Potato lipoxygenase genes: their role in tuber formation and defense responses against pathogens

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Potato lipoxygenase genes:
Their role in tuber formation and defense responses against pathogens

by

Mikhailo V. Kolomiets

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Horticulture
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iv</td>
</tr>
<tr>
<td>GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Dissertation Organization</td>
<td>1</td>
</tr>
<tr>
<td>Research Objectives</td>
<td>2</td>
</tr>
<tr>
<td>Literature Review</td>
<td>2</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>10</td>
</tr>
<tr>
<td>POTATO (Solanum tuberosum L. cv. Superior) Lipoxygenase Expresed During the Early Stages of Tuberization (Accession Nos. U60200 and U60201)</td>
<td>18</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>20</td>
</tr>
<tr>
<td>Nucleotide Sequence of a cDNA Clone for a Lipoxygenase From Abscisic Acid-Treated Potato Leaves (Accession No. U60202)</td>
<td>22</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>24</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>24</td>
</tr>
<tr>
<td>Potato Lipoxygenase Genes Potlx-1 and Potlx-2 Are Involved Specifically in Potato Tuberization</td>
<td>26</td>
</tr>
<tr>
<td>Summary</td>
<td>26</td>
</tr>
<tr>
<td>Introduction</td>
<td>27</td>
</tr>
<tr>
<td>Results</td>
<td>29</td>
</tr>
<tr>
<td>Discussion</td>
<td>33</td>
</tr>
<tr>
<td>Experimental Procedures</td>
<td>39</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>43</td>
</tr>
<tr>
<td>References</td>
<td>43</td>
</tr>
<tr>
<td>A Novel Potato Lipoxygenase Gene (Potlx-3) Induced in Response to Pathogen Attack But Not Wounding</td>
<td>63</td>
</tr>
<tr>
<td>Results</td>
<td>67</td>
</tr>
<tr>
<td>Discussion</td>
<td>72</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>78</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>82</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>83</td>
</tr>
<tr>
<td>General Conclusions</td>
<td>100</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>102</td>
</tr>
</tbody>
</table>
Lipoxygenase (LOX) is involved in the synthesis of jasmonic acid, methyl jasmonate, traumatin, and C-6 volatile compounds that serve hormone-like regulatory and defense-related roles in plants. To study possible lipoxygenase involvement in potato tuberization and defense responses against pests, we have isolated, sequenced, and characterized three lipoxygenase cDNA clones that represent distinct LOX genes. Two of these genes, designated POTLX-1 and POTLX-2, have been isolated from an early tuber cDNA library. Northern hybridization studies indicated that accumulation of POTLX-1 and POTLX-2 mRNA was specific to developing tubers and roots and correlated positively with tuber growth. Hormonal treatment, pathogen infection, or wounding did not induce POTLX-1 and POTLX-2 mRNA accumulation in leaves. In situ hybridization showed that POTLX-1 transcripts accumulated in the apical and subapical regions of the new tuber and in the vascular tissue of the most actively growing part of the developing tuber. Thus, POTLX-1 transcripts were localized within the sites of the most active cell division that occurs during tuber initiation.

Our results indicate that POTLX-1 and/or POTLX-2 may have an important function in controlling tuber initiation and growth. The third lipoxygenase cDNA clone, designated POTLX-3, was isolated from an ABA-induced potato leaf cDNA library. This is a novel potato LOX gene as it shares relatively low sequence identity with other classes of potato LOX genes. POTLX-3 transcript accumulation was not detected in untreated, healthy potato organs, but it was induced in leaves treated with ethylene or methyl jasmonate. Neither wounding nor treatment of leaves with ABA, cytokinin, auxin, or gibberellic acid induced POTLX-3 transcript accumulation. However, POTLX-3 mRNA accumulation was induced in potato leaves infected with virulent or avirulent strains of Phytophthora infestans. During the resistance response, POTLX-3 mRNA was induced within 6 h and continued to accumulate for a week postinoculation. During the compatible interaction, mRNA accumulation was
induced 3 to 6 days postinoculation. *POTLX-3* also was induced during hypersensitive response development caused by *Pseudomonas syringae* pv. *phaseolicola*, and this indicates that *POTLX-3* expression may be associated with the hypersensitive response. Thus, *POTLX-3* may be involved specifically in defense responses against pathogens.
GENERAL INTRODUCTION

Dissertation Organization

This dissertation consists of four manuscripts. The first and second manuscripts already have been published in the *Plant Gene Register* of *Plant Physiology*, and they are formatted for that journal. The third manuscript is formatted for submission to *The Plant Journal*. The fourth manuscript will be submitted to *Molecular Plant-Microbe Interactions*, and it is formatted for that journal. This dissertation also includes Literature Review, General Conclusions, and Acknowledgements sections.

Research Objectives

Lipoxygenases are the first committed enzymes in a biosynthetic pathways that produce compounds such as jasmonic acid, methyl jasmonate, traumatin, fatty acid hydroperoxides, and volatile aldehydes. These compounds serve hormone-like regulatory and defense-related roles in plants. Due to the production of functionally diverse intermediate and final products, lipoxygenase has been implicated in a number of physiological processes such as senescence, growth and development, and pathogen, pest, and wound responses. These distinct lipoxygenase functions likely are mediated by individual lipoxygenase isoforms. Multiple lipoxygenase isoforms have been found in all plant species examined, and they are encoded by families of differentially regulated genes. The overall objective of this research project was to determine lipoxygenase function(s) in tuber development and defense responses against pests in potato (*Solanum tuberosum* L.). The specific objectives were to isolate and characterize the expression pattern of potato LOX genes that are involved specifically in potato tuberization processes and defense responses against pathogens.
Literature Review

Overview of plant lipoxygenases

Lipoxygenases (LOXs) (linoleate:oxygen oxidoreductase. EC 1.13.11.12 ) are nonheme, iron-containing enzymes that catalyze the oxygenation of polyunsaturated fatty acids or their esters that contain a cis,cis-1,4-pentadiene structure. LOX primary products are fatty acid hydroperoxides, and they are enzymatically converted into molecules that have regulatory activities in cell metabolism. In mammals, fatty acid hydroperoxides are precursors of biologically active molecules such as prostaglandins, leukotrienes, lipoxins, and thromboxanes, and these compounds are well known as intracellular stress messengers involved in inflammatory defense responses (Anderson, 1989). In plants, LOX-derived fatty acid hydroperoxides also are metabolized into biologically active molecules.

Traumatin, jasmonic acid (JA), and methyl jasmonate (MJ) have been studied more thoroughly than other LOX final products, and these substances serve hormone-like regulatory roles (Anderson, 1989; Koda, 1992). Traumatin induces cell division, and therefore, it may promote healing of damaged tissues in response to wounding or insect chewing (Anderson, 1989). JA and MJ are involved in a variety of physiological processes (Koda, 1992), apparently via regulation of gene expression (Reinbothe et al., 1994). This includes induction of the genes that encode phenylalanine ammonia lyase (Gundlach et al., 1992), protease inhibitors (Farmer and Ryan, 1992), vegetative storage proteins and chalcone synthase (Reinbothe et al., 1994), proline-rich cell wall proteins (Creelman et al., 1992), and LOX itself (Melan et al., 1994). In addition, the C-6 volatile compounds derived from lipoxygenases possess antimicrobial activities (Croft et al., 1993). Recently, Blechert et al. (1995) have provided evidence that besides JA, another LOX-derived product, dihydrojasmonic acid, as well as their 18-carbon oxylipin precursors, 12-oxophytodienoic and 12-oxophytoenoic acid, can act as defense signals. The physiological functions of many other LOX-derived final
products are still to be elucidated (Anderson, 1989; Siedow, 1991; Vick and Zimmerman, 1987).

In higher plants, the most common substrates of lipoxygenases are linolenic and linoleic acids, fatty acids most likely derived from membrane phospholipids (Conconi et al., 1996; Siedow, 1991). LOX-catalyzed incorporation of molecular oxygen into these fatty acids occurs either at position 9 or 13 of their carbon chains, and two distinct fatty acid monohydroperoxides are formed. These hydroperoxidation products then traverse separate biosynthetic pathways that cause accumulation of compounds that may have distinct physiological functions in plants (Anderson, 1989). Distinct LOX isozyme forms preferentially catalyze either reaction (Siedow, 1991), and some LOX isozymes catalyze the introduction of molecular oxygen exclusively into the C-9 or C-13 position of fatty acids (Doderer et al., 1992; Peng et al., 1994; Siedow, 1991).

Multiple LOX isozyme forms exist in all plant species that have been studied, and their expression is under strict developmental, organ- and tissue-specific, and stress-regulated control (Heitz et al., 1997; Kausch and Handa, 1997; Melan et al. 1994; Saravitz and Siedow, 1995; Royo et al., 1996). Because different LOX isozymes produce functionally diverse final products, it is not surprising that LOXs were implicated in physiological processes as diverse as senescence, growth and development, and wound- and pathogen-induced defense responses (Siedow, 1991). It is possible that individual isozyme forms within a given plant organ, tissue, or cell type may have a distinct physiological role (Peng et al., 1994; Saravitz and Siedow, 1995; Stephenson et al., 1998). LOX multigene families likely serve as a means to carry on these diverse functions by elaborate regulation of their expression in specific tissues and cells in response to various developmental and environmental cues (Eiben and Slusarenko, 1994; Stephenson et al., 1998). With the exception of a chloroplast-specific isoform encoded by AtLOX2 gene from Arabidopsis (Bell et al., 1995) and the tobacco LOX1 gene (Rance et al., 1998), physiological roles of individual LOX isozymes have
not been established. Possible LOX involvement in potato growth and development, especially in tuberization, and pathogen-, insect-, and wound-triggered defense responses is the focus of this research, and, therefore, is the focus of this literature review.

Role of LOX in plant growth and development

Overview

Several lines of correlative evidence suggest that LOX may play an important role in plant growth and development. In many plant species, it was observed that rapidly growing tissues have the greatest LOX activity. Furthermore, there is a positive correlation between LOX activity within an organ and its rate of elongation (Siedow, 1991). LOX isozyme profiles change quantitatively and qualitatively during soybean leaf development (Saravitz and Siedow, 1995) and during seed germination in cucumbers (Feussner et al., 1996). In addition, many LOX genes are regulated differentially during Arabidopsis seedling development (Melan et al., 1994), tomato fruit ripening (Ferrie et al., 1994; Kausch and Handa, 1997), pea carpel development (Rodriguez-Conception and Beltran, 1995), and potato tuber development (Bachem et al., 1996). Despite these studies on the involvement of LOXs in plant development, virtually nothing is known about the role that specific LOXs might play in controlling the early stages of potato tuber formation.

LOX involvement in potato tuberization

Potato tuber induction and growth are complex developmental processes that require interaction of many environmental and genetic factors. Under inductive conditions of long and cool nights, longitudinal growth of stolons ceases, and tubers form as a result of subapical radial cell expansion in the stolon tip (Ewing and Struik, 1992). This step in tuber development is associated with numerous anatomical and biochemical changes that include a
switch in the plane of cell division from longitudinal to radial and rapid cell enlargement, starch biosynthesis, accumulation of the major storage proteins such as patatin and proteinase inhibitors, fluctuations in phytohormone levels (Ewing and Struik, 1992), and phosphorylation/dephosphorylation of specific polypeptides (MacIntosh et al., 1996). The molecular mechanisms involved in the regulation of tuberization are understood poorly. However, these orchestrated changes likely are controlled by differential gene expression as evidenced by RNA fingerprinting (Bachem et al., 1996) and by developmental changes in the profiles of total proteins and in vitro translation products (Hannapel, 1991; Taylor et al., 1991).

Hormonal regulation of tuber development has been studied in more detail. Gibberellins inhibit tuber formation, whereas abscisic acid or cytokinins may promote tuberization (Ewing and Struik, 1992). The roles of ethylene and auxin have not been established clearly. Recently, novel hormone-like compounds such as JA, MJ, tuberonic acid (TA), and tuberonic acid glycoside have been implicated in tuber induction (Koda et al., 1991; Koda, 1992). These hormone-like compounds have strong tuber-inducing activity in vitro (Koda et al., 1991; Koda, 1992) and may function by disrupting the arrangement of cortical microtubules to allow radial cell expansion that leads to tuber formation (Matsuki et al., 1992; Takahashi et al., 1995). Because LOX is an enzyme required for JA and MJ (Anderson, 1989) and possibly TA (Koda, 1992) biosyntheses, it is conceivable that LOXs play a functional role in tuber development. To our knowledge, though, no direct testing of LOX involvement in tuberization processes has been conducted. One of the objectives of our research was to isolate and characterize cDNAs that encode LOX isozymes involved specifically in potato tuberization.
Role of LOX in defense responses

Overview

Generally, it is accepted that LOX involvement in defense responses most likely is due to LOX participation in the biosynthesis of JA, MJ, and the C-6 volatile compounds (Siedow, 1991; Slusarenko, 1996). Correlative evidence suggests a role for LOX in stress responses. Biochemical studies indicated that LOX protein and activity levels are modulated in response to both biotic and abiotic stresses like mechanical wounding, insect chewing, and pathogen attack. For example, lipoxygenase activity is increased when plants are wounded (Hildebrand et al., 1988). Increases in LOX activity in response to pathogen infection have been reported for a number of plant-pathogen systems (Siedow, 1991). LOX activity increased only upon infection by an avirulent pathogen (Ohta et al., 1991), or it was induced rapidly by infection with an avirulent pathogen and slowly with the virulent strain (Croft et al., 1990). LOX activity also is induced by treatment of cell cultures or plants with elicitors. When potato tuber disks were treated with the fungal (*Phytophthora infestans*) elicitor arachidonic acid (AA), the activity of LOX increased almost 2-fold within 1/2 to 3 hours after treatment (Bostock et al., 1992). In addition, LOX genes are activated transcriptionally by wounding, pathogens, or their elicitors (Bell and Mullet, 1991; Bohland et al., 1997; Melan et al., 1993; Peng et al., 1994). Abscisic acid (ABA), JA, MJ, and linolenic acid have been implicated as intracellular signals that mediate LOX gene induction in defense responses (Bell and Mullet, 1991; Bohland et al., 1997; Melan et al., 1993).

In recent years, interest in LOX has been stimulated by the possibility that this enzyme may be a key component in the wound- and pathogen-inducible lipid-based signal-transduction pathway that leads to the induction defense responses (Fig. 1; Farmer and Ryan, 1992). In this model, wounding or pathogen infection leads to the production of systemic and/or
Fig. 1. Proposed model for the intracellular signal transduction pathway that regulates systemic and localized insect-induced and pathogen-induced expression of proteinase inhibitor and other plant defense genes (Farmer and Ryan, 1992).

Localized signals that interact with receptors on the leaf cell plasma membrane. Occupied receptors initiate a signaling cascade that activates lipases to release free linolenic acid from the plant membrane (Conconi et al., 1996). By the action of LOX and several other enzymes, linolenic acid is converted rapidly to JA and MJ. These compounds then act as signaling molecules to induce expression of defense-related proteins to ward off invading pathogens and herbivores.

**LOX involvement in pathogen-induced defense responses**

Plants commonly exert resistance to pathogens via development of an active defense mechanism known as the hypersensitive response (HR), a form of programmed cell death (Dangl et al., 1996). In the HR, an infection event is followed by rapid death of plant cells
localized around the infection site, and this leads to the formation of necrotic lesions. This reaction limits pathogen spread and prevents further damage to the remainder of the plant organ. Occurrence of the HR is linked tightly to increased expression of many defense genes required for synthesis of phytoalexins, antimicrobial proteins, phenolic compounds, and proteins involved in reinforcing cell walls (Lamb and Dixon, 1990). Transcriptional activation of these defense genes requires synthesis and transduction of specific signaling molecules that include the final products of LOX-catalyzed reactions, JA and MJ (Enyedi et al., 1992). In soybean, potato, and tomato, occurrence of the HR is associated with increased activity of LOX (Slusarenko, 1996). LOX activity increased in rice leaves after infection with an incompatible race of rice blast fungus but not with a compatible race (Ohta et al., 1991). These enzymes may be the primary cause of the HR because they produce organic free radicals and reactive oxygen species that are involved in cell membrane degradation during resistance responses that lead to hypersensitive local cell death (Croft et al., 1990; Keppler and Novacky, 1987). In addition, oxygenated unsaturated fatty acids, which depend on LOX activity for their synthesis, act as antifungal compounds against rice blast fungus (Kato et al., 1986).

Direct evidence for LOX involvement in the HR at the molecular level, however, is difficult to obtain because many LOX isozyme forms exist in plants, and each one may have a specific role in development, senescence, and wound- or pathogen-induced defense responses (Siedow, 1991). None of the previously identified plant LOX genes encode a specific LOX isoform that was shown conclusively to be essential for development of the HR. One of the objectives of this research was to isolate and characterize a potato LOX gene that might be involved specifically in defense responses against pathogens. Because late blight is perhaps the most important potato disease, we have chosen Phytophthora infestans, the causal agent for this disease, as a model pathogen in this study. The results of this study may provide information about the mechanism(s) of plant disease defense not only in the potato-P. infestans
system but also in a broader range of host-pathogen interactions. Potentially, this could lead to innovative approaches for the biotechnology of improved plant resistance.

Potato and Late Blight

Per capita consumption of potatoes is about 65 kg (143 lbs) annually, and only wheat flour makes a greater contribution to the diet of U.S. citizens. It is the leading vegetable crop in both the U.S. and the world, and on a global basis, consumption of potato ranks fourth behind rice, wheat, and corn. Worldwide, the most serious disease of potato is late blight, which is caused by the fungus *Phytophthora infestans*. This disease has received considerable attention since severe epidemics in the 1840s caused the Irish potato famine. Even today, late blight causes significant losses in spite of intensive and expensive efforts to control it. For example, it has been estimated that in 1996, farmers in New York state alone suffered yield losses amounting to $100 million dollars while spending an equivalent amount on fungicides to control *P. infestans*. This disease has become an even greater threat in recent years as new strains of the pathogen have migrated throughout the potato-growing regions of the world (Fry et al., 1993). The new strains are a concern because they have greater fitness, and perhaps, greater aggressiveness than the strains they have replaced. The new strains also allow the fungus to reproduce sexually, and this results in production of oospores capable of long periods of dormancy. Finally, many of the new strains are resistant to the systemic fungicide metalaxyl, which is used widely in late blight control. These circumstances suggest that late blight of potatoes could become much more difficult to control in the future. A promising approach to solving this problem is to determine exactly how the potato plant defends itself against *P. infestans*. This information, combined with the efforts of breeders, potentially could be used to improve resistance through genetic engineering.
Potato LOX genes

Potato LOXs are encoded by a multigene family. Recently, several LOX genes have been isolated from potato tubers, roots, and leaves (Casey, 1995; Geerts et al., 1994; Kolomiets et al., 1996a; Kolomiets et al., 1996b; Royo et al., 1996). Royo et al. (1996) have suggested that potato LOX genes be divided into three distinct classes based upon their deduced amino acid sequences, and these three classes also differ by their organ-specific expression. Lox 1 was expressed in tubers and roots, Lox 2 was leaf-specific, and Lox 3 was expressed in leaves and roots. To this point, no clear physiological roles have been established for any of them.

Literature Cited


POTATO (SOLANUM TUBEROSUM L. CV. SUPERIOR) LIPOXYGENASE GENES EXPRESSED DURING THE EARLY STAGES OF TUBERIZATION (ACCESSION NOS. U60200 AND U60201)

A paper published in the Plant Gene Register of Plant Physiology

Mikhailo V. Kolomiets, David J. Hannapel, and Richard J. Gladon

Plant lipoxygenases (LOXs) (linoleate:oxygen oxidoreductase, EC 1.13.11.12) are a class of nonheme iron-containing enzymes that catalyze the oxygenation of polyunsaturated fatty-acids such as linolenic and linoleic acids. The primary products of this reaction, fatty acid monohydroperoxides, are precursors of biologically active compounds such as traumatin, jasmonic acid (JA), and methyl jasmonate (MJ), which serve hormone-like regulatory roles (Anderson, 1989). Another group of LOX-derived final products, C-6 volatile compounds, possesses antimicrobial activities (Croft et al., 1993). Due to apparent LOX participation in the biosynthetic pathways for JA, MJ, and the C-6 volatile compounds, LOXs are believed to have a role in senescence, seed germination, plant growth and development, and in wound- and pathogen-induced defense responses (Siedow, 1991). In potato, LOXs are of additional interest because JA, MJ, and structurally similar tuberonic acid and tuberonic acid glycoside have been shown to be tuber-inducing substances (Koda et al., 1991).

We report here the isolation, partial characterization, and complete nucleotide sequence of two cDNA clones, designated POTLX-1 and POTLX-2, representing closely related (96%
nucleotide sequence homology) but distinct LOX genes. Both genes were isolated from a lambda ZAPII cDNA library constructed from mRNA extracted from axillary bud tubers that were 4 days old (Hannapel, 1991; Kang and Hannapel, 1995). The probe for screening this library, a 0.85-kb potato LOX fragment, was generated by using RT-PCR with primers based on conserved regions of known plant LOX sequences. The high sequence homology of these cDNA clones to other cloned plant LOXs provided evidence that they were lipoxygenases.

POTLX-1 contains 2772 nucleotides with an open reading frame of 2583 nucleotides, a 5' untranslated region of 6 nucleotides, and 183 nucleotides of 3' untranslated sequence. A putative polyadenylation signal typical of plant genes (AATAAA) begins at position 2745 in the 3' untranslated region. The open reading frame encodes a polypeptide of 861 aa, which is similar in length to other plant LOXs, indicating that the cDNA may represent a complete coding sequence. Plant LOX genes often exist as highly conserved multigene families not only within a given plant species but also among different species (Ferrie et al., 1994; Saravitz and Siedow, 1995). Thus, it is not surprising that the POTLX-1 sequence exhibits high homology to LOX cDNAs from potato and other plant species. The homology ranged from 64% for soybean GMLIPOX1 (GenBank X67304) to 98.2% for potato StLOX1 (GenBank X79107).

The POTLX-2 cDNA clone contains 2837 nucleotides with an open reading frame of 2580 nucleotides (encodes 860 aa), a 5' untranslated region of 46 nucleotides, and 211 nucleotides of a 3' untranslated sequence. The presence of an in-frame stop codon (TAA) in the eleventh codon upstream from the putative initiation codon (ATG) indicates that the
coding sequence is complete and that the first ATG codon at position 47 is in fact the
initiation codon. This cDNA sequence showed the greatest homology to potato
linoleate:oxygen oxidoreductase (99%, GenBank S73865), tomato loxA (95.2%, GenBank
U09026), and potato StLOX1 (94.9%, GenBank X79107). Although there is extremely high
nucleotide sequence homology of POTLX-2 to one of the potato LOX genes (GenBank
S73865), the amino acid sequence of the two differs by 11 aa.

Preliminary results of northern hybridization studies with polyadenylated mRNA
indicated that the expression of these genes may be restricted to developing tubers and roots
only. Accumulation of these LOX transcripts was not detected in leaves, flowers, stems,
shoot tips, or axillary buds.

Literature Cited

Anderson JM (1989) Membrane derived fatty acids as precursors to second messengers. In
WF Boss, DJ Morre, eds, Second Messengers in Plant Growth and Development. Alan
R Liss. New York, pp 181-212

Croft KPC, Juttner F, Slusarenko AJ (1993) Volatile products of the lipoxygenase
pathway evolved from Phaseolus vulgaris (L.) leaves inoculated with Pseudomonas


Plant lipoxygenase (LOX) (linoleate:oxygen oxidoreductase, EC 1.13.11.12) is an iron-containing enzyme that catalyzes the incorporation of molecular oxygen into polyunsaturated fatty acids such as linolenic and linoleic acids. Depending upon the carbon chain position at which oxygenation occurs, the primary product of this reaction is one or more fatty acid monohydroperoxides. These hydroperoxidation products then are metabolized into compounds that may have distinct physiological functions in plants (Anderson, 1989; Siedow, 1991). Traumatin, jasmonic acid (JA), and methyl jasmonate (MJ) have been studied more thoroughly than other LOX final products, and these substances serve hormone-like regulatory roles (Anderson, 1989). In addition, the C-6 volatile compounds derived from lipoxygenases possess antimicrobial activities (Croft et al., 1993). Multiple LOX isozyme forms exist, and different products are formed (Siedow, 1991; Ferrie et al., 1994; Saravitz and Siedow, 1995). Therefore, it is not surprising that LOXs have been proposed to play a role in many physiological processes such as senescence, seed germination, plant growth and development, and wound- and pathogen-induced defense responses (Siedow, 1991).
The primary objective of this research was to isolate a cDNA clone that represents a wound- and abscisic acid (ABA)-responsive LOX isoform that may be involved in potato defense responses against pests and pathogens. For this, we have used a lambda gt11 cDNA library constructed from mRNA extracted from ABA-treated potato leaves (Hildmann et al., 1992). ABA can induce the same set of defense genes as wounding (Hildmann et al., 1992; Pena-Cortes et al., 1996). To obtain a probe for screening this library, PCR was conducted with primers based on conserved regions of known plant LOX sequences available from the GenBank database. Template cDNA was obtained by reverse transcription of total RNA extracted from wounded potato leaves. The resulting 0.85-kb fragment was sequenced and exhibited high homology to other plant lipoxygenases, which suggested its identity as a lipoxygenase. By using this fragment as a probe, we have isolated a cDNA clone, designated POTLX-3, that represents a novel potato LOX gene expressed in ABA-treated potato leaves. Automated dideoxy sequencing of both strands of the cDNA clone was performed on an ABI 377 sequencer (Applied Biosystems, Inc., Foster City, CA, USA) at the Nucleic Acid Facility, Iowa State University.

POTLX-3 contains 2831 nucleotides, and this consists of an open reading frame of 2586 nucleotides, a 5' untranslated region of 38 nucleotides, and 207 nucleotides of 3' untranslated sequence that includes a 37-nucleotide polyadenylated tail. The presence of an in-frame stop codon (TAA) in the fourth codon upstream from the putative initiation codon (ATG) indicates that the coding sequence is complete and that the first ATG codon at position 47 is the initiation codon. The open reading frame encodes a polypeptide of 862 amino acids with
a calculated molecular mass of 97.8 kD and a pI of 5.6. The high sequence homology of our cDNA clone to other reported plant LOX genes (EMBL and GenBank databases) provided evidence that POTLX-3 is a lipoxygenase. POTLX-3 showed the greatest homology to tobacco Lox1 (81.4% identity, GenBank X84040), potato linoleate:oxygen oxidoreductase (79.4% identity, GenBank S73865), potato StLOX1 (78.4% identity, GenBank X79107), and tomato loxA (77.9% identity, GenBank U09026).

Acknowledgments

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Literature Cited


Ferrie BJ, Beaudoin N, Burkhart W, Bowsher CG, Rohstein SJ (1994) The cloning of
two tomato lipoxygenase genes and their differential expression during fruit ripening.
Plant Physiol 106:109-118

Hildmann T, Ebneth M, Pena-Cortes H, Sanchez-Serano JJ, Willmitzer L, Prat S
(1992) General roles of abscisic and jasmonic acids in gene activation as a result of
mechanical wounding. Plant Cell 4:1157-1170

deficient plants do not accumulate proteinase inhibitor II following systemin treatment.
Planta 198:447-451

Saravitz DM, Siedow JN (1995) The lipoxygenase isoymes in soybean (Glycine max (L.)
Merr.) leaves. Changes during leaf development, after wounding, and following

Mol Biol 42:145-188
POTATO LIPOXYGENASE GENES POTLX-1 AND POTLX-2 ARE INVOLVED SPECIFICALLY IN TUBER DEVELOPMENT

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Summary

Plant lipoxygenases (LOXs) are a functionally diverse class of enzymes implicated in physiological processes such as growth, senescence, and wound- and pathogen-induced defense responses. To study the possible involvement of LOX in potato tuberization, the mRNA accumulation patterns of two early tuber LOX cDNAs, designated POTLX-1 and POTLX-2, have been characterized during tuber initiation and growth. Analysis of the deduced amino acid sequence revealed that POTLX-1 and POTLX-2 are members of the potato Lox1 gene family that likely encode 9-LOX isozymes that are not targeted to chloroplasts. RNA blot hybridization studies indicated that accumulation of POTLX-1 and POTLX-2 was specific to developing tubers and roots only and that mRNA accumulation correlated positively with tuber initiation and growth. Hormonal treatments, pathogen infection, or wounding did not induce POTLX-1 and POTLX-2 mRNA accumulation in leaves. In situ hybridization showed that POTLX-1 transcripts accumulated in the apical and subapical regions of the new tuber and in the vascular tissue of the most actively growing part of the developing tuber. Transcript accumulation was not detected in the part of the new tuber immediately adjacent to the attached stolon. Thus, POTLX-1 transcripts were localized within the sites of the most active cell division that occurs during tuber initiation. Our results indicate that POTLX-1 and/or POTLX-2 may have an important function in controlling tuber initiation and growth.
Introduction

The induction and growth of potato tubers are complex developmental processes that require interaction of several environmental and genetic factors. Under inductive conditions, longitudinal stolon growth ceases, and tubers form as a result of subapical radial cell expansion in the stolon tip (Peterson et al., 1985). This step in tuber development is associated with numerous anatomical changes that include rapid cell enlargement and a switch in the plane of cell division from longitudinal to radial (Ewing and Struik, 1992). These orchestrated changes likely are controlled by differential gene expression as shown by RNA fingerprinting (Bachem et al., 1996) and developmental changes in the profiles of total protein and in vitro translation products (Hannapel, 1991; Taylor et al., 1991). Genes regulated developmentally during tuber initiation and growth include patatin (Hendriks et al., 1991), the proteinase inhibitors (Hendriks et al., 1991; Suh et al., 1991), tubulins (Taylor et al., 1991), S-adenosylmethionine decarboxylase (Taylor et al., 1992), starch biosynthesis enzymes (Preiss, 1991), calmodulin (Jena et al., 1989), and lipoxygenases (Bachem et al., 1996; Kolomiets et al., 1996a; Royo et al., 1996). Despite our understanding of the molecular events associated with tuber development, the gene(s) that control subapical changes in cell growth characteristics of tuber initiation have not been identified yet.

Plant LOXs (EC 1.13.11.12) are dioxygenases that catalyze oxygenation of polyunsaturated fatty acids such as linolenic and linoleic acids. Distinct LOX isozymes preferentially introduce molecular oxygen into either the C-9 or the C-13 position of linoleic and linolenic acids (Siedow, 1991). Subsequently, two distinct fatty acid monohydroperoxides are formed and directed into separate biosynthetic pathways that result in accumulation of compounds with distinct physiological functions (Anderson, 1989; Siedow, 1991). The 13-monohydroperoxides are precursors of biologically active compounds such as traumatin, jasmonic acid (JA), and methyl jasmonate (MJ), and these compounds serve
hormone-like regulatory and defense-related roles in plants (Siedow, 1991). The 9-
monohydroperoxides are converted into compounds whose physiological function is not
known (Siedow, 1991).

Multiple LOX isozyme forms exist in all plant species that have been studied, and their
expression is under strict developmental and stress-regulated controls (Heitz et al., 1997;
Saravitz and Siedow, 1995; Siedow, 1991; Stephenson et al., 1998). Because different LOX
isozymes produce functionally diverse final products, it is not surprising that LOXs were
implicated in such widespread physiological processes as senescence, growth and
development, and wound- and pathogen-induced defense responses (Hildebrand, 1989).
Several lines of correlative evidence suggest that LOX may play an important role in plant
growth and development. In many plant species, rapidly growing tissues have the greatest
LOX activity (Siedow, 1991), and there is a positive correlation between LOX activity within
an organ and its rate of elongation. LOX isozyme profiles change quantitatively and
qualitatively during soybean leaf development (Saravitz and Siedow, 1995) and during seed
germination in cucumbers (Feussner et al., 1996). In addition, many LOX genes are regulated
differentially during Arabidopsis seedling development (Melan et al., 1994), tomato fruit
ripening (Ferrie et al., 1994; Kausch and Handa, 1997), potato tuber development (Bachem et
al., 1996), and pea carpel development (Rodriguez-Conception and Beltran, 1995).
Nonetheless, there is still little evidence that demonstrates a functional role for specific LOXs
during the early stages of potato tuber formation. Recent studies have shown that novel
hormone-like compounds such as JA, MJ, tuberonic acid (TA), and tuberonic acid glycoside
are involved in tuber induction (Koda, 1992). Because LOX is an enzyme required for JA and
MJ synthesis (Anderson, 1989) and has been implicated in TA production (Koda, 1992), it is
conceivable that this LOX-dependent pathway plays an important role in regulating potato tuber
development.
Potato LOXs are encoded by a large multigene family. Recently, several LOX cDNAs have been isolated from potato tubers, roots, and leaves (Casey, 1995; Geerts et al., 1994; Kolomiets et al., 1996a; Kolomiets et al., 1996b; Royo et al., 1996). Royo et al. (1996) proposed that potato LOX genes be divided into three distinct classes based on their deduced amino acid sequences and by their patterns of expression. Lox1 genes were expressed in tubers and roots, Lox2 were leaf-specific, and Lox3 were expressed in leaves and roots. LOX expression has been detected in developing tubers, and several groups have proposed that LOXs are involved in potato tuber growth (Bachem et al., 1996; Kolomiets et al., 1996a; Royo et al., 1996). Previously, we have reported the identification of two potato tuber LOX cDNAs designated POTLX-1 and POTLX-2 (Kolomiets et al., 1996a). Our overall objective was to elucidate the function of POTLX-1 and POTLX-2 in potato tuberization. We have shown that accumulation of the mRNA corresponding to these genes was specific to developing tubers and roots and that POTLX-1 transcript accumulation correlated positively with the activation of cell division during the early stages of tuber formation. These results support the hypothesis that tuber-specific LOXs may be involved in regulating potato tuberization processes.

Results

Characterization of POTLX-1 and POTLX-2

Two full-length LOX cDNA clones were isolated from an early stage tuber cDNA library (Kolomiets et al., 1996a). These cDNAs represent two closely related but distinct LOX genes, and their predicted amino acid sequences share 95.6% identity and 96.9% similarity (Table 1). POTLX-1 and POTLX-2 proteins were compared with other plant LOXs by using the GAP program of the Genetics Computer Group (GCG) of the University of Wisconsin (Madison, WI, USA). The protein sequences compared in Table 1 represent the best matching LOXs
from several species from different families. POTLX-1 and POTLX-2 showed the greatest match (more than 78% identity) to LOXs from solanaceous species. The sequence of the potato T8 protein from the Lox1 family (Royo et al., 1996) had the greatest identity match with POTLX-1 (96.6%) and with POTLX-2 (96.9%). The identity match was lower for nonsolanaceous LOXs from Arabidopsis, soybean, and pea. POTLX-1 and POTLX-2 showed the least sequence identity match (58.4%) to barley LoxA (van Mechelen et al., 1995).

Protein sequence analysis of POTLX-1 and POTLX-2 revealed amino acid sequence motifs that are conserved highly among plant and mammalian LOXs (data not shown). The predicted peptides of POTLX-1 and -2 contained the conserved 39-amino acid motif essential for enzyme activity, the three conserved His residues and one Ile residue implicated in iron binding, and the 11 highly conserved amino acids that constitute a cavity that accommodates the fatty acid substrate (Boyington et al., 1993; Steczko et al., 1992). Several LOXs, including potato Lox2 and Lox3 (Royo et al., 1996), their tomato homologs TomLOXC and TomLOXD (Heitz et al., 1997), and the Arabidopsis AtLOX2 (Bell et al., 1995) possess an N-terminal amino acid sequence extension believed to be involved in chloroplast targeting. This sequence extension is absent in POTLX-1 and POTLX-2, and interestingly, POTLX-1 and -2 shared the least sequence homology with these and other known chloroplast-targeted LOX genes (data not shown). Amphiphilic structure recognition analysis of POTLX-1 and POTLX-2 sequences by using the PSORT protein-sorting program (Nakai and Kanehisa, 1992) revealed that, similar to the LOXs listed in Table 1, POTLX-1 and POTLX-2 do not exhibit the typical features of a chloroplast-targeted polypeptide. Therefore, based on amino acid sequence analysis, POTLX-1 and POTLX-2 likely represent LOXs that are not transported to chloroplasts.

Southern analysis revealed one to two intense bands and numerous fainter bands that hybridized to the POTLX-1 probe (Figure 1). These results indicated that POTLX-1 and POTLX-2 represent low-copy number genes in a multigene family in the potato genome.
Bands of lower signal intensity may represent other closely related genes previously identified in the Loxl family (Royo et al., 1996).

**Pattern of POTLX-1 and POTLX-2 mRNA accumulation**

To study POTLX-1 and POTLX-2 expression at the RNA level, northern hybridization analysis was conducted by using the POTLX-1 probe. The POTLX-1 probe cross-hybridized to POTLX-2 sequences, and it was impossible to distinguish between POTLX-1 and POTLX-2 transcripts. Therefore, we will refer only to POTLX-1 in the presentation of our results. Figure 2 shows organ-specific accumulation of POTLX-1 mRNA in the potato plant. POTLX-1 transcripts (approximately 2.8 kb) were detected only in underground organs. The highest levels of mRNA occurred in actively growing tubers, whereas lower levels were detected in nontuberizing stolons and roots. Transcript accumulation was not detected in leaves, flowers, or stems. Identical results were obtained in a separate experiment in which northern analyses were conducted by using poly(A)^+ RNA extracted from the same organs (data not shown). We have examined whether POTLX-1 mRNA accumulation could be induced in leaves in response to wounding, pathogen infection, and other stimuli such as MJ, abscisic acid (ABA), and other hormones. These stimuli were shown to effect expression of some plant LOXs and other tuber-abundant potato proteins such as proteinase inhibitor II (Pin2). Figure 3 shows that POTLX-1 and Pin2 mRNA accumulation in tubers are similar, but in contrast to Pin2, POTLX-1 was not induced in leaves by wounding. In addition, POTLX-1 was not upregulated in leaves infected with the fungal pathogen Phytophthora infestans or treated with sucrose, ABA, MJ, ethylene, gibberellic acid (GA3), auxin (NAA), or cytokinin (BA) (data not shown).

We investigated LOX involvement in potato tuberization processes by examining the POTLX-1 expression pattern during tuber development. Total RNA was isolated from stolons and tubers at the developmental stages indicated in Figure 4 and from mature tubers and
subjected to northern hybridization analysis (Figure 5). Although *POTLX-1* transcripts were detected in nontuberizing unswollen stolons, their steady-state levels increased in newly tuberizing, swollen stolons. *POTLX-1* mRNA increased in abundance as tubers enlarged and reached maximum levels in large, actively growing tubers. *POTLX-1* mRNA accumulation decreased in mature tubers, when tuber growth ceased, and transcripts were not detected in mature tubers stored at 6°C. These results were reproduced in a separate experiment that used poly(A)^+ RNA isolated from organs taken at the same developmental stages (data not shown). To determine in which part of a tuberizing stolon the induction of *POTLX-1* mRNA accumulation occurred, poly(A)^+ RNA was isolated from different sections of new tubers and unswollen stolon tips as shown in Figure 6. The greatest amount of *POTLX-1* transcripts accumulated in the tips of new tubers (Figure 6). Low, basal transcript levels were detected in proximal and middle stolon sections and remained low in unswollen stolon tips until induced to tuberize.

*Localization of POTLX-1 transcripts in a developing tuber*

*In situ* hybridization was utilized to determine the exact location of *POTLX-1* mRNA accumulation in developing tubers. These localization studies were conducted on new tubers because *POTLX-1* mRNA is most abundant at this stage of development (Figure 6), and the organ is small enough (about 2 mm in diameter) to allow use of a light microscope. Transverse (Figure 7a-d) and longitudinal (Figure 7e-h) sections through new tubers were hybridized to antisense digoxigenin-labeled *POTLX-1* riboprobes. An orange-brown color indicates a positive hybridization signal. A comparison of micrograph panels a through d and f through h with micrograph panel e clearly demonstrates that accumulation of *POTLX-1* transcripts occurred in the distal portion of the new tuber (Figure 7). *POTLX-1* mRNA was not detected in the proximal portion of the new tuber adjacent to the attached stolon (Figure 7a, e, and f). Micrograph panels e, g, and h show that mRNA accumulation was most abundant in vascular
bundles and in the apical dome of the new tuber. No hybridization signal was apparent in the
cortex, pith (Figure 7g and h), or the lamina of primary leaves (Figure 7d and h). Transverse
sections through the new tuber hybridized to the \textit{POTLX-1} antisense probe confirmed the
location of \textit{POTLX-1} transcripts in vascular bundles of the most actively growing, distal
portion of the new tuber (Figure 7b and c). \textit{POTLX-1} mRNA was not detectable in the
endodermis (Figure 7b), a distinct cell layer that is present only at the early stages of tuber
development (Cutter, 1978). Figure 7i shows a longitudinal section (corresponding to
micrograph panel e) incubated with the sense \textit{POTLK-1} probe used as a negative control.
There was no background hybridization signal observed in this section. Similar results were
observed consistently by using the sense probe with all other tissue sections shown here.

\textbf{Discussion}

Previously, we reported the sequence of two potato LOX genes designated \textit{POTLX-1} and
\textit{POTLX-2} that were isolated from an early-stage tuber cDNA library (Kolomiets \textit{et al.}, 1996a).
To better understand LOX involvement in potato tuberization processes, we have characterized
the pattern and localization of their expression at the mRNA level during tuber development.
Analysis of the deduced amino acid sequences revealed that \textit{POTLX-1} and \textit{POTLX-2} are
members of the potato Lox1 gene family that likely encode 9-lipoxygenase isozymes and that
are not targeted to chloroplasts. Transcript accumulation of these genes was limited to
developing tubers and roots and was not inducible in leaves by wounding, pathogen infection,
sucrose, or hormonal treatments. \textit{POTLX-1} and \textit{POTLX-2} mRNA accumulation was induced
strongly during tuber initiation and growth, and this suggests possible involvement in tuber
development. Localization studies showed that \textit{POTLX-1} transcripts were detected in the most
actively growing part of a developing tuber, specifically in the tissues where most cell divisions
occur during the early stages of tuber formation. To our knowledge, this is the first report of LOX transcript localization in specific tissues of a developing tuber.

The high overall amino acid similarity to previously characterized LOXs and the presence of the conserved amino acid motifs and residues essential for enzyme functionality confirmed the identity of POTLX-1 and POTLX-2 as lipoxygenases. Several potato LOX genes have been grouped into three classes based upon their deduced amino acid sequence similarity (Royo et al., 1996). Because our gene products exhibited the greatest sequence match (more than 95% sequence identity) with the T8 LOX isozyme and other representatives of the potato Lox 1 class (Royo et al., 1996), we propose that POTLX-1 and POTLX-2 are members of the Lox 1 class. Genomic Southern blot analysis revealed multiple bands that hybridized to the POTLX-1 probe, confirming the existence of a family of LOX genes closely related to POTLX-1. Under our hybridization conditions, the POTLX-1 probe cross-hybridized to POTLX-2 sequences and other members of the Lox 1 class due to high sequence conservation. Many plant species contain multiple, closely related LOX isozymes that are encoded by different genes (Saravitz and Siedow, 1995; Siedow, 1991; Stephenson et al., 1998). These multiple genes likely serve as a means to carry on the diverse functions ascribed to LOXs by elaborate regulation of expression in specific tissues and cells in response to various cues (Eiben and Slusarenko, 1994; Stephenson et al., 1998).

The physiological role of specific LOX isozymes apparently depends on enzymatic properties such as substrate and positional specificity (Siedow, 1991) as well as their subcellular compartmentalization (Stephenson et al., 1998). Distinct LOX isozymes prefer either linoleic or linolenic acids as substrates, and depending on the site of oxygenation, they form either 9- or 13-lipid hydroperoxides as their primary products (Siedow, 1991). Recently, Royo et al. (1996) have reported that the potato LOX isozyme encoded by the T8 cDNA prefers linoleic acid as a substrate, and preferentially catalyzes formation of 9-lipid hydroperoxides (96%) over 13-lipid hydroperoxides (2.1%). It is probable that these same
enzymatic properties are shared by other Lox1 members due to their high sequence similarity (Royo et al., 1996). Therefore, we predict that POTLX-1 and POTLX-2 gene products have predominantly 9-lipoxygenase activity with linoleic acid as their preferred substrate. The physiological function of 9-lipoxygenases in plants is not known, but apparently, they are associated with the production of 9-lipid hydroperoxides and their derivatives. Some identified products derived from 9-lipid hydroperoxides are C9 oxoacids (such as 9-oxo-nonanoic acid) and volatile aldehydes (such as cis-3-nonenal and trans-2-nonenal) (Galliard and Chan, 1980). No physiological activity has been assigned yet to any of these 9-hydroperoxide derivatives.

LOX-derived products such as JA, MJ, and TA seem to play an important role in potato tuberization. These hormone-like compounds have strong tuber-inducing activity in vitro (Koda, 1992) and may function by disrupting the arrangement of cortical microtubules that allow radial cell expansion that leads to tuber formation (Matsuki et al., 1992; Takahashi et al., 1995). It has been established that the 13-hydroperoxide of linolenic acid is the only precursor for JA biosynthesis (Siedow, 1991). Due to their predicted enzymatic properties, it is unlikely that POTLX-1 and POTLX-2 are involved in the synthesis of JA or other jasmonates like TA. Furthermore, unlike the chloroplast-targeted LOXs from potato (Royo et al., 1996), tomato (Heitz et al., 1997), and Arabidopsis (Bell et al. 1995), POTLX-1 and POTLX-2 lack the amino-terminal sequence that functions as a chloroplast transit peptide (Heitz et al. 1997), indicating that POTLX-1 and POTLX-2 likely are not targeted to chloroplasts. This decreases the possibility that these new LOXs function in JA biosynthesis, because chloroplasts are one site of the initial, LOX-catalyzed step of JA biosynthesis (Bell et al., 1995; Creelman and Mullet, 1997; Vick and Zimmerman, 1987). An analysis of amino acid sequences (PSORT), which evaluates the subcellular localization of proteins based on the sequence features related to protein sorting signals, predicted with 58% certainty that POTLX-1 and POTLX-2 were associated with peroxisomes. Such compartmentalization is consistent with the previous findings that tuber-abundant LOX isozymes were present in microsomal organelles (Mulliez et
al., 1987). If these two LOXs are located in the peroxisomes, they could be involved in the mobilization of lipid reserves to provide energy for actively growing tissues (Matsui et al., 1992; Siedow, 1991). Lipid mobilization during the early stages of seedling growth is the major function ascribed to glyoxysomes, a class of peroxisomes (Olsen and Harada, 1995).

We have demonstrated that, similar to other members of the Lox1 family (Geerts et al., 1994; Royo et al., 1996), POTLX-1 and POTLX-2 mRNA accumulation was specific to underground organs with the highest transcript levels found in actively growing tubers and the lowest levels detected in roots. Although Royo et al. (1996) and Geerts et al. (1994) have reported low Lox1 mRNA levels in leaves, we did not detect POTLX-1 and POTLX-2 transcript accumulation in potato leaves even when using poly(A)+ RNA. Furthermore, in contrast to other LOXs that are induced by a number of external stimuli (Bell and Mullet, 1991; Melan et al., 1993; Peng et al., 1994), POTLX-1 and POTLX-2 mRNA accumulation could not be induced in leaves by wounding, pathogen infection, or treatment with MJ, ABA, auxin, gibberellic acid, cytokinin, or sucrose. Unlike POTLX-1 and POTLX-2, expression of other tuber-specific genes like the proteinase inhibitors and patatin can be induced in leaves by MJ, ABA, sucrose, and wounding (Ewing and Struik, 1992; Suh et al., 1991). These results show that POTLX-1 and POTLX-2 are clearly specific to underground organs, and this suggests that the transcriptional activator(s) of these genes are present in tubers and/or roots but not leaves. Because they are not induced by wounding or pathogens, POTLX-1 and POTLX-2 probably are not involved in defense responses against pests and pathogens in leaves. However, some Lox1 isozymes may have a protective role in tubers and roots as their corresponding genes are induced by wounding and MJ in roots (Royo et al., 1996) and stored mature tubers (Geerts et al., 1994). On the contrary, Geerts et al. (1994) reported that treatment with MJ or wounding of actively growing tubers caused a pronounced decline in the expression level of the same (or different but related) gene(s) that was induced in stored tubers by these stimuli.
Our results show that steady-state levels of \textit{POTLX-1} and \textit{POTLX-2} mRNA are under strict developmental control during tuber initiation, growth, and maturation. \textit{POTLX-1} mRNA levels increased when tuberization began and peaked in large, actively growing tubers. This tuberization-associated induction of \textit{POTLX-1} and \textit{POTLX-2} transcript accumulation occurred exclusively in the tips of tuberizing stolons where radial cell expansion takes place to form a tuber (Fig. 6). Only basal, low-abundance levels of \textit{POTLX-1} and \textit{POTLX-2} were detected in middle or proximal stolon sections of tuberizing stolons and in nontuberizing stolon tips. Later in tuber development, when growth ceased and tubers became dormant, \textit{POTLX-1} and \textit{POTLX-2} transcript accumulation returned to these basal levels. This positive correlation between LOX mRNA accumulation and tuber growth and development suggests that isozymes encoded by these two genes are involved in tuber growth processes. The \textit{POTLX-1} probe provided a combined expression pattern for both \textit{POTLX-1} and \textit{POTLX-2}, as well as other Lox1 members. It is conceivable that some of these genes are expressed constitutively in roots, nontuberizing stolons, and mature tubers, whereas others are induced during tuberization. In support of this hypothesis, Bachem \textit{et al.} (1996) showed by using RNA fingerprinting that two other highly conserved potato LOX genes are expressed differentially during the early stages of tuber development \textit{in vitro}. In another study, specific members of the highly conserved vegetative LOX gene family were regulated differentially in response to sink limitation and were localized in discrete, subcellular compartments (Stephenson \textit{et al.}, 1998).

Localization studies showed that \textit{POTLX-1} transcripts accumulated most abundantly in vascular tissues in the subapical region of new tubers. The primary source of new cells for tuber radial expansion is the vascular bundles, and more specifically, parenchyma associated with both internal phloem (perimedulary zone) and external phloem (Cutter, 1978; Peterson \textit{et al.}, 1985). Therefore, the sites of \textit{POTLX-1} mRNA accumulation coincide with the sites where most cell divisions occur during early tuber formation, suggesting the involvement of
this LOX in cell division. Hybridization signals were not detected in the vascular bundles of the most proximal part of the new tuber (immediately adjacent to the attached stolon). This indicates that POTLX-1 function is not associated with vascular tissues in general, but rather, is specific to the processes occurring in the most actively growing part of the developing tuber. No POTLX-1 mRNA was detected in poly(A)⁺ RNA isolated from actively growing apical shoots. Thus, POTLX-1 is not associated with vegetative growth, in general, but is specific to tuber radial cell growth.

Our findings from both RNA blot and in situ hybridization analyses implicate POTLX-1 and POTLX-2 in the processes of tuber initiation and growth. Based on these preliminary findings and previous work on LOXs, there are several possibilities for the potential function of POTLX-1 and POTLX-2. One hypothesis is that POTLX-1 and POTLX-2 may be involved in the mobilization of lipid reserves to provide energy for cell growth during tuberization. This function would be consistent with the predicted localization of POTLX-1 and POTLX-2 isozymes in peroxisomes where energy is generated via mobilization of fatty acids (Olsen and Harada, 1995). A role in lipid mobilization has been proposed for LOX isozymes that are highly induced during the early stages of seedling growth in several plant species (Feussner et al., 1996; Siedow, 1991; Hildebrand, 1989). LOXs found in actively growing tissues also have been implicated in the disruption of cellular membranes and facilitation of the transport of storage products during cell growth (Hildebrand, 1989). This membrane disruption also may increase membrane permeability that may result in increased levels of Ca²⁺, a process that is required for tuberization (MacIntosh et al., 1996). Another possibility is that these LOX isozymes may be involved in the production of JA and TA due to some residual 13-lipoxygenase activity ascribed to the Lox1 family (Royo et al., 1996). Alternatively, because POTLX-1 and POTLX-2 are likely 9-lipoxygenases, it is probable that some of the 9-hydroperoxide derivatives may have a specific role, as yet unclear, in tuber growth regulation. Lastly, POTLX-1 and POTLX-2 could be involved in ABA synthesis, a hormone that may
have a role in tuber initiation (Vreugdenhil and Struik, 1989). Several lines of evidence have suggested a role for LOX in an alternate pathway for the synthesis of ABA from the carotenoid violaxanthin (Creelman et al., 1992; Vick and Zimmerman, 1987). To determine more accurately the physiological function of POTLX-1 and POTLX-2 isozymes in potato tuber development, phenotypic and biochemical analysis of down-regulated antisense potato mutants will be necessary. Transgenic plants, in which the function of these LOX isozymes is impaired, can provide more definitive information concerning the biochemical mechanisms modulated by these isozyme forms during tuberization.

**Experimental procedures**

**Plant material**

Potato (*Solanum tuberosum* L. cv. Superior) plants were grown in an environmentally controlled (20 to 22°C, 16 h daylength) greenhouse under standard conditions for one week. Plants then were transferred to short days (8 h daylength) for three to four weeks to induce tuberization, and they were harvested to obtain stolons and tubers at appropriate stages of development. Four morphogenically distinct stages of tuber formation were recognized (Figure 4) and described. Stolons not induced for tuberization were identified by a hook at their tip. Swollen stolons have begun tuberization and are characterized by a straightened hook and visible subapical swelling. Only stolons in which the swollen part was less than twice the diameter of the remainder of the stolon were selected for this stage. New tubers (≤1 g fresh mass) have a recognizable tuber appearance and a swollen subapical region (2 to 5 mm) that is more than twice the diameter of the remainder of the stolon. Larger, actively growing tubers (≥5.0 g fresh mass) were harvested from plants five to six weeks old. Mature tubers used for northern analysis were dormant and harvested from plants that senesced in the greenhouse. They were then stored in the dark at 21°C for one month. Stored tubers in Figure 5 were
mature tubers that had been stored at 6°C for at least one month. Roots, stems, leaves, and flowers were collected from flowering plants. The fourth, fifth, and sixth fully expanded leaves from five-week-old plants were used for wounding experiments. Leaflets were wounded by crushing the lamina between veins with a hemostat.

Isolation and Analysis of LOX cDNA Clones

An early-stage tuber cDNA library from potato (Kang and Hannapel, 1995) was screened for LOX cDNAs by using a nick-translated 0.85-kb potato LOX fragment. This fragment was generated by using RT-PCR with primers based on conserved regions of known plant LOX sequences (Kolomiets et al., 1996b). The screening procedure based on the plaque purification technique was conducted as described by Polking et al. (1995). Six putative LOX cDNA clones were isolated from more than 500,000 pfu screened, and their pBluescript® SK(-) phagemids were excised in vivo. Restriction analysis revealed that two clones had inserts of approximately 2.2 kb, whereas the other four were approximately 2.8 kb, the expected size of a full-length plant lipoxygenase cDNA. Based on restriction mapping, the four latter clones were grouped into two types. Two clones, one from each group, were subcloned into pGEM®-11Zf(+) and sequenced completely by using automated dideoxy sequencing at the Nucleic Acid Facility, Iowa State University. The two clones were designated POTLX-1 and POTLX-2 (Kolomiets et al., 1996a). The amino acid sequence comparison was conducted by using the GAP and FASTA programs of the Genetics Computer Group (GCG) of the University of Wisconsin (Madison, WI, USA). The predicted subcellular localization of POTLX-1 and POTLX-2 proteins was determined by the PSORT protein-sorting program (Nakai and Kanehisa, 1992) available at http://psort.nibb.ac.jp.
Genomic Southern Analysis

Genomic DNA was extracted from ‘Superior’ leaves according to the CTAB (cetyltrimethylammonium bromide) method (Rogers and Bendich, 1985). Ten micrograms of genomic DNA were digested with restriction endonucleases, electrophoretically separated on a 0.9% agarose gel, denatured, and transferred to a MagnaGraph nylon membrane (Micron Separations Inc., Westboro, MA, USA). Membranes were hybridized at high stringency in a 50% formamide hybridization buffer at 42°C for 48 h. The hybridization buffer was 50% formamide, 6X SSC (1X SSC: 0.15 M NaCl, 0.015 M Na-citrate), 3.3X Denhardt’s solution (1X Denhardt’s: 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 25 mM Na-phosphate buffer (pH 7.0), 0.115 mg/ml salmon sperm DNA. Membranes were washed in 2X SSC, 0.1% SDS at 23°C for 10 min, 1X SSC, 0.1% SDS at 65°C for 30 min. followed by 0.1X SSC, 0.1% SDS at 65°C for 30 min. For autoradiography, membranes were dried and exposed to X-ray film with intensifying screens for six days at -80°C. We used a 32P-labeled 2.1 kb Clal fragment of the POTLX-1 cDNA clone as the only probe for Southern and northern analyses, and we refer to it as the POTLX-1 probe. Construction of a gene-specific probe was not possible due to high sequence identity between POTLX-1 and POTLX-2 cDNAs (96% nucleotide sequence identity) throughout their entire length. The POTLX-1 probe cross-hybridized to POTLX-2 sequences even under high stringency conditions (data not shown).

Northern Analysis

Total RNA was extracted from various organs of ‘Superior’ plants according to the phenol/chloroform extraction procedure described by Dix and Rawson (1983). Poly(A)+ RNA was obtained from total RNA by chromatography on oligo-dT cellulose (Sambrook et al., 1989). RNA was size-fractionated electrophoretically through a 1% agarose gel that contained 5 mM methyl-mercury hydroxide and transferred onto a MagnaGraph nylon membrane.
(Micron Separations Inc., Westboro, MA, USA). Equal loading of RNA samples and uniform transfer onto a nylon membrane was confirmed by visualizing RNA stained with ethidium bromide under UV light. Membranes were hybridized with the \textit{POTLX-I} probe under conditions identical to those described for Southern analysis. Membranes were washed in 1X SSC, 0.1\% SDS at 23°C for 15 min, in 0.1X SSC, 0.1\% SDS at 23°C for 30 min. and 0.1X SSC, 0.1\% SDS at 65°C for 30 min. RNA blots were exposed to X-ray film using intensifying screens for 2 to 4 days. In Figure 3, a duplicate blot was hybridized to a \textsuperscript{32}P-labeled p755 cDNA fragment that represented a potato proteinase inhibitor II (\textit{Pin2}) gene isolated from a tuber cDNA library (Hannapel, unpublished data). This probe was used as a positive control for wounding and hormone induction. Blots presented are representative examples of at least two independent experiments.

\textit{In Situ Hybridization Analysis}

New tubers (≈2 mm across the swollen part) were fixed (Cañas \textit{et al.}, 1994), embedded in paraffin, and cut by a rotary microtome into 8-μm longitudinal and transverse sections. Sections were incubated with either sense or antisense digoxygenin-UTP-labeled RNA probes transcribed \textit{in vitro} from the full length \textit{POTLX-I} cDNA clone according to manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN, USA). RNA transcripts were hydrolyzed partly in 0.2 M Na-carbonate and 0.2 M Na-bicarbonate at 65°C for 1 h. Tissue sections were deparaffinized, hybridized, and washed as described by Hart \textit{et al.} (1998). Hybridization was performed with 0.6 μg of either sense or antisense riboprobes for 24 h. Sections were washed and incubated with antidigoxygenin antibody bound to alkaline phosphatase for immunological detection. Color reactions with 6.6 μg/ml nitro-blue tetrazolium salt, an alkaline phosphatase substrate, were conducted for 4 h. Accumulation of \textit{POTLX-I} mRNA bound to the antisense probe was seen as a brown/orange stain when viewed
under dark-field illumination. The micrographs in Figure 7 were obtained by using dark-field illumination on a Leitz Orthoplan light microscope.

Acknowledgments

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References


Table 1. Comparison of deduced amino acid sequences of POTLX-1 and POTLX-2 genes with those of other plant LOX genes

<table>
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* POTLX-2 is GenBank accession number U60201

Percentage identity and similarity were calculated with the GAP program of the Genetics Computer Group (GCG) of the University of Wisconsin (Madison, WI, USA). The predicted amino acid sequences of POTLX-1 and POTLX-2 were compared with other plant LOX gene products available in the GenBank database: POTLX-1 and POTLX-2 (Kolomiets et al., 1996a); potato T8 (Royo et al., 1996); tomato tomloxA (Ferrie et al., 1994); tobacco LOXI (Veronesi et al., 1995); Arabidopsis AtLox1 (Melan et al., 1993); soybean LOX3 (Yenofsky et al., 1988); pea PSLIPOCY (Ealing and Casey, 1988); and barley LoxA (van Mechelen et al., 1995).
Figure Captions

Figure 1. Southern blot analysis of potato genomic DNA with POTLX-I probe. Genomic DNA (10 µg per lane) was digested with EcoRI (lane E), HindIII (lane H), PstI (lane P), and BamHI (lane B). The membrane was probed with the 32P-labeled 2.1 kb ClaI fragment of the POTLX-I cDNA clone. DNA size markers (kb) are indicated on the left.

Figure 2. Northern blot analysis of organ-specific accumulation of POTLX-I mRNA. Blots of total RNA (10 µg per lane) extracted from leaves, stems, flowers, roots, stolons, and actively growing tubers were hybridized with the 32P-labeled 2.1 kb ClaI fragment of the POTLX-I cDNA clone. Transcript size is approximately 2.8 kb. Equal loading of RNA samples into each lane was confirmed by visualizing the RNA with ethidium bromide and UV light.

Figure 3. Northern blot analysis of POTLX-I and Pin-2 mRNA accumulation after wounding potato leaves. Total RNA was isolated from wounded leaves of Solanum tuberosum cv. Superior plants harvested 0 to 72 h after wounding. The 0 h RNA sample was extracted from leaves harvested immediately after wounding. RNA samples (18 µg per lane) from wounded leaves were loaded on duplicate gels, and northern blots were probed with 32P-labeled inserts of either POTLX-I cDNA or proteinase inhibitor II (Pin-2) cDNA. Total RNA (10 µg per lane) from large, growing tubers was included as a positive control for both probes.

Figure 4. Morphological stages of tuber development in vivo. Underground organs were photographed from 3-week-old Solanum tuberosum cv. Superior plants grown under short-day conditions (8h/16h, day/night). Descriptions of the stages are
presented in the order of development: unswollen stolons have a hook characteristic of stolons not induced for tuberization; swollen stolons are induced for tuberization as shown by a straightened hook and initial swelling in the subapical region; new tubers have a recognizable tuber appearance with the swollen subapical region more than twice the diameter of the attached stolon; and large tubers are growing actively and have more than 5.0 g fresh mass. Magnification of the insert photographs is 1.6 X.

**Figure 5.** Northern blot analysis of \textit{POTLX-I} mRNA accumulation during potato tuber development.

Total RNA was extracted from stolon and tuber tissues at different stages of tuber development (see Figure 4 for the description of the stages): unswollen stolon (US); swollen stolon (SS); new tuber (NT); large, actively growing tuber (LT); mature tuber stored at room temperature for several months (MT); mature tuber stored in refrigerated storage room at 6°C for two months (ST). Equal amounts of total RNA (10 µg per lane) were subjected to electrophoresis, blotted onto a nylon membrane, and hybridized with the \(^{32}\text{P}-\)labeled 2.1 kb ClaI fragment of the \textit{POTLX-I} cDNA clone.

**Figure 6.** Northern blot analysis of localization of \textit{POTLX-I} mRNA accumulation in developing stolons and new tubers.

To determine the location of the greatest \textit{POTLX-I} gene expression in developing tubers, poly (A)+RNA was extracted from three stolon regions of developing new tubers, and from unswollen stolon tips as shown. Proximal stolon sections, middle stolon sections, new tuber tips (each approximately 1 cm long), and entire unswollen stolon tips were used. An equal amount of poly (A)+RNA (2 µg per lane) was loaded in each lane, and the consistency of loading was determined by visualizing RNA after staining with ethidium bromide. Hybridization was conducted with the \(^{32}\text{P}-\)labeled 2.1 kb ClaI fragment of the \textit{POTLX-I} cDNA
clone. Note that POTLX-1 expression in the unswollen tip is about the same as in other parts of the stolon unless tuberization has started (new tuber tip). Magnification is 1.6 X for the photograph of new tuber, and 4.2 X for the photograph of the unswollen stolon tip.

**Figure 7.** In situ localization of POTLX-1 transcripts in developing tubers.

Thin sections (8 μm) through a new tuber were hybridized with digoxygenin-labeled RNA probes that were synthesized from a full-size POTLX-1 cDNA in either sense or antisense orientation. Accumulation of POTLX-1 mRNA bound to the antisense digoxygenin-labeled probe is visualized by alkaline phosphatase activity and is seen as an orange-brown stain on the dark-field micrographs.

(a) - (d) Transverse sections through a new tuber were hybridized with POTLX-1 antisense riboprobe. Approximate location of the transverse sections is indicated by corresponding letters with arrows in micrograph panel e. Hybridization signals were detected in sections (b) and (c) but not in (a) and (d). The arrows in (b) indicate endodermis. The scale bar in (d) is 125 μm. Magnification in (a), (b), and (c) is the same as in (d).

(e) a longitudinal section through a new tuber was hybridized with POTLX-1 antisense probe. Scale bar = 500 μm.

(f) Close-up micrograph of the longitudinal section hybridized to the antisense probe shown in micrograph panel e. The photographed area covers the junction of the stolon and the proximal portion of the new tuber. Note that the hybridization signal is not detected in this portion of the new tuber.

(g) Close-up micrograph of the longitudinal section hybridized to the antisense probe shown in micrograph e. The photographed area covers the subapical portion of the new tuber.

(h) Close-up micrograph of the longitudinal section hybridized to the antisense probe shown in micrograph e. The photographed area covers the apical region of the new tuber. The arrow
indicates the apical dome where POTLX-1 transcripts accumulate to high levels. Scale bar = 250 μm.

(i) In situ hybridization negative control: longitudinal section through a new tuber was hybridized with POTLX-1 sense probe. No hybridization signal was detected. Scale bar = 500 μm.

Magnification in (f) and (g) is the same as in micrograph panel h. Abbreviations: ct, cortex; p, pith; vt, vascular tissue; l, newly formed leaf.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
A NOVEL POTATO LIPoxyGENASE GENE (POTLX-3) INDUCED BY PATHOGEN ATTACK BUT NOT WOUNDING

A paper to be submitted to *Molecular Plant-Microbe Interactions*

Mikhailo V. Kolomiets, Richard J. Gladon, David J. Hannapel, and Edward J. Braun

Lipoxygenase (LOX)-mediated products often function in pathogen-induced defense responses. We studied LOX involvement in defense responses against pathogens by characterizing the pattern of mRNA accumulation of a potato (*Solanum tuberosum* L.) LOX gene designated POTLX-3. POTLX-3 shares relatively low sequence homology with other classes of potato LOX genes, and its expression pattern at the mRNA level is unique compared with other potato LOXs. POTLX-3 transcript accumulation was not detected in untreated, healthy potato organs, but it was induced in leaves treated with ethylene or methyl jasmonate. Neither wounding nor treatment of leaves with ABA, cytokinin, auxin, or gibberellic acid induced POTLX-3 transcript accumulation. However, POTLX-3 mRNA accumulation was induced when potato leaves were infected with either virulent or avirulent strains of *Phytophthora infestans*, the causal agent of late blight. During the resistance response, POTLX-3 was induced within 6 h, and its mRNA continued to accumulate for a week after inoculation. In contrast, when a plant was susceptible to *P. infestans*, mRNA accumulation was induced only 3 to 6 days after inoculation. POTLX-3 mRNA accumulation also was induced during hypersensitive response development caused by the incompatible pathogen *Pseudomonas syringae* pv. *phaseolicola*, and this indicated that POTLX-3 expression may be associated with the hypersensitive response. Thus, POTLX-3 is not involved in wound-inducible defense responses, and it may be involved specifically in defense responses against pathogen infection.
Lipoxygenases (EC 1.13.11.12) are dioxygenases that catalyze the hydroperoxidation of polyunsaturated fatty acids or their esters that contain a cis,cis-1,4-pentadiene moiety. In higher plants, the natural substrates for these enzymes are linolenic and linoleic acids, fatty acids that are most likely derived from membrane phospholipids (Siedow 1991; Conconi et al. 1996). The primary products of LOX action, fatty acid hydroperoxides, are highly toxic to cell membranes and normally do not accumulate in healthy tissues (Galliard and Chan 1980). These hydroperoxidation products are metabolized enzymatically into compounds that may have distinct physiological functions (Anderson 1989; Siedow 1991). Traumatin, jasmonic acid (JA), and methyl jasmonate (MJ) have been studied more thoroughly than other LOX final products, and these substances serve hormone-like regulatory and defense-related roles (Anderson 1989; Creelman and Mullet 1997; Koda 1992). JA and MJ are involved in several physiological processes, and they function as signals in transduction pathways that regulate expression of many genes (Reinbothe et al. 1994). This includes induction of genes that encode defense-related proteins such as phenylalanine ammonia lyase (Gundlach et al. 1992), protease inhibitors (Farmer and Ryan 1992), pathogenesis-related (PR) proteins and chalcone synthase (Reinbothe et al. 1994), proline-rich cell wall proteins (Creelman et al. 1992), and LOX itself (Melan et al. 1993). Recently, Blechert et al. (1995) showed that other LOX-mediated products such as 12-oxophytodienoic and 12-oxophytoenoic acid and their derivative, dihydrojasmonic acid, act as defense signals. In addition, oxygenated unsaturated fatty acids (Kato et al. 1986) and the C-6 volatile aldehydes (Croft et al. 1993) derived from LOX-catalyzed reactions possess antimicrobial activities.

LOX involvement in the production of a variety of functionally diverse compounds implicates these enzymes in a wide range of physiological processes like growth and development, senescence, and wound- and pathogen-induced defense responses (Siedow 1991). Multiple LOX isozymes exist in many plant species, and they are encoded by families of genes that respond differentially to specific developmental, hormonal, and environmental
stimuli (Shibata 1996). For example, several potato cDNA clones encoding distinct LOX isoforms have been identified, and their expression is organ-specific and differentially regulated during tuber development and in response to wounding and MJ treatments (Casey 1995; Geerts et al. 1994; Kolomiets et al. 1996a; Kolomiets et al. 1996b; Royo et al. 1996). Individual isozyme forms within a given plant organ, tissue, or cell type may have a distinct physiological role (Peng et al. 1994; Saravitz and Siedow 1995; Stephenson et al. 1998). With the exception of the chloroplast-specific AtLOX2 isoform from Arabidopsis (Bell et al. 1995) and tobacco LOX1 (Rance et al. 1998), physiological roles of individual plant LOX isozymes have not been established.

Correlative evidence has suggested a role for LOX in biotic and abiotic stress responses. Biochemical studies indicated that LOX protein and activity levels were modulated in response to mechanical wounding, insect chewing, and pathogen attack. For example, lipoxygenase activity and protein content increased when plants were wounded (Hildebrand et al. 1988; Saravitz and Siedow 1995). A LOX activity increase in response to infection has been reported for several plant-pathogen systems, and LOX activity correlated with plant resistance against pathogens (Slusarenko 1996). Increased LOX activity occurred in rice leaves after infection with an incompatible race of rice blast fungus but not with a compatible race (Ohta et al. 1991). In several other host-pathogen combinations, LOX activity was induced more rapidly and to greater levels in an incompatible response than in a compatible one (Slusarenko 1996). LOX activity also is induced by treatment of cell cultures or plants with elicitors. When potato tuber disks were treated with the fungal (Phytophthora infestans) elicitor arachidonic acid (AA). the activity of LOX increased almost 2-fold in 0.5 to 3 hours after treatment (Bostock et al. 1992). In addition, LOX genes were activated transcriptionally by wounding, pathogens, or their elicitors (Bell and Mullet 1991; Bohland et al. 1997; Melan et al. 1993; Peng et al. 1994; Veronesi et al. 1996). Abscisic acid (ABA), JA, MJ, and linolenic acid have been implicated
as intracellular signals that mediate LOX gene induction in defense responses (Bell and Mullet 1991; Bohland et al. 1997; Melan et al. 1993).

It has been suggested that LOX is involved in the development of an active resistance mechanism known as the hypersensitive response (HR), a form of programmed cell death (Croft et al. 1990; Keppler and Novacky 1987; Koch et al. 1992). In the HR, an infection event is followed by rapid death of plant cells localized around the infection site, and this leads to necrotic lesion formation. This reaction limits pathogen spread and prevents further damage to the remainder of the plant organ. In several plant-pathogen systems, occurrence of the HR is linked tightly to increased activity, protein, or mRNA levels of LOXs (Slusarenko 1996). LOX-deficient potato cells failed to develop the HR in response to arachidonic acid treatment (Vaughn and Lulai 1992). Peroxidation of membrane lipids has been postulated to be a causative factor in the localized cell death associated with the HR (Keppler and Novacky 1987). LOX activity may be involved in lipid peroxidation via the production of highly reactive radicals such as singlet oxygen and superoxide anions (Croft et al. 1990; Roy et al. 1994). These free oxygen radicals, if not scavenged, initiate the chain reaction of lipid peroxidation of cell membranes that leads to membrane damage, and consequently, to rapid cell death (Keppler and Novacky 1987; Levine et al. 1994). Alternatively, LOX may have a role in the HR due to participation in the synthesis of signalling molecules like MJ and JA that regulate expression of defense genes associated with HR development and phytoalexin accumulation (Enyedi et al. 1992).

Direct evidence for LOX involvement in the resistance and/or the HR reactions at the molecular level, however, is difficult to obtain because multiple LOX isozymes may be involved in wound- and pathogen-induced defense responses, and each one may have a specific role in these processes (Bohland et al. 1997; Saravitz and Siedow 1995; Saravitz and Siedow 1996). For example, a recent study of LOX isozyme profiles in the wheat-rust fungus pathosystem revealed that several LOX species were induced differentially during the HR
evoked by the pathogen, its specific glycopeptide elicitor, other elicitors like chitosan and chitin oligosaccharides, and MJ. The objective of this research was to isolate and characterize the expression pattern of a potato LOX gene that might be involved specifically in defense responses against pathogens. Several potato LOX genes have been reported recently, and they were implicated in wound-induced defense responses and tuber development (Geerts et al. 1994; Royo et al. 1996; Kolomiets et al. 1998), but none have been shown to be involved specifically in pathogen-induced defense responses. Here we report the pattern of mRNA accumulation of a novel potato LOX cDNA, designated POTLX-3 (Kolomiets et al. 1996b), that is not expressed constitutively in any potato organ and is not wound-inducible. However, it is induced in leaves when treated with MJ and ethylene and during the development of the HR elicited by pathogen attack.

RESULTS

Characterization of POTLX-3

The full-length potato LOX cDNA clone, designated POTLX-3, has been isolated from an abscisic acid (ABA)-induced leaf cDNA library (Kolomiets et al. 1996b). This cDNA clone encodes a polypeptide of 862 amino acids with a calculated molecular mass of 97.8 kilodaltons, and the size and mass of this polypeptide is similar to that of other nonchloroplast-targeted plant LOXs. A comparison of POTLX-3 with other plant LOXs from potato and several other species revealed considerable amino acid sequence similarity with LOXs from solanaceous species (Table 1). POTLX-3 showed the greatest match (85.5% identity and 89.7% similarity) to tobacco LOX1 (Veronesi et al. 1995), which recently was shown to be essential for resistance against pathogens (Rance et al. 1998). The identity match was somewhat lower (less than 80%) for the representatives of the Lox1 isozyme family from potato (POTLX-1 and POTLX-2) (Kolomiets et al. 1996a) and tomato (TomLoxA) (Ferrie et
al. 1994; Heitz et al. 1997). The members of two other potato LOX families, H1 and H3, which contain chloroplast-targeting signal peptides (Royo et al. 1996), shared the least identity match (43.1% and 46.1%, respectively) with POTLX-3. In comparison, the sequence identity was greater than 59% for nonsolanaceous LOXs from *Arabidopsis* and barley (Table 1).

The amino acid sequence motifs that are highly conserved among plant and animal LOXs and that are necessary for LOX function also were present in POTLX-3 (data not shown). The predicted POTLX-3 polypeptide contained the conserved 39-amino acid motif and the three conserved His residues and one Ile residue that are essential for iron binding and enzyme activity. It also contained the 11 highly conserved amino acids possibly involved in the accommodation of the fatty acid substrate (Boyington et al. 1993; Steczko et al. 1992). Unlike the members of the Lox2 and Lox3 families from potato (Royo et al. 1996) and tomato (Heitz et al. 1997), POTLX-3 protein does not possess an N-terminal transit peptide that is believed to be involved in chloroplast targeting. The PSORT protein-sorting analysis (Nakai and Kanehisa 1992), which predicts subcellular localization of proteins based on the sequence features related to protein sorting signals, reinforced the idea that POTLX-3 likely is not transported to the chloroplast. This analysis predicted peroxisomes and/or the cytoplasm as the most probable sites (48% and 45% certainty, respectively) for POTLX-3 localization. Thus, amino acid sequence analysis indicated that POTLX-3 represents a novel, enzymatically active potato LOX that probably is not transported to chloroplasts.

Southern hybridization on potato genomic DNA was performed to evaluate the complexity of *POTLX*-3-related genes in the potato genome. Under the hybridization conditions utilized, the *POTLX*-3 probe did not cross-hybridize with the sequences of two other potato LOXs, *POTLX*-1 and *POTLX*-2, that share the greatest sequence identity (Table 1). Southern analysis revealed one to two bands that hybridized to the *POTLX*-3 probe (Fig. 1), and this indicated the existence of 1 to 2 copies of *POTLX*-3 or closely related genes in the potato genome.
Effect of wounding and hormonal treatments on *POTLX-3* mRNA levels

We studied organ-specific expression of *POTLX-3* at the mRNA level in potato plants by performing northern blot analysis on total and poly(A)^+^ RNA isolated from leaves, stems, flowers, roots, tubers, and stolons. *POTLX-3* transcripts were not detected in any of these organs (data not shown). Although *POTLX-1*, *POTLX-2*, and other members of the potato Lox1 multigene family are regulated developmentally during tuber formation (Kolomiets et al. 1998; Royo et al. 1996), no *POTLX-3* mRNA accumulation was detected in tubers at several stages of development (data not shown). Induction of LOX genes by wounding, including potato Lox1, Lox2, and Lox3, has been reported previously (Royo et al. 1996). To determine if *POTLX-3* can be induced by wounding, we examined *POTLX-3* mRNA accumulation in leaves that had been wounded mechanically. No accumulation of *POTLX-3* transcripts occurred in leaves for up to 72 h after wounding, even though *Pin2* mRNA levels were detected as early as 1 h after wounding and persisted for 72 h (Fig. 2). These data indicated that *POTLX-3* likely is not involved in the wound-induced signal transduction pathway(s) that regulate proteinase inhibitor gene expression.

Northern analysis also was performed to study whether *POTLX-3* mRNA accumulation could be induced by phytohormones known to affect expression of other plant LOX and defense-related genes. Total RNA was extracted from detached leaves that had been treated with physiological concentrations of ethylene, ABA, MJ, gibberellic acid (GA₃), auxin (NAA), cytokinin (BA), or water as a control. Steady-state levels of *POTLX-3* mRNA were induced only by MJ and ethylene (Fig. 3). In leaves treated with MJ, mRNA accumulation was detected in 6 h, reached maximal levels at 12 h, and decreased drastically after 24 h. Induction by ethylene also occurred within 6 h, but maximal levels of *POTLX-3* mRNA were attained only at 24 h and rapidly decreased at 48 h. Although potato Lox2 and Lox3 genes were induced by exogenously applied ABA (Royo et al. 1996), *POTLX-3* mRNA accumulation was not detected in detached leaves treated with 100 μM ABA for 48 h (Fig. 3) or up to 120 h (data
similarly, treatments with either water (control), NAA, GA₃ (Fig. 3) or cytokinin (BA) (data not shown) had no effect on the POTLX-3 mRNA accumulation. Induction of proteinase inhibitor-II (Pin-2) transcript accumulation was used as a positive control for ABA, MJ, and NAA treatments (Hannapel, unpublished data; Hildmann et al. 1992).

POTLX-3 mRNA accumulation in response to pathogen attack

Because developmental and hormonal regulation of POTLX-3 expression is similar to pathogenesis-related (PR) proteins, especially the PR-1 and PR-5 groups (Xu et al. 1994), we hypothesized that POTLX-3 may be involved specifically in pathogen-induced defense responses. To test this hypothesis, we examined whether POTLX-3 could be induced in potato leaves challenged by pathogens. Because late blight, caused by Phytophthora infestans, is perhaps the most harmful potato disease (Fry et al. 1993) and LOX activity was induced strongly by inoculation with this pathogen (Slusarenko 1996), we chose P. infestans as the model pathogen in this study.

To examine POTLX-3 expression during both compatible and incompatible potato-P. infestans interactions, two potato cultivars, 'Superior' and 'Kennebec', were inoculated with sporangial suspensions of two P. infestans strains, US940507 or ME93-2A, in a factorial combination. 'Kennebec' harbors resistance gene R-1, and is resistant to US940507 (incompatible interaction), but is susceptible to ME93-2A (compatible interaction), whereas both strains are compatible on 'Superior'. Northern analysis of total RNA extracted from infected leaves revealed that POTLX-3 transcript accumulation was induced by infection with both P. infestans strains in all combinations. POTLX-3 mRNA accumulation was induced rapidly and to high levels during the incompatible interaction of 'Kennebec' leaves with P. infestans US940507 (Fig. 4A). POTLX-3 transcripts were detected at 6 h, increased in abundance at 12 h, and reached maximal levels at 24 h postinoculation. At days 2 and 3 postinoculation, transcript levels steadily declined and stayed the same from 3 to 7 days.
POTLX-3 transcripts were not detected in mock-inoculated control potato leaves incubated under identical conditions (data not shown). By day 2, when POTLX-3 transcript accumulation started to decrease, the first symptoms of necrotic lesion development characteristic of the HR were clearly visible.

During compatible interactions, large water-soaked lesions first were observed four days postinoculation, and leaves collapsed completely by day 7. In contrast to the resistance response, POTLX-3 transcripts accumulated more slowly during the compatible interaction between ‘Superior’ and P. infestans ME93-2A (Fig. 4B). Transcript accumulation first was detected at 3 days, reached a maximum at 6 days, and declined rapidly at 7 days postinoculation. In general, this overall delayed pattern of POTLX-3 transcript accumulation was consistent across all compatible interactions examined. In all three compatible interactions, a low, inconsistent amount of POTLX-3 transcript was detected between 6 and 24 h (e.g., Fig. 4B, 12 h). Subsequent stripping and rehybridization of the blots to a wheat 18S rRNA gene probe confirmed that RNA loading between samples was similar (Fig. 4).

To determine whether POTLX-3 gene induction is a specific response to P. infestans infection or a general host response associated with the HR, leaves of ‘Superior’ plants were challenged with the incompatible bacterial pathogen Pseudomonas syringae pv. phaseolicola. This pathogen causes halo blight disease on common bean (Phaseolus spp.), and it is a nonpathogenic on potato, in which it induces development of necrotic lesions typical of the HR. The leaflet areas infiltrated with P. syringae pv. phaseolicola showed symptoms of incompatible-type tissue collapse after 6 h and were completely collapsed by 24 h. The timing of the visual appearance of hypersensitive cell collapse was preceded by pronounced induction of POTLX-3 mRNA accumulation (Fig. 5A). Transcripts were detected 3 h after inoculation and reached maximal levels by 6 h. After 9 h, when HR necrotic lesions were clearly visible, POTLX-3 mRNA levels started to decline steadily and were very low by 24 h postinoculation. POTLX-3 transcripts were not detected in control, mock-inoculated potato plants (Fig. 5B).
DISCUSSION

Correlative evidence exists that strongly implicates LOX activity in potato resistance mechanisms against pathogens (Bostock et al. 1992; Slusarenko 1996), but no potato LOX genes involved specifically in defense against pathogens have been identified. We have characterized the pattern of \textit{POTLX-3} mRNA accumulation in potato organs and in response to wounding, hormonal stimuli, and pathogen infection so that we could determine its role in defense responses. Amino acid sequence analysis revealed that \textit{POTLX-3} represents a novel potato LOX gene that encodes an isozyme that likely is not targeted to chloroplasts. The pattern of \textit{POTLX-3} mRNA accumulation is distinct from the other potato LOX genes. \textit{POTLX-3} mRNA was not detected in any healthy potato organ, and was not inducible in leaves by wounding or ABA treatment suggesting no role in either development or wound-induced defense responses. However, \textit{POTLX-3} transcript accumulation was induced strongly in leaves treated with ethylene or MJ or inoculated with bacterial or fungal pathogens. These results suggest \textit{POTLX-3} has a specific role in defense responses against pathogens.

The polypeptide deduced from \textit{POTLX-3} showed high overall amino acid sequence similarity with plant LOXs from both dicots and monocots. These data and the presence of the highly conserved amino acid motifs and residues required for LOX enzyme activity established \textit{POTLX-3} as a lipoxygenase. To some extent, the involvement of individual LOX isozymes in physiological processes of plants depends on their subcellular compartmentalization (Stephenson et al. 1998; Siedow 1991). Among all the plant LOX sequences available from the databases, \textit{POTLX-3} shared the least amino acid sequence similarity with the chloroplast-targeted LOXs from potato (H1 and H3 in Table 1) (Royo et al. 1996), tomato (Heitz \textit{et al.}, 1997), rice (Peng \textit{et al.} 1994), and \textit{Arabidopsis} (Bell \textit{et al.} 1995) (data not shown). The \textit{Arabidopsis} AtLOX2 isozyme is required for wound-induced JA biosynthesis (Bell \textit{et al.} 1995), and a similar role has been proposed for other wound- and pathogen-inducible
chloroplast-localized LOXs (Heitz et al. 1997; Peng et al 1994; Royo et al. 1996). Amino acid sequence analysis showed that POTLX-3 does not possess a chloroplast transit peptide found in these LOXs, and this indicates that POTLX-3 likely is not targeted to chloroplasts. This diminishes the probability that POTLX-3 is involved in JA biosynthesis because chloroplasts seem to be the primary site where the LOX-catalyzed step in JA biosynthesis occurs (Bell et al. 1995; Creelman and Mullet 1997; Vick and Zimmerman 1987).

Potato LOX genes have been grouped into three classes on the basis of the similarity of their deduced amino acid sequences (Royo et al. 1996). The Lox1 class comprises several genes that share more than 95% sequence similarity, including two tuber-specific genes, POTLX-I and POTLX-2 (Kolomiets et al. 1996a). The Lox2 and Lox 3 classes share limited similarity to each other and to the Lox1 isozymes (less than 65%), and are represented by single genes HI and H3, respectively (Table 1; Royo et al. 1996). Relatively low sequence similarity of POTLX-3 with the proteins from these three classes (Table 1) indicates that POTLX-3 represents a novel potato LOX gene not associated with any of these families. POTLX-3 did not cross-hybridize with POTLX-I or POTLX-2 that are members of the Lox1 family (data not shown). Southern analysis showed that, unlike potato Lox1 and Lox2 genes that exist as multigene families (Royo et al. 1996), POTLX-3 is most likely represented by one to two genes in the potato genome.

Developmental and hormonal regulation of POTLX-3 mRNA accumulation also was unique compared with other potato LOXs. The three classes of potato genes showed clear organ-specific expression: Lox1 was expressed in tubers and roots, Lox2 was leaf-specific, and Lox3 was expressed in leaves and roots (Royo et al. 1996). Unlike these other potato LOX genes, POTLX-3 mRNA was not expressed constitutively in any healthy potato organ and was not induced during tuber development. These results suggested that POTLX-3 apparently is not involved in developmental processes. This conclusion is supported by our
finding that \textit{POTLX-3} was not inducible by auxin. A role in plant growth and development has been proposed for an auxin-inducible soybean LOX (Liu et al. 1991).

Unlike Lox2 and Lox3 genes from potato (Royo et al. 1996) and many other plant LOX (Heitz et al. 1997: Melan et al. 1993), mechanical wounding of potato leaves did not induce \textit{POTLX-3} gene expression. Consistent with this, ABA, a required signaling molecule in wound-induced signal transduction pathways (Pena-Cortes et al. 1995) and a strong inducer of a number of wound-inducible LOX genes including potato Lox2 and Lox3 (Royo et al. 1996), did not induce \textit{POTLX-3} transcript accumulation. These results suggest that \textit{POTLX-3} probably is not involved in wound-induced defense responses, and apparently, is not a LOX isozyme implicated in the octadecanoid wound-inducible signal transduction pathway that leads to activation of proteinase inhibitors (Farmer and Ryan 1992). That ABA did not induce \textit{POTLX-3} transcript accumulation was unexpected as the \textit{POTLX-3} cDNA clone was retrieved originally from an ABA-induced potato leaf library. One explanation could be that \textit{POTLX-3} expression in leaves was induced by secondary ethylene that was produced by the original ABA treatment of the potato leaves (Riov et al. 1990).

MJ and ethylene serve as signals in both wound- and pathogen-induced defense pathways that lead to activation of a number of defense-related genes (Enyedi et al. 1992; Farmer and Ryan 1992; Gundlach et al. 1992). Potato and tomato plants sprayed with JA and MJ showed increased resistance against \textit{P. infestans} (Cohen et al. 1993). \textit{POTLX-3} mRNA accumulation was induced highly in potato leaves after treatment with physiological concentrations of MJ or ethylene. These results are consistent with the observation that MJ and ethylene synergistically induce expression of other pathogen-induced defense genes such as those encoding PR-1 and PR-5 proteins (Xu et al. 1994). Accumulation of \textit{POTLX-3} transcripts was not induced in leaves treated with salicylic acid (data not shown). Similar to
**POTLX-3.** *Arabidopsis* defensin genes, which are involved in defense against fungal pathogens, were induced by MJ and ethylene but not wounding (Penninckx et al. 1996). Because defensin genes could not be induced by salicylic acid, the MJ and ethylene pathway was suggested as a separate salicylic acid-independent pathway operative during pathogen-induced resistance response. In summary, developmental and hormonal regulation of *POTLX-3* mRNA accumulation is different from that of most other plant LOX genes. For example, Melan et al. (1993) showed that the *Arabidopsis* LOX gene *AtLox1* is similar to *POTLX-3* in that it can be induced by MJ and pathogens, however it differs because it is also ABA-inducible.

LOX activity, protein, and mRNA levels increase in response to pathogen challenge or elicitor treatments in a number of plant species (Melan et al. 1993; Ohta et al. 1991; Peng et al. 1994; Koch et al. 1992). We demonstrated that *POTLX-3* transcript accumulation was induced in leaves inoculated by both compatible and incompatible strains of *P. infestans*. *POTLX-3* transcripts accumulated more rapidly and to higher levels during an incompatible interaction. The timing of the most abundant accumulation of *POTLX-3* transcripts preceded the formation of necrotic lesions characteristic of the HR, suggesting that *POTLX-3* may play a role in localized cell death associated with the HR. In contrast, during compatible interactions, *POTLX-3* transcripts accumulated later and to lower levels, and the timing of expression coincided with the timing of massive tissue collapse. In other studies that compared compatible and incompatible interactions, it was generally observed that LOX mRNA accumulation reaches a greater level more rapidly in resistant than susceptible plants (Koch et al. 1992; Melan et al. 1993; Veronesi et al. 1996).
In support of the putative role of POTLX-3 in the HR, we showed that its mRNA accumulation was induced by infiltration with the incompatible bacterial pathogen *P. syringae* pv. *phaseolicola*. The greatest level of induction preceded the visual appearance of hypersensitive necrotic lesions, and this is consistent with putative POTLX-3 involvement in the development of the HR. Lending support to this hypothesis is a report on the phenotype of transgenic tobacco plants in which expression of a specific pathogen-inducible LOX1 gene was suppressed by an antisense construct (Rance et al. 1998). The resulting decrease of LOX activity was sufficient to abolish the HR and resistance of tobacco against *Phytophthora parasitica* var. *nicotianae*. Because POTLX-3 protein shared the greatest sequence similarity (about 90%. Table 1) with this tobacco gene and the pattern of mRNA accumulation is similar (Veronesi et al. 1996), we propose that POTLX-3 may have a similar function in potato.

An important result of this study is that POTLX-3 likely is involved specifically in defense against pathogens but not wounding. The picture that emerges is that some LOX isozymes may operate in both wound- and pathogen-induced defense signal transduction pathways (Melan et al. 1993), whereas others, like POTLX-3 and tobacco LOX1 (Veronesi et al. 1996; Rance et al. 1998), may have a specialized function in pathogen-induced defense responses only. Two related but distinct isozymes of 3-hydroxy-3-methylglutaryl-coenzyme A reductase have been shown to function in separate wound- and pathogen-responsive pathways that produce different final isoprenoid compounds (Choi et al. 1994). Such isozyme specialization in diverse defenses likely involves a different network of signalling molecules (Lee et al. 1996; Penninckx et al. 1996). Wound-induced pathways seem to require ABA and JA as signals (Pena-Cortes et al. 1995), whereas some of the pathogen-induced
defense reactions are mediated by JA and ethylene but not by ABA (Lee et al. 1996; Xu et al. 1994). Interestingly, ABA has been shown to induce susceptibility and to inhibit arachidonic acid induction of both the hypersensitive response and LOX activity in potato tubers (Bostock et al. 1992; Henfling et al. 1980).

Because POTLX-3 mRNA maximal accumulation preceded the onset of HR caused by both incompatible pathogens P. infestans and P. syringae, we hypothesize that the POTLX-3 isozyme may be specifically involved in ethylene- and MJ-mediated development of the HR. One possible mechanism for POTLX-3 isozyme involvement in the HR can be the production of free radicals and reactive oxygen species associated with LOX activity that leads to cell membrane damage (Croft et al. 1990; Keppler and Novacky 1987). Other potential functions of POTLX-3 in disease resistance could be associated with the production of antimicrobial substances (Croft et al. 1993; Kato et al. 1986) or signalling molecules such as JA and MJ capable of inducing defense-related genes. The POTLX-3 isozyme may have a specific function in potato-P. infestans interactions by mediating the elicitor activity of arachidonic and eicosapentaenoic acids. These two P. infestans-derived elicitors are LOX substrates, and they induce the HR and phytoalexin accumulation due to LOX action on them (Bostock et al. 1992; Castoria et al. 1992; Preisig and Kuc 1987). Consistent with this function, potato LOX converted arachidonic acid into highly reactive intermediate 5-S-hydroperoxyeicosatetraenoic acid (5-S-HPTE) that induced phytoalexin accumulation to much greater levels than the elicitor itself (Castoria et al. 1992). To determine unequivocally whether POTLX-3 is essential for HR development and plant resistance mechanisms, it will be necessary to suppress its expression in transgenic plants. Such mutant plants also would provide a valuable tool to study the biochemical mechanisms of LOX involvement in defense responses against pathogens.
MATERIALS AND METHODS

Plants, wounding, and hormonal treatments of leaves.

Potato (*Solanum tuberosum* L.) plants were grown from seed tubers in a greenhouse at 20 to 22°C under a 16-h daylength. Plants five to six weeks old were used for all experiments. Cultivar Superior was used for studies of organ-specific expression, wounding, and hormone treatments. The fourth, fifth, and sixth fully expanded leaves were used for wounding experiments, and leaflets were wounded by crushing the lamina between veins with a hemostat. Leaf-petiole cuttings were used for hormone treatments, and they were incubated with the cut end placed in 100 ml of either water, 100 μM abscisic acid (ABA), 100 μM methyl jasmonate (MJ), 100 μM gibberellic acid (GA₃), 50 μM auxin (NAA), or 100 μM cytokinin (BA). For ethylene treatment, the cut ends of leaf-petiole cuttings were kept in water and incubated in hermetically sealed 4 L jars that contained 10 μL/L ethylene. Treatment with MJ (a volatile compound) was conducted in hermetically sealed 4 L jars. All treatments were incubated under constant light at 22°C, and treated leaves were harvested after 6, 12, 24, and 48 h, frozen in liquid N₂, and stored at -80°C. Each treatment contained 5 leaf-petiole cuttings, and each part of the experiment was conducted at least two times. All chemicals were purchased from Sigma (St. Louis, MO) except MJ, which was purchased from Bedoukian Industries (Danbury, CT).

Pathogens, inoculum production, and inoculation methods.

‘Superior’ and ‘Kennebec’ (resistance gene R-1) were used for inoculations with *Phytophthora infestans*. *P. infestans* strains ME93-2A (US Genotype US8, Mating Type A2, compatible on ‘Kennebec’ and ‘Superior’) and US940507 (US Genotype US1, Mating Type A1, incompatible on ‘Kennebec’ and compatible on ‘Superior’) were obtained from Dr. W. E. Fry, Department of Plant Pathology, Cornell University. The fungi were grown on Rye B
Agar for two weeks at 22°C for sporangia production, and the sporangia were harvested by washing the plates with sterile water (Erwin and Ribeiro 1996). Sporangial suspensions were diluted to approximately 10,000 sporangia per ml. Inoculations were performed by using a detached leaflet assay. Recently expanded leaflets from the fourth, fifth, and sixth leaves from the plant apex were placed in petri dishes that contained water agar. Droplets (10 µl) of the sporangial suspension were distributed evenly over the abaxial surface of the leaflets.

Inoculated leaflets were incubated in dark overnight in a growth chamber at 18°C and then maintained in light at 18°C for the remainder of the incubation period. Samples were taken after 6, 12 h and 24 h, and at 24-h intervals for 7 days after inoculation, frozen in liquid N₂, and stored at -80°C.

For infiltration with the incompatible bacterial pathogen, *Pseudomonas syringae pv. phaseolicola*, the fourth, fifth, and sixth fully expanded leaves of 'Superior' plants five weeks old were used. For inoculum production, cells were harvested from bacterial cultures grown on plate count agar (Difco) at 22°C for 48 h. Cells were suspended in sterile distilled water at a final concentration of 1.2 X 10⁸ CFU ml⁻¹. The abaxial surface of the leaflets was infiltrated with this bacterial suspension by using a hypodermic syringe with a short piece of latex tubing attached. Infiltration was conducted by forcing the liquid into the intercellular spaces through the stomates. Plants with infiltrated leaves were incubated for 1, 3, 6, 12, 18, or 24 h in the greenhouse. Control leaves were infiltrated with sterile water (mock-inoculated). After each incubation time, entire leaflets were collected and frozen immediately in liquid N₂ and stored at -80°C until used for RNA extractions.

**Isolation and analysis of POTLX-3 cDNA clone**

A lambda gtl 1 cDNA library constructed from mRNA extracted from ABA-treated potato leaves (Hildmann et al. 1992), a generous gift from Dr. Salome Prat (Center of Research and Development, Barcelona, Spain), was screened for LOX cDNAs by using a nick-translated
0.85-kb potato LOX fragment. To obtain a potato LOX fragment to use as a probe for screening this library, RT-PCR was conducted with primers based on regions of low degeneracy from known plant LOX sequences. Two PCR primers (5'-CAGCCATATCTCCCAAGTGAA and 5'-TCTCGAGGCATATGTTTT) were synthesized at the DNA Synthesis Facility, Iowa State University. Template cDNA for PCR was obtained by reverse transcription of total RNA extracted from wounded potato leaves. Wounded leaves were used because some plant LOXs can be induced by both wounding and pathogens (Melan et al. 1993). PCR amplification of this cDNA produced one band of around 850 bp that is an expected size based on known plant LOX sequences. This product was cloned into a pCR1 TA cloning vector (Invitrogen, San Diego, CA), sequenced, and identified as a potato LOX fragment due to high sequence identity with other plant LOX genes.

This fragment was used as a heterologous probe to screen the library under low stringency conditions (40% formamide hybridization solution, hybridization carried out at 40°C, and washing at 58°C). The remainder of the screening procedure has been described previously (Polking et al. 1995). Four putative LOX cDNA clones were isolated from more than 500,000 pfu screened. Two of these were approximately 2.8 kb, the expected size of a full-length plant lipoxygenase cDNA. These longest two clones were subcloned into pGEM®-1 lZf(+) (Promega Corp., Madison, WI). Partial sequencing and sequence analysis indicated that they were identical. Therefore, only one of these clones, pABA-1, was sequenced completely by using automated dideoxy sequencing at the Nucleic Acid Facility, Iowa State University. The clone was designated POTLX-3 (Kolomiets et al. 1996b). The amino acid sequence comparison was conducted by using the GAP and FASTA programs of the Genetics Computer Group (GCG) of the University of Wisconsin (Madison, WI). The predicted subcellular localization of POTLX-3 protein was determined by the PSORT protein-sorting program (Nakai and Kanehisa, 1992), available on the Nakai server (http://psort.nibb.ac.jp).
Genomic Southern Analysis

Genomic DNA was extracted from ‘Superior’ leaves according to the CTAB (cetyltrimethylammonium bromide) method (Rogers and Bendich, 1985). Ten micrograms of genomic DNA was digested with restriction endonucleases, electrophoretically separated on a 0.9% agarose gel, denatured, and transferred to a MagnaGraph nylon membrane (Micron Separations Inc., Westboro, MA). Membranes were hybridized at high stringency in a 50% formamide hybridization buffer at 42°C for 48 h. The hybridization buffer was 50% formamide, 6X SSC (1X SSC: 0.15 M NaCl, 0.015 M Na-citrate), 3.3X Denhardt’s solution (1X Denhardt’s: 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 25 mM Na-phosphate buffer (pH 7.0), 0.115 mg/ml salmon sperm DNA. Membranes were washed in 2X SSC, 0.1% SDS at 23°C for 10 min, 1X SSC, 0.1% SDS at 65°C for 30 min, followed by 0.1X SSC, 0.1% SDS at 65°C for 30 min. For autoradiography, membranes were dried and exposed to X-ray film with intensifying screens for six days at -80°C. We used a ^{32}P-labeled 1.4 kb EcoRI fragment of the POTLX-3 cDNA clone as the hybridization probe for Southern and northern analyses, and we refer to it as the POTLX-3 probe. The POTLX-3 probe did not cross-hybridize to POTLX-1 or POTLX-2 sequences (data not shown), the two other LOX cDNA clones that we have isolated from potato tubers (Kolomiets et al. 1996a).

Northern analysis

Total RNA was extracted from leaf tissue of ‘Superior’ or ‘Kennebec’ plants according to the phenol/chloroform extraction procedure (Dix and Rawson 1983). Total RNA used for northern blots presented in Figs. 2 and 5 was isolated by using TRI REAGENT® according to the manufacturer’s manual (Molecular Research Center, Inc., Cincinnati, OH). Various amounts of RNA were size-fractionated electrophoretically through a 1% agarose gel that contained 5 mM methyl-mercury hydroxide and transferred onto a MagnaGraph nylon
membrane (Micron Separations Inc., Westboro, MA). Equal loading of RNA samples and uniform transfer onto a nylon membrane was confirmed by visualizing RNA stained with ethidium bromide under UV light. Membranes were hybridized with the POTLX-3 probe under hybridization conditions identical to those described for Southern analysis. Membranes were washed in 1X SSC, 0.1% SDS at 23°C for 15 min. in 0.1X SSC, 0.1% SDS at 23°C for 30 min, followed by a final wash in 0.1X SSC, 0.1% SDS at 65°C for 30 min. For autoradiography, RNA blots were exposed to X-ray film by using intensifying screens for 2 to 4 days. In Fig. 2, a duplicate blot was hybridized to a 32P-labeled p755 cDNA fragment that represented a potato proteinase inhibitor II (Pin2) gene isolated from a tuber cDNA library (Hannapel, unpublished data). This probe was used as a positive control for wounding and hormonal induction. A 1.2 kb wheat 18S ribosomal RNA probe was used to confirm uniform loading of RNA on blots in Figs. 4 and 5. Blots presented are representative examples of at least two independent experiments.

Figure preparation

Photographs taken from autoradiographic images were digitized at a resolution of 141.7 pixels per cm (360 pixels per inch) by using an Arcus II flat bed scanner (AGFA, Wilmington, MA). Images in Fig. 3 were assembled by using Adobe Photoshop 4.0 (Adobe Systems Inc., Mountain View, CA) to combine the lanes from digitized images of separate northern blot gels. Figures were labeled and prepared for final photoimaging by using Power Point 4.0 (Microsoft Corp., Redmond, WA).

ACKNOWLEDGMENTS

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LITERATURE CITED


eicosatetraynoic acid, and disulfiram of hypersensitive resistance elicited by arachidonic acid
or poly-l-lysine in potato tuber. Plant Physiol. 84:891-894.

between Phytophthora parasitica var. nicotianae race 0 and tobacco is suppressed in
USA 95:6554-6559.

Reinbothe, S., Mollenhauer, B., and Reinbothe, C. 1994. JIPs and RIPs: the regulation of
plant gene expression by jasmonates in response to environmental cues and pathogens.
Plant Cell 6:1197-1209.


Rogers, S.O., and Bendich, A.J. 1985. Extraction of DNA from milligram amounts of fresh,
herbarium, and mummified plant tissues. Plant Mol. Biol. 5:69-76.

lipoxygenase in the presence of NADH and NADPH. Biochim. Biophys. Acta 1214:171-
179.

Royo, J., Vancanneyt, G., Perez, A.G., Sanz, C., Stormann, K., Rosahl, S. and Sanchez-
activities and different organ-specific and wound-regulated expression patterns. J. Biol.
Chem. 271:21012-21019.

max (L.) Merr.] leaves. Changes during leaf development, after wounding, and following


Table 1. Comparison of percentage identity and percentage similarity of deduced amino acid sequences of \textit{POTLX-3} gene with those of other plant LOX genes\textsuperscript{a}

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<th>GenBank accession number</th>
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\textsuperscript{a} Percentage identity and percentage similarity were calculated with the GAP program of the Genetics Computer Group (GCG) of the University of Wisconsin (Madison, WI).

\textsuperscript{b} The predicted amino acid sequence of \textit{POTLX-3} was compared with other plant LOX gene products available in the GenBank database: tobacco \textit{LOX1} (Veronesi et al. 1995); \textit{POTLX-1} and \textit{POTLX-2} (Kolomiets et al. 1996a); tomato \textit{tomloxA} (Ferrie et al. 1994); \textit{Arabidopsis AtLoxI} (Melan et al. 1993); barley \textit{LoxA} (van Mechelen et al. 1995); and potato \textit{H1} and \textit{H3} (Royo et al. 1996).

\textsuperscript{c} \textit{POTLX-3} is GenBank accession number U60202.
Figure Captions

Fig. 1. Southern blot analysis of potato genomic DNA with POTLX-3 probe. Genomic DNA (10 µg per lane) was digested with EcoRI (lane E), XbaI (lane X), HindIII (lane H), and PstI (lane P). The membrane was probed with the 32P-labeled 1.5 kb EcoRI fragment of the POTLX-3 cDNA clone. DNA size markers in kilobases are indicated on the left.

Fig. 2. Northern blot analysis of POTLX-3 and Pin-2 mRNA accumulation after wounding potato leaves. Total RNA was isolated from wounded leaves of Solanum tuberosum ‘Superior’ plants harvested 0 to 72 h after wounding. The 0 h RNA sample was extracted from leaves harvested immediately after wounding. RNA samples (18 µg per lane) from wounded leaves were loaded on duplicate gels, and northern blots were probed with 32P-labeled inserts of either POTLX-3 cDNA or proteinase inhibitor II (Pin-2) cDNA. Total RNA (18 µg per lane) from leaves treated with ethylene for 24 h was included as a positive control. Hybridization to the Pin2 probe was conducted as a positive control for wounding. POTLX-3 transcript size is approximately 2.8 kb. Equal loading of RNA samples into each lane was confirmed by visualizing the RNA gel stained with ethidium bromide.

Fig. 3. Northern blot analysis of POTLX-3 mRNA accumulation in potato leaves treated with various phytohormones. Leaf-petiole cuttings were incubated for 6, 12, 24, and 48 h in either water (control), 100 µM ABA, 100 µM MJ, 100 µM gibberellic acid (GA3), 50 µM auxin (NAA), or in hermetically sealed 4 L jars that contained 10 µl/L ethylene. An equal amount of total RNA (12 µg per lane) was loaded in each lane, and the consistency of loading was determined by visualizing RNA under UV light after staining with ethidium bromide. Hybridization was conducted with the 32P-labeled 1.5 kb EcoRI fragment of the POTLX-3
cDNA clone. Blots subsequently were stripped and rehybridized with probe derived from a proteinase inhibitor II \((\text{Pin-2})\) gene.

**Fig. 4.** Northern blot analysis of \(\text{POTLX-3}\) mRNA accumulation in response to \(\text{Phytophthora infestans}\) inoculation. Detached leaflets of ‘Kennebec’ (resistance gene R-1) were inoculated with a sporangial suspension of \(\text{P. infestans}\) US940507 (incompatible interaction resulting in resistance) (A). ‘Superior’ leaflets were inoculated with \(\text{P. infestans}\) ME93-2A (compatible interaction that resulted in susceptibility) (B). Total RNA was extracted from inoculated leaflets that were incubated in a growth chamber at 18°C for the indicated times. An equal amount of total RNA (18 μg per lane) was loaded in each lane, and transferred onto a nylon membrane. The blots were hybridized with the \(^{32}\text{P}-\text{labeled} 1.5\ \text{kb} \text{EcoRI}\) fragment of the \(\text{POTLX-3}\) cDNA clone. The blots were stripped and rehybridized to a 1.2 kb wheat 18S ribosomal RNA probe to confirm that similar quantities of total RNA were present in each lane.

**Fig. 5.** Northern blot analysis of \(\text{POTLX-3}\) mRNA accumulation in response to \(\text{Pseudomonas syringae pv. phaseolicola}\) inoculation. ‘Superior’ leaflets were inoculated with \(\text{P. syringae pv. phaseolicola}\) bacterial suspension \((1.2 \times 10^8 \text{ CFU ml}^{-1})\) (A) or with sterile water (mock-inoculated) (B). Plants with inoculated leaves were incubated for 0, 3, 6, 12, 18, or 24 h in the greenhouse, and total RNA was extracted from the entire leaflet at each time. Blots of total RNA (18 μg per lane) were hybridized with the \(^{32}\text{P}-\text{labeled} 1.5\ \text{kb} \text{EcoRI}\) fragment of the \(\text{POTLX-3}\) cDNA clone. Equal loading of RNA samples into each lane was confirmed by stripping and rehybridizing the blots with a 1.2 kb wheat 18S ribosomal RNA probe.
Fig. 2
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Fig. 3
Fig. 4
Fig. 5
GENERAL CONCLUSIONS

Three full-length cDNA clones that represent distinct LOX genes have been isolated from potato, and their expression pattern at the mRNA level was characterized to explore their possible involvement in tuberization processes and in defense responses against pests and pathogens. The high sequence identity of these cDNA clones to other plant LOXs provided evidence that they were lipoxygenases.

Two of these cDNAs, designated POTLX-1 and POTLX-2, were isolated from a lambda ZAPII cDNA library constructed from mRNA extracted from axillary bud tubers that were 4 days old. They represent closely related (96% nucleotide sequence match) but distinct LOX genes. The open reading frame of POTLX-1 encodes a polypeptide of 861 amino acids with a calculated pI of 5.46. The POTLX-2 cDNA clone encodes a polypeptide of 860 amino acids with a calculated pI of 5.61. We studied the possible involvement of LOX in potato tuberization by characterizing mRNA accumulation patterns of POTLX-1 and POTLX-2 during tuber initiation and growth. Analysis of the deduced amino acid sequence revealed that POTLX-1 and POTLX-2 are members of the potato Loxl gene family that likely encode 9-LOX isozymes that are not targeted to chloroplasts. RNA blot hybridization studies indicated that accumulation of POTLX-1 and POTLX-2 was specific to developing tubers and roots and that mRNA accumulation correlated positively with tuber initiation and growth. Treatment with hormones, pathogen infection, or wounding did not induce POTLX-1 and POTLX-2 mRNA accumulation in leaves. In situ hybridization showed that POTLX-1 transcripts accumulated in the apical and subapical regions of the new tuber and in the vascular tissue of the most actively growing part of the developing tuber. Transcript accumulation was not detected in the part of the new tuber immediately adjacent to the attached stolon. Thus, POTLX-1 transcripts were
localized within the sites of the most active cell division that occurs during tuber initiation. Our results indicate that *POTLX-1* and/or *POTLX-2* may function in controlling tuber initiation and growth.

To study lipoxygenase involvement in defense responses against fungal pathogens, we have isolated and characterized a potato lipoxygenase cDNA from ABA-treated leaves designated *POTLX-3*. The open reading frame of the *POTLX-3* cDNA encodes a polypeptide of 862 amino acids with a calculated molecular mass of 97.8 kilodaltons and a pI of 5.6. *POTLX-3* shares a relatively low sequence identity match with other classes of potato LOX genes, and its expression pattern is unique among plant LOXs. *POTLX-3* transcript accumulation was not detected in untreated, healthy potato organs, but it was induced in leaves treated with ethylene or methyl jasmonate. Neither wounding nor treatment of leaves with ABA, cytokinin, auxin, or gibberellic acid induced *POTLX-3* transcript accumulation. However, *POTLX-3* mRNA accumulation was induced when potato leaves were infected with either virulent or avirulent strains of *Phytophthora infestans*, the causal agent of late blight. During the resistance response, *POTLX-3* was induced within 6 h, and its mRNA continued to accumulate for a week after inoculation. In contrast, when a plant was susceptible to *P. infestans*, mRNA accumulation was induced 3 to 6 days after inoculation. *POTLX-3* also was induced during hypersensitive response development caused by the incompatible pathogen *Pseudomonas syringae* pv. *phaseolicola*, and this indicated that *POTLX-3* expression may be associated with the hypersensitive response. Thus, *POTLX-3* is not involved in wound-inducible defense responses and may be involved specifically in defense responses against pathogen infection.
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