffatt, 1986) that carried the plasmid pSB161 encoding $tRNA^{Arg}$ of *E. coli* (kindly provided by Prof. Dr. Mattes, Stuttgart, Germany). The latter strain has been reported to yield higher amounts of proteins encoded by sequences containing the rare Arg codons AGG and AGA (Brinkmann et al., 1989). Transformed bacteria were grown to mid-log phase and induced by addition of isopropylthio-β-galactoside to a final concentration of 1 mM. The cultures were subsequently incubated at 17°C for 16 h because low-temperature cultivation has been shown to increase the amount of enzymically active protein (Shirano and Shibata, 1990). Bacteria were harvested by centrifugation, resuspended in 50 mM Tris-HCl (pH 7.5), 10% glycerol, 0.1% Tween 20, 1 mM EDTA, 0.5 M NaCl and subjected to sonication. Supernatants of the crude sonicated extracts were used to determine enzyme activity.

Determination of LOX Activity

LOX activity was determined by two methods. Spectrophotometric measurements of the increase in A_{235}, caused by the formation of conjugated diene structures, was performed as described by Shimizu et al. (1990). Bacterial extracts were incubated with 100 μM linoleic acid in 100 mM sodium acetate buffer for the pH range from 4.0 to 6.5 and 100 mM Tris-HCl buffer for pH 6.5 to 9.0. Tween 20 was added to a final concentration of 0.005%. Consumption of oxygen with linoleic, linolenic, or arachidonic acid as substrates in 100 mM sodium acetate buffer (pH 6.0), 0.1% Tween 20 was determined with a Clark oxygen electrode (Delieu and Walker, 1972). Protein extracts from tuber discs were prepared by adding 100 mM sodium phosphate buffer (pH 6.0), 0.1% Tween 20 to ground tissue. After cellular debris was removed by centrifugation the supernatant was used to determine enzyme activity with the Clark oxygen electrode.

Wound Induction, RNA Extraction, and Northern Analysis

For wounding experiments, tubers were used that were either taken from 8-week-old potato plants, cv Desirée, growing in the greenhouse ("growing"; 15°C, 16 h light) or that had been stored for at least 2 months at 15°C in the dark ("stored"). Tubers were cut with a cork borer, and discs of 2 to 3 mm thickness and 10 mm diameter were generated, which were subsequently incubated in Petri dishes at 28°C in the dark. Aerobic incubation was performed with no additional substances present in the Petri dish, whereas for the anaerobic incubation tuber discs were submersed in 10 mL of 50 mM sodium phosphate buffer (pH 7.0). Linolenic and arachidonic acid in ethanol were added to a final concentration of 100 μM and MeJA was present at a concentration of 50 μM. Ethanol alone did not influence gene expression.

RNA was isolated according to the method of Logemann et al. (1987) with the following alterations: after the tissue was ground and resuspended in extraction buffer (8 M guanidinium chloride, 20 mM EDTA, 20 mM Mes, pH 7.0), phenol-chloroform-isoamyl alcohol was added. After the suspension was centrifuged, 0.05 volume of 1 N acetic acid and 0.7 volume of 100% ethanol were added to the aqueous phase. The precipitate was washed once with 70% ethanol, once with 3 M sodium acetate (pH 4.8), and once with 70% ethanol. The dried pellet was resuspended in water.

Northern analyses were performed according to the method of Lehrach et al. (1977). Hybridizations of the filters were carried out as described for genomic Southern analyses. The probes used were either an internal 1.4-kb *Eco*RI fragment of the StLOX1 cDNA or the 0.6-kb *Pst*I fragment from the PI-II cDNA clone (Sanchez-Serrano et al., 1986).

The experiments were repeated at least four times, and representative results are shown in the figures.

Western Analysis

Total protein was extracted with 100 mM sodium phosphate buffer (pH 7.0) from potato tuber discs incubated as described above. Protein extracts were run on 10% denaturing polyacrylamide gels and transferred onto nitrocellulose filters (Schleicher & Schuell) by electroblotting. Detection of LOX proteins was performed as described by Sambrook et al. (1989) using polyclonal antiserum raised in rabbits against the N-terminal 539 amino acids of the protein expressed in bacteria carrying the pET-LOX5 plasmid.

RESULTS

Characterization of a LOX cDNA

A nearly full-length cDNA clone containing LOX-homologous sequences was obtained from an expression library prepared from RNA of potato roots (*S. tuberosum* L. cv Desirée; Feltkamp et al., 1994). The clone λStLOX1 had an insert of 2.8 kb, which was subcloned and sequenced (Fig. 1). The longest open reading frame encoded 857 amino acids. Although the potato cDNA clone did not encode a full-length LOX, comparison of the length of the deduced amino acid sequence with those of other plant LOXs suggested that only a few amino acids were missing. The similarity of the deduced amino acid sequence to LOXs from soybean (Shibata et al., 1987, 1988; Yenofsky et al., 1988), pea (Ealing and Casey, 1989), and rice (Ohta et al., 1992) varies from 74 to 79%. The 3′ untranslated region of the cDNA comprises 145 bp with putative polyadenylation signals located 90 and 100 bp upstream of a 46-bp poly(A) tail.

A second cDNA clone (λStLOX1–5) was isolated on the basis of its homology to the 5′ half of λStLOX1 and contained 1.6 kb of LOX sequences. The 5′ end of this cDNA clone lacked four nucleotides compared to λStLOX1. Partial sequence analysis furthermore revealed identity with the sequence of λStLOX1 (data not shown), and it is therefore assumed that the two cDNA clones were derived from transcripts of the same gene.

Genomic Southern analysis, using an internal 1.4-kb *Eco*RI fragment as a probe, revealed several hybridizing bands (Fig. 2), indicating that LOX is encoded by a multigene family in potato. Restriction fragment length polymorphism mapping with the same fragment demonstrated the existence of at least two loci for LOX genes in the potato genome (C. Gebhardt, personal communication).

Functional Analysis of Potato LOX by Expression in *E. coli*

The LOX cDNA sequences were expressed in *E. coli* to analyze enzyme activity. Because the cDNA clone did not contain an initiation codon, a translational fusion was constructed in which the ATG was provided by the major capsid protein of phage T7. The 2.8-kb fragment of the λ clone StLOX1 was cloned into the vector pET3b (Rosenberg et al., 1987). The resulting clone, pET-LOX1, encodes a fusion protein consisting of 13 amino acids of the T7 protein, 3 amino acids encoded by linker sequences, and the 857 amino acids of the LOX cDNA clone (Fig. 3A). In addition, we used cDNA clone StLOX1–5, which contained only 1.6 kb of LOX sequences corresponding to 539 amino acids and which lacked four nucleotides at the 5′ end compared to clone StLOX1, to create a second fusion protein with different amino acids at the junction (Fig. 3A). To this end we first subcloned the LOX sequences of clone StLOX1–5 into the pET3a vector (pET-LOX5) and subsequently exchanged the sequences encoding the N terminus in pET-LOX1 for those encoded by pET-LOX5. The resulting plasmid was termed pET-LOX2 and differs from pET-LOX1 in the codons at the junction site and the lack of the Gln codon, which represents the first codon in λ clone StLOX1 (compare to Fig. 1).

Expression of the fusion proteins of pET-LOX1 and pET-LOX2 in *E. coli* led to the appearance of a weak band of about 95 kD on Coomassie-stained polyacrylamide gels, whereas pET-LOX5 resulted in a strong band of about 60 kD (data not shown). Because of its abundant expression, the latter polypeptide was used to raise antisera. Enzyme activity was determined in crude extracts of bacteria expressing the three fusion proteins by measuring the oxygen consumption with a Clark electrode using linoleic acid, linolenic acid, or arachidonic acid as substrates (Fig. 3B). Extracts from bacteria carrying pET-LOX1 or pET-LOX2 showed high enzyme activity with no significant differences in substrate specificity. Whereas linoleic and linolenic acid were good substrates for both proteins encoded by pET-LOX1 and pET-LOX2, oxygen consumption with arachidonic acid as a substrate was lower. Extracts derived from bacteria with pET-LOX5 did not contain LOX activity, indicating that the 539 amino acids encoded by this clone are not sufficient for enzyme activity.

LOX activity was also determined by measuring the increase in A_{235} upon incubation of bacterial extracts with linoleic acid. This substrate was efficiently converted under these conditions, the enzyme activity being 168 mkat hydroperoxylinoleic acid per kg of protein at pH 6.0 (Fig. 3C). Extracts prepared from bacteria containing the pET vector alone did not cause a significant increase in A_{235}. The reaction was carried out in buffers of different pHs to determine the optimum pH for LOX activity. Under these conditions, extracts from bacteria carrying pET-LOX1 exhibited highest activity at pH values of 5.5 to 7.0.

Expression of LOX Genes in Potato Plants

Total RNA from different organs of potato plants was isolated and subjected to northern analysis. An internal 1.4-kb *Eco*RI fragment of the cDNA clone StLOX1 was used as a probe, and hybridization was observed predominantly with RNA from tubers and to a lesser extent with RNA from roots, whereas little LOX mRNA was detectable in leaves, petioles, or stems (Fig. 4A). The size of the transcripts was approximately 2.8 kb. The same filter was hybridized to a cDNA insert encoding a potato histone to verify that equal amounts of RNA were loaded on the gel (data not shown). A polyclonal antiserum raised against the N-terminal 539 amino acids of LOX was used in western analyses, and a strongly reacting band representing a 95-kD polypeptide as well as a faster migrating band was detected in extracts from potato

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GCCAAATTGTGGGTGGACTAATTGGTGGCCATCATGACTCAAAGAAAGTTAAAGGAACTGTGGTGATGATGAAGAAAAATGCTCTAGATTTTACTGATCTTGCTGGTTCTTTGACTGATA  120
  Q  I  V  G  G  L  I  G  G  H  H  D  S  K  K  V  K  G  T  V  V  M  M  K  K  N  A  L  D  F  T  D  L  A  G  S  L  T  D  K

AAATCTTTGAGGCCCTTGGCCAAAAGGTTTCTTTTCAATTAATTAGTTCTGTTCAAAGTGATCCTGCAAATGGTTTACAAGGGAAACACAGCAATCCAGCTTATTTGGAGAACTTTCTCT  240
  I  F  E  A  L  G  Q  K  V  S  F  Q  L  I  S  S  V  Q  S  D  P  A  N  G  L  Q  G  K  H  S  N  P  A  Y  L  E  N  F  L  F

TTACTCTAACACCATTAGCAGCAGGTGAAACAGCCTTTGGTGTCACATTTGATTGGAATGAGGAGTTTGGAGTTCCAGGTGCATTTATCATCAAAAATACGCATATCAATGAGTTCTTTC  360
  T  L  T  P  L  A  A  G  E  T  A  F  G  V  T  F  D  W  N  E  E  F  G  V  P  G  A  F  I  I  K  N  T  H  I  N  E  F  F  L

TCAAGTCACTCACACTTGAAGATGTGCCTAATCATGGCAAGGTCCATTTTGTTTGCAATTCTTGGGTTTATCCATCTTTTAGATACAAGTCAGATCGAATTTTCTTTGCAAATCAGCCAT  480
  K  S  L  T  L  E  D  V  P  N  H  G  K  V  H  F  V  C  N  S  W  V  Y  P  S  F  R  Y  K  S  D  R  I  F  F  A  N  Q  P  Y

ATCTCCCAAGTGAAACACCAGAGCTTTTGCGCAAATACAGAGAAAATGAATTACTAACATTAAGAGGAGATGGAACTGGAAAGCGCGAGGCGTGGGATAGGATTTACGACTATGATGTCT  600
  L  P  S  E  T  P  E  L  L  R  K  Y  R  E  N  E  L  L  T  L  R  G  D  G  T  G  K  R  E  A  W  D  R  I  Y  D  Y  D  V  Y

ACAATGACTTAGGTAATCCTGATCAAGGTGAACAAAATGTTAGAACTACCTTAGGAGGTTCTGCTGACTACCCGTATCCTCGGAGAGGAAGAACTGGTAGACCACCAACACGAACAGATC  720
  N  D  L  G  N  P  D  Q  G  E  Q  N  V  R  T  T  L  G  G  S  A  D  Y  P  Y  P  R  R  G  R  T  G  R  P  P  T  R  T  D  P

CTAAAAGTGAAAGCAGGATTCCACTTATTCTGAGCTTAGACATCTATGTACCGAGAGATGAGCGTTTTGGTCACTTGAAGATGTCAGACTTCCTAACATATGCTTTGAAATCCATTGTTC  840
  K  S  E  S  R  I  P  L  I  L  S  L  D  I  Y  V  P  R  D  E  R  F  G  H  L  K  M  S  D  F  L  T  Y  A  L  K  S  I  V  Q

AATTCATCCTCCCTGAATTACATGCCCTGTTTGATGGTACCCCTAACGAGTTCGATAGTTTTGAGGATGTACTTAGACTATATGAAGGAGGGATCAAACTTCCTCAAGGACCTTTATTTA  960
  F  I  L  P  E  L  H  A  L  F  D  G  T  P  N  E  F  D  S  F  E  D  V  L  R  L  Y  E  G  G  I  K  L  P  Q  G  P  L  F  K

AGGCTCTCACTGCTGCTATACCTCTGGAGATGATGAAAGAACTCCTTCGAACAGACGGTGAAGGAATATTGAGATTTCCAACTCCTCTAGTGATTAAAGATAGTAAAACCGCATGGAGGA 1080
  A  L  T  A  A  I  P  L  E  M  M  K  E  L  L  R  T  D  G  E  G  I  L  R  F  P  T  P  L  V  I  K  D  S  K  T  A  W  R  T

CTGATGAAGAATTCGCAAGAGAAATGCTAGCTGGAGTTAATCCTATCATAATTAGTAGACTTCAAGAATTTCCTCCAAAAAGCAAGCTAGATCCCGAAGCATATGGAAATCAAAACAGTA 1200
  D  E  E  F  A  R  E  M  L  A  G  V  N  P  I  I  I  S  R  L  Q  E  F  P  P  K  S  K  L  D  P  E  A  Y  G  N  Q  N  S  T

CAATTACTGCAGAACACATAGAGGATAAGCTGGATGGACTAACGGTTGATGAGGCGATGAACAATAATAAACTTTTCATTTTGAACCATCATGATGTTCTTATACCATATTTGAGGAGGA 1320
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TAAACACTACAACAACGAAAACATATGCCTCGAGAACTTTGCTCTTCTTGCAAGATAATGGATCTTTGAAGCCACTAGCAATTGAATTGAGTTTGCCACATCCAGATGGAGATCAATTTG 1440
  N  T  T  T  T  K  T  Y  A  S  R  T  L  L  F  L  Q  D  N  G  S  L  K  P  L  A  I  E  L  S  L  P  H  P  D  G  D  Q  F  G

GTGTTATTAGTAAAGTGTATACTCCAAGTGATCAAGGTGTTGAGAGCTCTATCTGGCAATTGGCCAAAGCTTATGTTGCGGTGAATGACTCTGGTGTTCATCAACTAATTAGTCATTGGT 1560
  V  I  S  K  V  Y  T  P  S  D  Q  G  V  E  S  S  I  W  Q  L  A  K  A  Y  V  A  V  N  D  S  G  V  H  Q  L  I  S  H  W  L

TGAATACACATGCGGTGATTGAGCCATTTGTGATTGCAACAAACAGGCAACTAAGTGTGCTTCACCCTATTCATAAGCTTCTATATCCTCATTTCCGGGACACAATGAATATTAATGCTA 1680
  N  T  H  A  V  I  E  P  F  V  I  A  T  N  R  Q  L  S  V  L  H  P  I  H  K  L  L  Y  P  H  F  R  D  T  M  N  I  N  A  M

TGGCAAGACAGATCCTAATCAATGCTGGTGGGGTTCTTGAGAGTACAGTTTTTCCATCCAAATTTGCCATGGAAATGTCAGCTGTCGTTTACAAAGATTGGGTTTTCCCTGATCAAGCCC 1800
  A  R  Q  I  L  I  N  A  G  G  V  L  E  S  T  V  F  P  S  K  F  A  M  E  M  S  A  V  V  Y  K  D  W  V  F  P  D  Q  A  L

TTCCGGCTGATCTTGTTAAAAGGGGAGTAGCAGTTGAGGACTCGAGTTCTCCTCATGGTGTTCGTTTACTGATAGAGGACTATCCATACGCTGTTGATGGCTTAGAAATATGGTCTGCAA 1920
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TCAAAAGTTGGGTGACAGACTACTGCAGCTTCTACTATGGATCGGACGAAGAGATTCTGAAAGACAATGAACTCCAAGCCTGGTGGAAGGAACTCCGAGAAGTGGGACATGGTGACAAGA 2040
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  N  E  P  W  W  P  E  M  E  T  P  Q  E  L  I  D  S  C  T  T  I  I  W  I  A  S  A  L  H  A  A  V  N  F  G  Q  Y  P  Y  A

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  F  D  K  F  G  K  K  L  T  D  I  E  K  Q  I  I  Q  R  N  G  D  N  I  L  T  N  R  S  G  P  V  N  A  P  Y  T  L  L  F  P

CAACAAGTGAAGGTGGACTTACAGGCAAAGGAATTCCCAACAGTGTGTCAATATAGAAGGTCGACACCCGAAAATGAAGAAAGCTGGAGTTTGAATAAATCTTGAAATAAATTTTCATTA 2640
  T  S  E  G  G  L  T  G  K  G  I  P  N  S  V  S  I  *

TTATGTTAAGTGTAATCTCCCTTTGTTTGCTTCATTTCTGTATGTTTGATTCTCCTAAGTTTACTGTATTTTCATTTC(A)46  2764
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Figure 1. Nucleotide sequence and deduced amino acid sequence of the insert of the potato LOX cDNA clone λStLOX1.

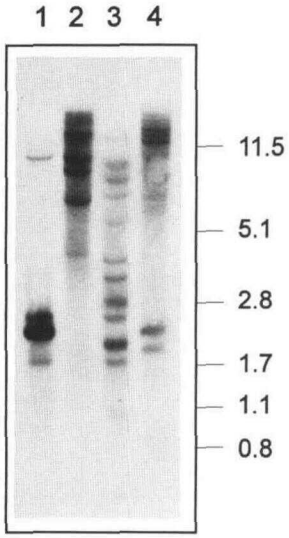

Figure 2. Genomic Southern analysis of the LOX genes of potato. Nuclear DNA of potato cv Desirée was digested with *Eco*RI (lane 1), *Eco*RV (lane 2), *Hin*dIII (lane 3), and *Sst*I (lane 4) and subjected to Southern analysis. A 1.4-kb internal *Eco*RI fragment of the potato LOX cDNA clone was radioactively labeled and used as a probe. The sizes (in kb) of the DNA mol wt standards are indicated at the right.

tubers taken from growing plants. Root extracts also contained proteins that were recognized by the antiserum, albeit to a lower extent (Fig. 4B). In leaves and stems, bands of the same size could be seen only after extended incubation (data not shown).

Expression of LOX Genes in Wounded Potato Tubers

LOX activity increases in potato tubers after wounding (Lulai, 1988). Using the cDNA clone StLOX1 as a probe we studied the effect of wounding on the accumulation of LOX transcripts under different conditions. Tubers that either were taken directly from growing potato plants (growing tubers) or had been stored for several weeks (stored tubers) were cut into slices. Tuber discs of similar size were incubated aerobically in the dark at 28°C for different time intervals and assayed for the presence of LOX transcripts and protein by northern and western analyses. As shown in Figure 5, there was a significant difference in the wound response depending on the state of the tuber. Growing tubers contained high levels of LOX mRNA, which started to decrease within 4 h and were significantly reduced after 16 h (Fig. 5A). In contrast, low levels of LOX transcripts were present in stored tubers, which increased upon wounding (Fig. 5B). The induction of LOX transcripts in discs from stored tubers was detectable within 8 h after wounding and reached a maximum after 24 h. Further incubation for up to 3 d led to a decrease of LOX mRNA. Analysis of LOX protein present in discs from growing tubers did not show significant differences in the labeling of the 95-kD band (Fig. 5, C and D), indicating that LOX protein is not rapidly degraded upon wounding. However, similar to the mRNA levels, LOX enzyme activity decreased in growing tubers after wounding (Fig. 5E) and increased in stored tubers (Fig. 5F).

The same or identical RNA blots were assayed for the presence of PI-II mRNA, which accumulates systemically in leaves of potato plants after wounding (Green and Ryan, 1972; Pena-Cortes et al., 1988). Because data concerning the behavior of PI-II in wounded tubers are conflicting (see "Discussion"), we were interested in analyzing the expression of PI-II genes in wounded tubers under different conditions. With the PI-II cDNA (Sanchez-Serrano et al., 1986) as a probe, we observed a gradual decrease of PI-II transcripts in growing tubers (Fig. 5A) as well as in discs from stored tubers (Fig. 5B).

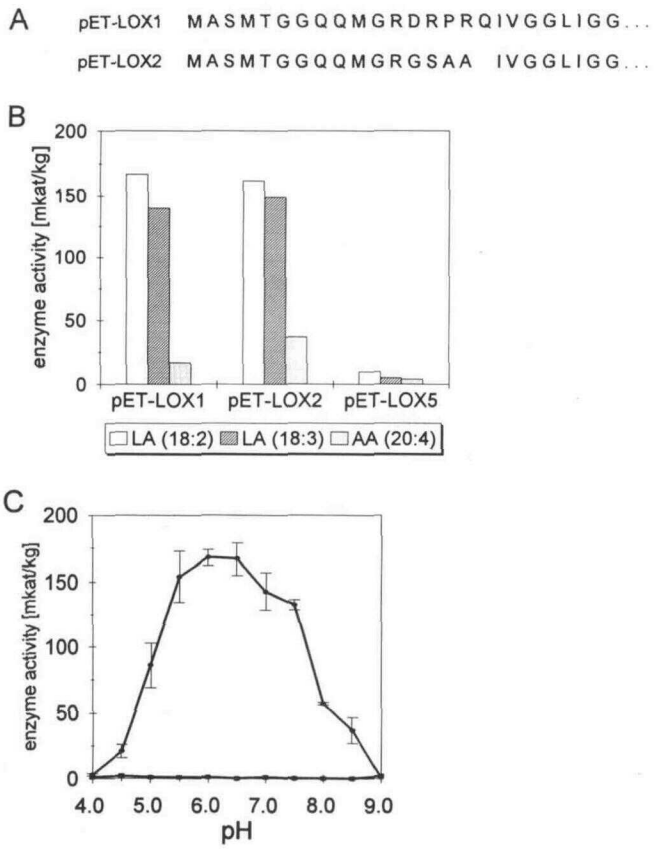

Figure 3. Expression of the potato LOX in *E. coli*. A, N-terminal amino acids of the fusion protein encoded by pET-LOX1 and pET-LOX2. The 2.8-kb *Not*I fragment from the λ clone λStLOX1 was subcloned into the pET3b expression vector resulting in a fusion of 13 amino acids of the major capsid protein of phage T7 and 3 amino acids derived from linker sequences to the first amino acid of the potato LOX, respectively. pET-LOX2 is a fusion in pET3b lacking one amino acid of the LOX sequence and containing different amino acids at the junction. B, Enzyme activity in extracts from LOX-overproducing bacteria carrying the plasmids as indicated, with linoleic acid (LA [18:2]), linolenic acid (LA [18:3]), or arachidonic acid (AA [20:4]) as substrates. Activity was measured with a Clark oxygen electrode and is given as consumption of oxygen in mkat/kg protein. C, pH optimum of LOX activity. Extract from bacteria harboring pET-LOX2 (circles) or the pET vector alone (squares) were analyzed for LOX activity in buffers with different pH values using linoleic acid as a substrate. Enzyme activity was determined by measuring the increase in A_{235}.

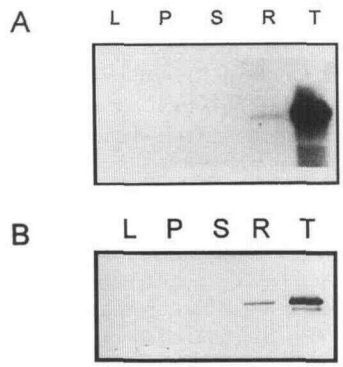

Figure 4. Analysis of potato LOX expression. A, Total RNA (7 μg) from leaf (L), petiole (P), stem (S), root (R), and tuber (T) tissue of 8-week-old potato plants, cv Desirée, was subjected to northern analysis. An internal 1.4-kb *Eco*RI fragment of the potato LOX cDNA clone was radioactively labeled and used as a probe. B, Western analysis with 5 μg of total protein from the same tissues as in A using antiserum raised against the 539 N-terminal amino acids of LOX.

Influence of MeJA on LOX Expression in Wounded Tubers

Additional experiments were carried out to assess the effect of different regulatory compounds on LOX expression after wounding. Tuber discs were incubated anaerobically in phosphate buffer and analyzed for the presence of LOX transcripts at different times (Fig. 6A). Growing tubers responded with a decrease in LOX mRNA levels between 4 and 12 h. Addition of linolenic acid to the buffer did not significantly alter this response. Addition of MeJA, however, resulted in a less pronounced decrease of LOX transcript levels, indicating that MeJA can prevent the wound-induced decrease of LOX mRNA levels in growing tubers. Hybridizing the same blot with a PI-II probe demonstrated a decrease in PI-II mRNA that was similar to that of LOX mRNA. The decrease of PI-II transcript levels in the presence of MeJA was also less pronounced than with linoleic acid or with no additional substance present.

Stored tubers had less or equal levels of LOX transcripts before and after wounding and subsequent incubation in buffer, linolenic acid, or arachidonic acid (Fig. 6B). This observation suggests that induction of LOX mRNA in stored tubers by wounding is specific for the conditions described in Figure 5, i.e. aerobic incubation. Addition of MeJA to the buffer, however, resulted in an increase of LOX transcripts 24 h after incubation of the tuber discs. Thus, MeJA can induce the accumulation of LOX transcripts in anaerobic discs of stored tubers after wounding. In contrast, PI-II mRNA levels decreased gradually in discs from stored tubers and did not increase if incubation took place in the presence of MeJA (Fig. 6B).

DISCUSSION

The isolation and characterization of a LOX cDNA clone from a root-specific expression library of *S. tuberosum* L.

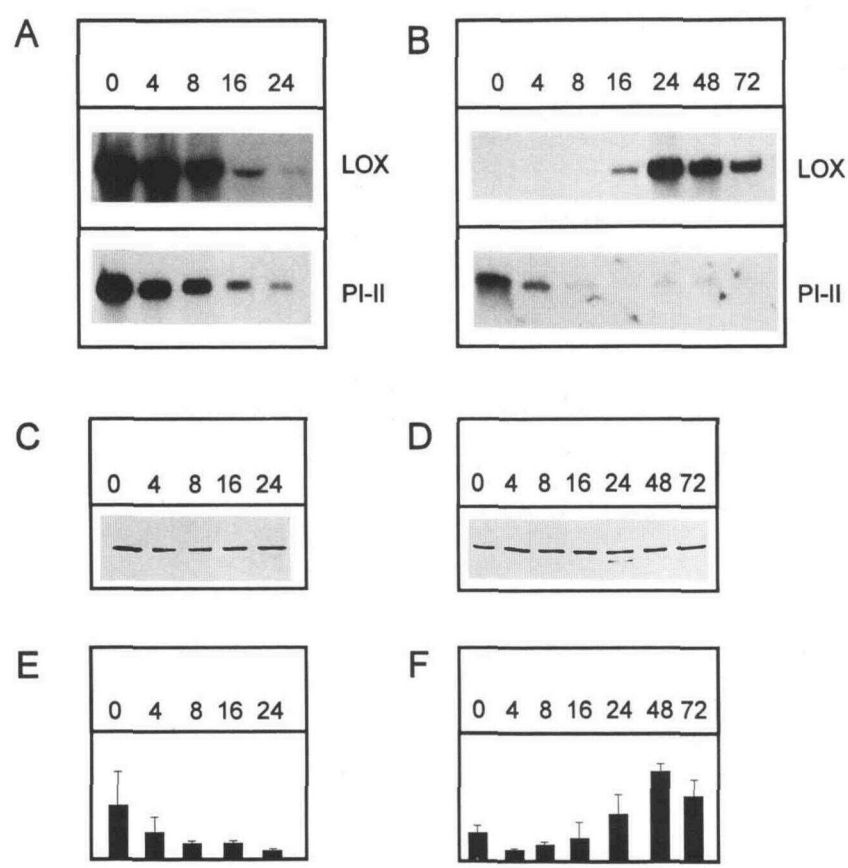

Figure 5. LOX expression in wounded tubers. Growing (A, C, and E) or stored (B, D, and F) tubers were cut into discs and incubated under aerobic conditions as described in "Materials and Methods." A and B, Total RNA was isolated at the times indicated (in h after wounding), and 6 μg (A) and 3 μg (B) of total RNA were loaded on the gel. Either the internal 1.4-kb *Eco*RI fragment of the potato LOX ("LOX") or the 0.6-kb *Pst*I fragment of the PI-II cDNA clone was radioactively labeled and used as probes. C and D, Western analysis of 5 μg of total protein of potato tubers after wounding and treatment as described for A and B using antiserum against the 539 N-terminal amino acids of LOX. E and F, LOX activity in protein extracts of potato tubers after wounding and treatment as described for A and B. Highest and lowest LOX activities were 12.3 and 1.8 mkat/kg (E, 0 and 24 h after wounding, respectively) and 20.8 and 2.4 mkat/kg (F, 48 and 4 h after wounding, respectively).

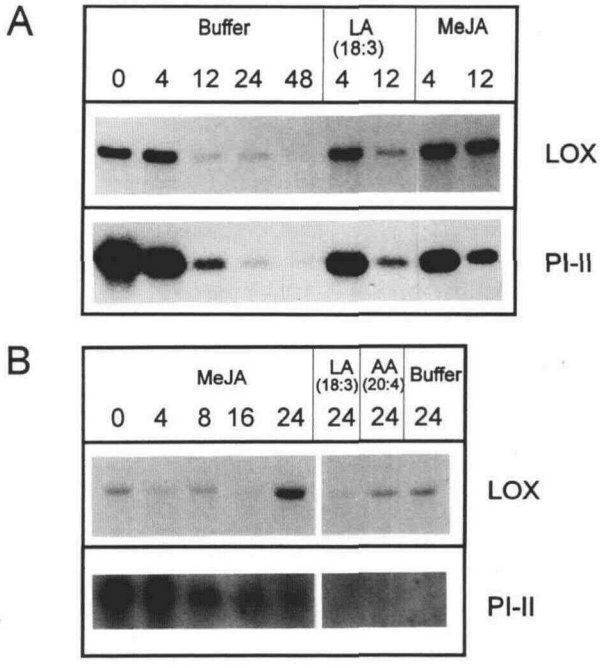

Figure 6. Influence of MeJA on LOX gene expression in wounded tubers. Discs from growing (A) or stored (B) tubers were incubated in 50 mM sodium phosphate buffer (pH 7.0) supplemented with the compounds indicated as described in "Materials and Methods." RNA was isolated at the times shown (in h after wounding), and 4 μg (A) or 11 μg (B) of total RNA was loaded on the gel. The filter was hybridized against the radioactively labeled inserts of the LOX or the PI-II cDNA clones.

allowed us to study LOX expression in potato plants and to compare the well-studied tuber LOXs with a root isoform. The analysis of crude protein extracts from bacteria expressing different LOX constructs shows that the potato LOX cDNA clone StLOX1 encodes a functional protein despite the lack of sequences encoding the N-terminal amino acids. No significant changes in enzymic functions were detected for the two different fusion proteins that were analyzed. Shirano and Shibata (1990) also did not observe a difference in pH optimum or heat stability with fusion proteins of rice LOX containing as N-terminal amino acids those encoded by the pET vectors. Moreover, the relative molecular mass, substrate specificity, and optimal pH for LOX activity of the potato cDNA-derived protein are similar to those of tuber LOXs (Shimizu et al., 1984, 1990; Mulliez et al., 1987; Reddanna et al., 1990). In particular, the less-efficient peroxidation of arachidonic acid in comparison to linoleic or linolenic acid that was observed in this study is in accordance with the substrate specificity reported for purified tuber LOXs (Berkeley and Galliard, 1976; Mulliez et al., 1987; Shimizu et al., 1990).

Using the cDNA sequences as a probe we were able to detect LOX transcripts predominantly in potato tubers and to a lower extent in roots. LOXs in tubers are assumed to play a role in defense against pathogens by being involved in the hypersensitive response (Bostock and Stermer, 1989; Vaughn and Lulai, 1992a, 1992b). It is interesting to note that potato tubers represent the plant tissue with the highest LOX activity (Pinsky et al., 1971). The role of LOX in root growth and development, however, is not clear. It is interesting that JA, one of the products of the LOX pathway, inhibits root longitudinal growth but stimulates adventitious root formation (reviewed by Sembdner and Parthier, 1993). A decrease in rooting ability was also observed in potato stolon explants that were incubated with MeJA or kinetin (Pelacho and Mingo-Castel, 1991), suggesting that MeJA has a negative effect on root development, as has been shown for cytokinins (Mathysse and Scott, 1984).

Wounding of potato tubers has various effects on metabolism and gene expression. In addition to an enhancement of RNase activities (Pitt and Galpin, 1971) or DNA polymerase activity (Watanabe and Imaseki, 1977), the level of polysomes has been shown to increase immediately after wounding (Ishizuka and Imaseki, 1989). Specific sets of genes are induced upon wounding, whereas other RNA species are destabilized (Butler et al., 1990; Ishizuka et al., 1991, and refs. therein).

The increase of LOX activity in wounded potato tubers (Lulai, 1988) is dependent on different parameters that influence LOX mRNA levels in tuber discs. First, accumulation of LOX mRNA can be observed only if stored tubers are used for the wounding experiment, whereas tubers that are taken directly from the plant do not respond to wounding by inducing the accumulation of LOX transcripts. Second, the wound inducibility is dependent on aerobic conditions. Incubation of tuber discs under aerobic conditions, which is frequently referred to as "ageing" of the tuber tissue, leads to a significant increase in LOX transcript levels, whereas submerging the tuber discs in buffer results in a failure to increase the amount of LOX mRNA. Although the changes in LOX enzyme activity resemble those in mRNA levels, the amount of total LOX protein is not significantly altered. Similarly, Bostock et al. (1992) reported an increase in LOX activity in wounded tubers in response to arachidonic acid that was not accompanied by a change in LOX protein levels.

Other genes or enzyme activities that are wound inducible in potato tubers are assumed to play a role in phytoalexin accumulation, suberization, or wound healing. Experiments describing the wound induction of PAL and extensin (Butler et al., 1990; Rumeau et al., 1990; Ishizuka et al., 1991), 3-hydroxy-3-methylglutaryl-CoA reductase (Stermer and Bostock, 1987; Choi et al., 1992), anionic peroxidase (Roberts et al., 1988), and a chitin-binding glycoprotein (Millar et al., 1992) were performed with tubers that were either stored or had been separated from the potato plant for a considerable time. Thus, the wound inducibility of these RNAs or enzyme activities is comparable to the increase of LOX transcript levels in stored tubers under aerobic conditions, although the onset of induction varies from 1 h after wounding (PAL) to 24 h after wounding (extensin, LOX). Because LOX transcripts are not wound inducible in growing tubers, it would be interesting to investigate whether the wound enhancement of the RNA levels or enzyme activities described above also occur in growing tubers.

The reason for the difference in wound-induced LOX gene expression of growing or stored tubers can only be subject to speculation at this time. The stored or dormant tuber repre-

sents a fundamentally different physiological state compared to the developing tuber of a growing potato plant. The implication of LOX involvement in a plant's response to wounding or pathogen attack suggests that the increase of LOX mRNA levels in stored tubers results from the need of a metabolically inactive storage organ to respond to wound stress. Growing tubers, in contrast, already contain high amounts of LOX and therefore may not need to activate LOX gene expression to start a signal transduction chain similar to the one postulated to lead to the expression of wound-inducible genes in tomato leaves (Farmer and Ryan, 1992).

The inability of LOX transcripts to accumulate upon wounding under anaerobic conditions resembles the behavior of several wound-induced genes such as PAL, Gly-rich protein, extensin, and histone H4 (Butler et al., 1990; Rumeau et al., 1990). Because RNAs that encode enzymes involved in anaerobic metabolism accumulated, a hierarchy of stress responses has been postulated in which the response to hypoxia precludes the plant's response to wounding (Butler et al., 1990).

In contrast to the RNAs described above, LOX transcripts are present predominantly in tubers. Other tuber-associated RNAs such as PI-II and patatin transcripts have been reported to be degraded after wounding under aerobic or hypoxic conditions (Sanchez-Serrano et al., 1986; Butler et al., 1990). Our data extend this analysis and show that the level of PI-II RNA decreases after wounding under aerobic or anaerobic conditions not only in stored tubers but also in tubers taken from growing plants. It is interesting that PI-II mRNA does not decrease after wounding in tubers that are still attached to the potato plant (Pena-Cortes et al., 1988).

Jasmonates are known effectors of LOX gene expression (Bell and Mullet, 1991; Grimes et al., 1992; Melan et al., 1993). In wounded tubers incubated under anaerobic conditions, the potato LOX mRNA is inducible by MeJA, whereas linolenic acid or arachidonic acid does not significantly alter the level of LOX transcripts. Whether the MeJA-induced LOX RNAs are derived from different genes that are specifically expressed or whether the same set of genes is switched on under these conditions can only be addressed after the isolation of gene-specific probes. It should be noted, however, that MeJA can also act at the translational level by repressing translational initiation from specific transcripts in barley (Reinbothe et al., 1993).

The PI-II genes, which have been shown to be inducible by jasmonates in leaves (Farmer and Ryan, 1990; Farmer et al., 1992), do not respond to MeJA in discs from stored tubers. However, similar to the LOX mRNAs, the decrease of PI-II mRNA in growing tubers incubated in buffer is delayed in the presence of MeJA. Thus, different tissues respond to jasmonates with an accumulation of or decrease in PI-II transcripts.

The isolation and characterization of a potato LOX cDNA clone are prerequisites to study the role of LOX in potato. Expression of sense and antisense constructs of the cDNA in transgenic potato plants and analysis of the effect on different physiological processes, such as wound and pathogen response and tuberization, could help to elucidate the role of LOXs in these processes. In addition, it will be of particular interest to study the regulation of LOX promoters and to identify sequences responsible for either predominant tuber expression, wound inducibility, or MeJA responsiveness.

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