

# A Leaf Lipoxygenase of Potato Induced Specifically by Pathogen Infection<sup>1</sup>

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Lipoxygenase (LOX) activity has been identified consistently during pathogen-induced defense responses. Here we report the involvement of a specific leaf LOX gene of potato (*Solanum tuberosum*), designated *POTLX-3* (GenBank/EMBL accession no. U60202), in defense responses against pathogens. The sequence of *POTLX-3* does not match any other LOX genes of potato and has the greatest match to a tobacco LOX gene that contributes to a resistance mechanism against *Phytophthora parasitica* var *nicotianae*. *POTLX-3* transcript accumulation was not detected in untreated, healthy potato organs or in wounded mature leaves. *POTLX-3* mRNA accumulation was induced in potato leaves treated with ethylene or methyl jasmonate or 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 hours, increased steadily through 24 hours, and its mRNA continued to accumulate for a week after inoculation. In contrast, when a plant was susceptible to *P. infestans*, induction of mRNA accumulation in response to inoculation was inconsistent and delayed. LOX activity assayed during an incompatible interaction in leaves peaked 3 days earlier than during a compatible interaction. *POTLX-3* mRNA accumulation also was induced during hypersensitive response development caused by the incompatible pathogen *Pseudomonas syringae* pv *phaseolicola*. Our results show that *POTLX-3* may be involved specifically in defense responses against pathogen infection.

Lipoxygenases (LOX; 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 (Siedow, 1991; Conconi et al., 1996). The primary products are fatty acid hydroperoxides that are metabolized enzymatically into compounds like traumatin, jasmonic acid (JA), and methyl jasmonate (MJ) (Anderson, 1989; Koda, 1992; Creelman and Mullet, 1997). These compounds have physiological functions (Anderson, 1989; Siedow, 1991) in growth and development, senescence, and wound- and pathogen-induced defense responses (Siedow, 1991).

LOX protein and activity levels were induced in response to mechanical wounding and insect attack (Hildebrand et al., 1988; Saravitz and Siedow, 1995). An increase in LOX activity in response to infection has been reported for several plant-pathogen systems, and LOX activity has been 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; Veronesi et al., 1996). LOX activity also is induced when cell cultures or plants are treated with elicitors. When potato (*Solanum tuberosum*) tuber discs were treated with the fungal elicitor arachidonic acid, the activity of LOX increased almost 2-fold in 0.5 to 3 h after treatment (Bostock et al., 1992). In addition, LOX genes were activated transcriptionally by wounding, pathogens, or their elicitors (Bell and Mullet, 1991; Melan et al., 1993; Peng et al., 1994; Veronesi et al., 1996; Bohland et al., 1997; Fidantsef and Bostock, 1998).

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 (Keppler and Novacky, 1987; Croft et al., 1990; Koch et al., 1992; Rusterucci et al., 1999). 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, HR occurrence is linked tightly to increased activity, protein, or mRNA levels of LOXs (Vaughn and Lulai, 1992; Slusarenko, 1996; Rusterucci et al., 1999). Transgenic studies have shown that expression of a tobacco LOX gene is involved directly in the HR and resistance to *Phytophthora parasitica* var *nicotianae* (Rance et al., 1998). Products of the 9-LOX pathway contributed to the development of hypersensitive cell death induced by an elicitor on tobacco leaves (Rusterucci et al., 1999).

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Identification of specific LOX genes involved in mechanisms of resistance is difficult, however, because multiple LOX isozymes are involved in wound- and pathogen-induced defense responses (Saravitz and Siedow, 1995, 1996; Bohland et al., 1997). For example, several potato cDNA clones that encode distinct LOX isoforms have been identified, and their expression is organ-specific and differentially regulated during tuber development and in response to wounding, pathogen infection, and MJ treatments (Geerts et al., 1994; Casey, 1995; Kolomiets et al., 1996a, 1996b; Royo et al., 1996; Fidantsef and Bostock, 1998). 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 (Bohland et al., 1997). Several potato LOX genes have been identified and implicated in wound-induced defense responses and in tuber development (Geerts et al., 1994; Kolomiets et al., 1996a; Royo et al., 1996), but none are involved specifically in pathogen-induced defense responses. Here we report the specific induction of a novel LOX gene of potato, designated *POTLX-3* (Kolomiets et al., 1996b), during an incompatible interaction with the pathogen *Phytophthora infestans* but not in response to wounding.

## RESULTS

### Characterization of *POTLX-3*

The full-length potato LOX cDNA clone, *POTLX-3* (Kolomiets et al., 1996b), encodes a polypeptide of 862 amino acids with a calculated molecular mass of 97.8 kD. 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 I). *POTLX-3* showed the greatest match (85.5% identity and 89.7% similarity) to tobacco *LOX1* (Veronesi et al., 1995), which 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 two other known leaf LOXs of potato, *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 non-solanaceous LOXs from *Arabidopsis* and barley (Table I).

The predicted *POTLX-3* polypeptide contained the conserved 39-amino acid motif, 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 in-

**Table I.** Comparison of percentage identity and percentage similarity of deduced amino acid sequences of *POTLX-3* gene with those of other plant LOX genes

Plant LOX Gene <sup>a</sup>	GenBank Accession No.	POTLX-3 <sup>b</sup>	
		Identity (%)	Similarity (%)
Tobacco, <i>LOX1</i>	X84040	85.5	89.7
Potato, <i>POTLX-2</i>	U60201	79.1	85.3
Tomato, <i>tomLoxA</i>	U09026	78.9	84.7
Potato, <i>POTLX-1</i>	U60200	77.9	84.1
<i>Arabidopsis</i> , <i>AtLox1</i>	L04637	69.0	76.0
Barley, <i>LoxA</i>	L35931	59.3	68.9
Potato, <i>H3</i>	X96406	46.1	57.1
Potato, <i>H1</i>	X96405	43.1	54.2

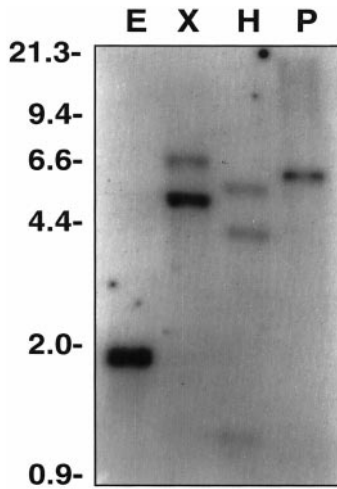
<sup>a</sup> The predicted amino acid sequence of *POTLX-3* (GenBank accession no. U60202) was compared with other plant LOX gene products available in the GenBank database: tobacco *LOX1* (Veronesi et al. 1995); *POTLX-1* and *POTLX-2* (Kolomiets et al. 1996a); tomato *tomLoxA* (Ferrie et al. 1994); *Arabidopsis AtLox1* (Melan et al. 1993); barley *LoxA* (van Mechelen et al. 1995); and potato *H1* and *H3* (Royo et al. 1996). <sup>b</sup> Percentage identity and percentage similarity were calculated with the GAP program of the Genetics Computer Group of the University of Wisconsin (Madison, WI).

involved in the accommodation of the fatty acid substrate (Steczko et al., 1992; Boyington et al., 1993). Like other plant 9-LOXs (Royo et al., 1996; Heitz et al., 1997), *POTLX-3* contains no transit peptide for chloroplast targeting and has the conserved space-saving amino acid pairing at positions 580 to 581 (Hornung et al., 1999). The PSORT protein-sorting analysis (Nakai and Kanehisa, 1992), which predicts subcellular localization of proteins, indicated peroxisomes and/or the cytoplasm as the most probable sites (48% and 45% certainty, respectively) for *POTLX-3* localization.

Southern hybridization of potato genomic DNA was performed to evaluate the complexity of *POTLX-3*-related genes in the potato genome. Under the hybridization conditions used, the *POTLX-3* probe did not cross-hybridize with the sequences of two other potato LOXs, *POTLX-1* and *POTLX-2* (Kolomiets et al., 1996a), that share the greatest sequence identity (Table I). Southern analysis revealed one to two bands that hybridized to the *POTLX-3* probe (Fig. 1), indicating 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<sup>+</sup>) RNA isolated from leaves, stems, flowers, roots, tubers, and stolons. *POTLX-3* transcripts were not detected in any of these organs during normal growth and development (data not shown). Although *POTLX-1*, *POTLX-2*, and other members of the potato *Lox1*



**Figure 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 <sup>32</sup>P-labeled 1.5-kb *EcoRI* fragment of the *POTLX-3* cDNA clone. DNA size markers in kilobases are indicated on the left.

multigene family are regulated developmentally during tuber formation (Kolomiets et al., 1996a; Royo et al., 1996), no *POTLX-3* mRNA accumulation was detected in tubers at several stages of development (data not shown). To determine if *POTLX-3* can be induced by wounding, we examined *POTLX-3* mRNA accumulation in mature 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).

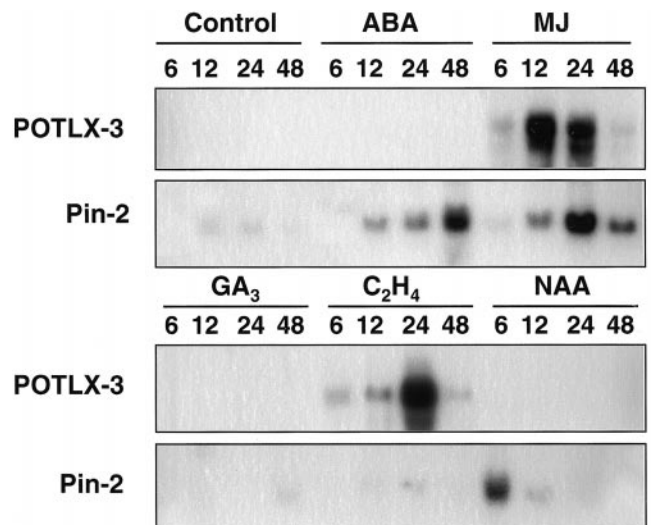


**Figure 2.** Northern-blot analysis of *POTLX-3* and *Pin-2* mRNA accumulation after wounding potato leaves. Total RNA was isolated from wounded leaves of potato cv Superior plants harvested 0 to 72 h after wounding. The 0-h RNA sample was extracted from leaves harvested immediately after wounding. RNA samples (18 µg per lane) from wounded leaves were loaded on duplicate gels, and northern blots were probed with <sup>32</sup>P-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.

Northern analysis also was performed to determine whether *POTLX-3* mRNA accumulation could be induced by phytohormones that affect expression of other plant LOX and defense-related genes. 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 at 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. Induction of proteinase inhibitor-II (*Pin-2*) transcript accumulation was used as a positive control for abscisic acid (ABA), MJ, and NAA treatments (Hildmann et al., 1992).

**POTLX-3 mRNA Accumulation and LOX Activity in Response to Pathogen Attack**

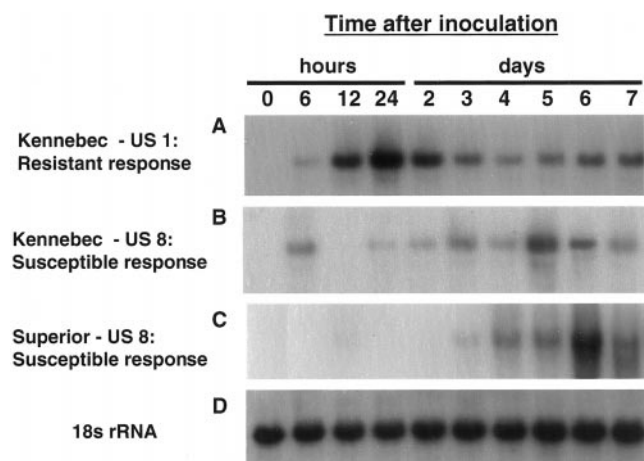
Because LOX activity is induced strongly by inoculation with *P. infestans*, we used this pathogen to study the dynamics of *POTLX-3* expression during the resistance response in potato. To examine *POTLX-3* expression during both compatible and incompatible potato-*P. infestans* interactions, two potato cultivars, cv Superior and cv Kennebec, were inoculated with sporangial suspensions of two *P. infestans* strains, US940507 (US1) or ME93-2A (US8), in a factorial combination. cv Kennebec harbors re-



**Figure 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 (*GA*<sub>3</sub>), 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 <sup>32</sup>P-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 (*Pin-2*) gene.

sistance gene R-1, and cv Kennebec is resistant to US940507 (incompatible interaction) and susceptible to ME93-2A (compatible interaction). Both strains are compatible on cv 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 cv 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 d 2 and 3 postinoculation, transcript levels steadily declined but were detectable from 3 to 7 d. *POTLX-3* transcripts were not detected in mock-inoculated control potato leaves incubated under identical conditions (data not shown). By d 2, when *POTLX-3* transcript accumulation started to decrease, the first symptoms of necrotic lesion development characteristic of the HR were clearly visible. *POTLX-3* mRNA accumulation was also induced early (12–48 h) in leaves during an incompatible interaction in several breeding lines of potato selected for resistance against the late blight US8 type (data not shown).

During compatible interactions, large water-soaked lesions first were observed 4 d postinoculation, and leaves collapsed completely by d 7. In contrast to the resistance response, *POTLX-3* transcripts



**Figure 4.** Northern-blot analysis of *POTLX-3* mRNA accumulation in response to *P. infestans* inoculation. Detached leaflets of cv Kennebec (resistance gene R-1) were inoculated with a sporangial suspension of *P. infestans* US940507 (US1), incompatible interaction resulting in resistance (A), or with ME93-2A (US8), compatible interaction (B). cv Superior leaflets were inoculated with *P. infestans* ME93-2A, compatible interaction that resulted in susceptibility (C). 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  $\mu$ g per lane) was loaded in each lane and transferred onto a nylon membrane. The blots were hybridized with the  $^{32}$ P-labeled 1.5-kb *Eco*RI fragment of the *POTLX-3* cDNA clone. The blots were stripped and rehybridized to a 1.2-kb wheat 18S ribosomal RNA probe (D) to confirm that similar quantities of total RNA were present in each lane.

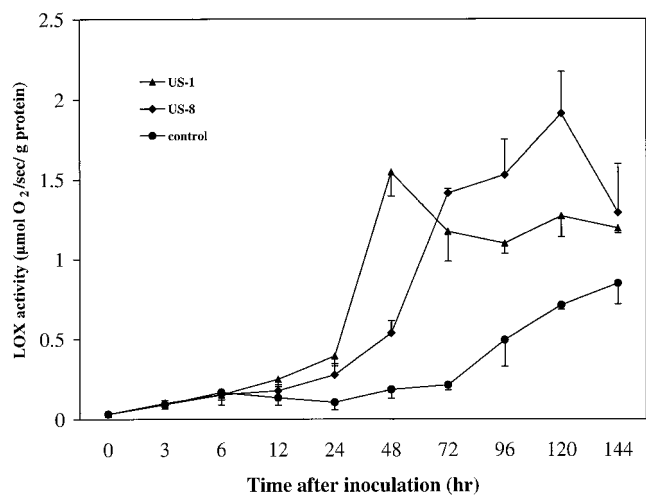
accumulated more slowly or inconsistently during the compatible interaction between cv Kennebec or cv Superior and *P. infestans* ME93-2A (Fig. 4, B and C, respectively). For cv Kennebec, transcript accumulation was inconsistent and delayed with the highest levels detected at d 5 (Fig. 4). For cv Superior, transcript accumulation was detected first at 3 d, reached a maximum at 6 d, and declined at 7 d postinoculation. This overall delayed pattern of *POTLX-3* transcript accumulation was consistent across all compatible interactions observed. Subsequent stripping and rehybridization of the blots to a wheat 18S rRNA gene probe confirmed that RNA loading between samples was comparable (Fig. 4D).

LOX activity was assayed in the same pathogen-infection system used for the RNA analysis. LOX activity in leaves of cv Kennebec inoculated with US1 (incompatible interaction) exhibited a peak at 48 h, whereas in response to inoculation with US8 (compatible interaction), LOX activity was relatively low at 48 h with peak activity occurring 3 d later at 120 h (Fig. 5). The LOX activity assay in cv Kennebec leaves was repeated several times with similar results. The increase in LOX activity during the compatible interaction and in control leaves after 72 h is probably the result of necrosis associated with infection and senescence. Transcript accumulation of two other LOXs of potato leaf, H1 and H3 (Royo et al., 1996), was not induced by pathogen infection (data not shown). Transcripts of *POTLX-1* and -2 are only detected in tubers, stolons, and roots (data not shown).

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 cv 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 non-pathogenic on potato in which it induces development of necrotic lesions typical of the HR. The leaflet areas infiltrated with *P. syringae* pv phaseolicola exhibited symptoms of incompatible-type tissue collapse after 6 h and were completely collapsed by 24 h. The timing of the visual appearance of HR lesions was preceded by pronounced induction of *POTLX-3* mRNA accumulation (Fig. 6A). 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 through 24 h postinoculation. *POTLX-3* transcripts were not detected in control, mock-inoculated potato plants (Fig. 6B).

## DISCUSSION

Correlative evidence exists that strongly implicates LOX activity in potato resistance mechanisms against pathogens (Bostock et al., 1992; Slusarenko, 1996; Weber et al., 1999). Specific pathogen-induced LOXs



**Figure 5.** LOX activity in potato leaflets inoculated with *P. infestans*. Detached leaflets of cv Kennebec (resistance gene R-1) were inoculated with a sporangial suspension of *P. infestans* US940507 (US1, incompatible interaction resulting in resistance, ▲) or with ME93-2A (US8, compatible interaction resulting in susceptibility, ◆). Control cv Kennebec leaflets were inoculated with water (no interaction, ●). LOX activity ( $\mu\text{mol O}_2 \text{ s}^{-1} \text{ g}^{-1} \text{ protein}$ ) in leaves was determined by measuring oxygen consumption in protein extracts from pooled samples by using a Clark electrode for detection (Royo et al., 1996). Linolenic acid was added as a substrate and the pH of the reaction mix was 6.8. Each data point represents the mean of three measurements.

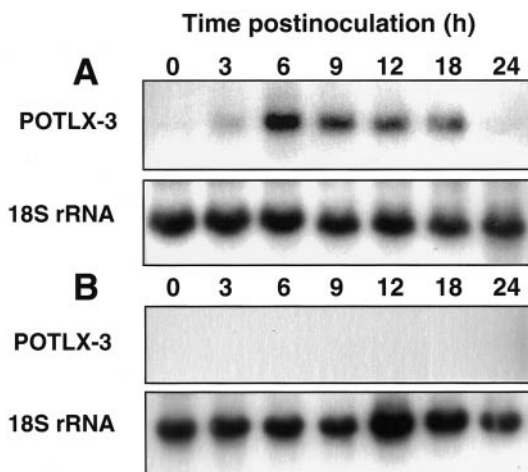
of leaves of potato and tobacco have been implicated in the production of antifungal compounds (Rance et al., 1998; Hamberg, 1999; Rusterucci et al., 1999; Weber et al., 1999), but no potato LOX genes involved specifically in defense against pathogens have been identified. Here we report the characterization of a novel leaf lipoygenase of potato and examine its role in defense responses.

The polypeptide deduced from *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 *POTLX-3* as a lipoygenase. To some extent, the involvement of individual LOX isozymes in physiological processes of plants depends on their subcellular compartmentalization (Siedow, 1991; Stephenson et al., 1998). Among all of the plant LOX sequences available from the databases, *POTLX-3* shared the least amino acid sequence similarity with the chloroplast-targeted LOXs from potato leaves (H1 and H3 in Table I) (Royo et al., 1996), tomato (Heitz et al., 1997), rice (Peng et al., 1994), and Arabidopsis (Bell et al., 1995) (data not shown). The Arabidopsis AtLOX2 isozyme is required for wound-induced JA biosynthesis (Bell et al., 1995), and a similar role has been proposed for other wound- and pathogen-inducible chloroplast-localized LOXs (Peng et al., 1994; Royo et al., 1996; Heitz et al., 1997). 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.

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-1* and *POTLX-2* (Kolomiets et al., 1996a). The Lox2 and Lox3 classes share limited similarity to each other and to the Lox1 isozymes (less than 65%) and are represented by single genes *H1* and *H3*, respectively (Table I; Royo et al., 1996). A comparison of the sequence of *POTLX-3* with the proteins from these three classes (Table I) indicates that *POTLX-3* represents a novel potato LOX gene. The best match (89.7% similarity) to *POTLX-3* is *LOX1* from tobacco, a lipoygenase involved in resistance against black shank via development of the HR (Rance et al., 1998). Southern analysis showed that, unlike potato Lox1 and Lox2 genes that exist as multigene families (Royo et al., 1996; Fidantsef and Bostock, 1998), *POTLX-3* is most likely represented by only one or two genes in the potato genome.

Developmental and inducible regulation of *POTLX-3* mRNA accumulation also was unique when compared with other potato LOXs. The known classes of potato genes showed clear organ-specific expression (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



**Figure 6.** Northern-blot analysis of *POTLX-3* mRNA accumulation in response to *P. syringae* pv *phaseolicola* inoculation. cv Superior leaflets were inoculated with *P. syringae* pv *phaseolicola* bacterial suspension ( $1.2 \times 10^8$  colony forming units  $\text{mL}^{-1}$ ) (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  $\mu\text{g}$  per lane) were hybridized with the  $^{32}\text{P}$ -labeled 1.5-kb *EcoRI* fragment of the *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.

tuber development. Unlike *Lox2* and *Lox3* genes from potato (Royo et al., 1996) and many other plant LOXs (Melan et al., 1993; Heitz et al., 1997), mechanical wounding of mature potato leaves did not induce *POTLX-3* gene expression. These results suggest that *POTLX-3* probably is not involved in the octadecanoid wound-inducible signal transduction pathway that leads to the activation of proteinase inhibitors (Farmer and Ryan, 1992; Royo et al., 1999) or that *POTLX-3* has a very low sensitivity to the wound signal. *POTLX-3* mRNA accumulation was induced in potato leaves after treatment with 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). Arabidopsis defensin genes, which are involved in defense against fungal pathogens, were also induced by MJ and ethylene but not wounding (Penninckx et al., 1996).

LOX activity and mRNA levels increased in response to pathogen challenge or elicitor treatments in a number of plant species (Ohta et al., 1991; Koch et al., 1992; Melan et al., 1993; Peng et al., 1994; Rusterucci et al., 1999). 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, more consistently, and to greater levels during an incompatible interaction. The greatest levels of LOX activity coincided with the appearance of HR lesions 48 h after inoculation. In contrast, during compatible interactions, *POTLX-3* transcript accumulation and LOX activity were delayed and coincided with the occurrence of massive tissue collapse 96 to 144 h postinoculation. In other studies that compared compatible and incompatible interactions, it was observed that LOX mRNA accumulation and activity reach a greater level more rapidly in resistant than susceptible plants (Koch et al., 1992; Melan et al., 1993; Veronesi et al., 1996). In the Veronesi et al. (1996) study with tobacco, during the resistance response, LOX activity peaked at 3 d, whereas in the susceptible response, activity peaked 1 d later. With the incompatible interactions of both *P. infestans* and *P. syringae*, the most abundant accumulation of *POTLX-3* transcripts preceded the formation of necrotic lesions characteristic of the HR, whereas LOX activity coincided with it, suggesting that *POTLX-3* may play a role in localized cell death associated with the HR in potato.

Several reports implicate 9-LOX activity in a causal relationship with the HR. The production of free polyunsaturated fatty acid hydroperoxides dependent on 9-LOX activity was identified during leaf necrosis in tobacco (Rusterucci et al., 1999). In this study, inhibition and activation of the 9-LOX pathway was shown to inhibit or to activate localized cell death. LOX involvement in the HR was further demonstrated in

transgenic tobacco plants in which expression of a specific pathogen-inducible 9-lipoxygenase was suppressed by antisense sequences (Rance et al., 1998). The resulting decrease of LOX activity was sufficient to abolish the HR and reduce the resistance of tobacco against *P. parasitica* var *nicotianae*. Because *POTLX-3* protein shared the greatest sequence similarity (approximately 90%, Table I) with this tobacco gene and the pattern of mRNA accumulation and LOX activity are similar (Veronesi et al., 1996), we propose that *POTLX-3* may have a similar function in potato.

Because the timing of *POTLX-3* transcript accumulation is correlated with the onset of HR lesions during incompatible interactions, it is plausible that *POTLX-3* may have a causal relationship with the development of the HR. Similar to the 9-LOX of tobacco, the *POTLX-3* isozyme may contribute to cell death by the massive production of free fatty acid hydroperoxides that are responsible for tissue necrosis (Deighton et al., 1999; Rusterucci et al., 1999). Free radicals and reactive oxygen species are produced from this pathway that can lead to cell membrane damage (Keppler and Novacky, 1987; Croft et al., 1990). Other potential functions of *POTLX-3* in disease resistance could be associated with the production of antimicrobial substances (Kato et al., 1986; Croft et al., 1993; Hamberg, 1999; Weber et al., 1999) or signaling 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 LOX activity may induce the HR and phytoalexin accumulation in this system (Preisig and Kuc, 1987; Bostock et al., 1992; Castoria et al., 1992). Consistent with this function, a potato LOX converted arachidonic acid into the 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).

Some LOX isozymes operate in both wound- and pathogen-induced defense signal transduction pathways (Melan et al., 1993; Royo et al., 1999), whereas others, like *POTLX-3* and tobacco LOX1 (Veronesi et al., 1996; Rance et al., 1998), appear to have a specialized function in pathogen-induced defense responses only. There is clear evidence that products of the 9-LOX pathway have antimicrobial and cytotoxic properties (Vaughn and Gardner, 1993; Adams et al., 1999; Weber et al., 1999). Hamberg (1999) showed that approximately 95% of the LOX activity in potato leaves produced 9-hydroperoxides of linoleic acid that could be converted into antifungal compounds. Specific local and temporal expression of the LOXs that regulate this pathway would be an efficient means of targeting and controlling pathogen spread early in the development of the disease. Weber et al. (1999) showed that a completely different profile of

oxylipins was produced in potato leaves depending on whether they were infected with *P. infestans* or were wounded. They propose the existence of a 9-LOX pathway in potato leaves, which produces antimicrobial compounds like the divinyl ether fatty acids they detected in infected leaves. It is quite plausible that *POTLX-3* serves this function in the defense against pathogen attack in potato.

## MATERIALS AND METHODS

### Plants, Wounding, and Hormonal Treatments of Leaves

Potato (*Solanum tuberosum*) plants were grown vegetatively from seed tubers in a greenhouse at 20°C to 22°C under a 16-h daylength. Plants 5 to 6 weeks old were used for all experiments. The cv 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  $\mu$ M ABA, 100  $\mu$ M MJ, 100  $\mu$ M gibberellic acid (GA<sub>3</sub>), 50  $\mu$ M auxin (NAA), or 100  $\mu$ M cytokinin (benzyladenine). 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  $\mu$ L/L ethylene. Treatment with MJ 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 N<sub>2</sub>, and stored at -80°C. Each treatment contained five leaf-petiole cuttings, and each part of the experiment was conducted at least two times. All chemicals were purchased from Sigma (St. Louis) except MJ, which was purchased from Be-doukian Industries (Danbury, CT).

### Pathogens, Inoculum Production, and Inoculation Methods

cv Superior and cv Kennebec (resistance gene R-1) were used for inoculations with *Phytophthora infestans*. *P. infestans* strains ME93-2A (U.S. Genotype US8, Mating Type A2, compatible on cv Kennebec and cv Superior) and US940507 (U.S. Genotype US1, Mating Type A1, incompatible on cv Kennebec) were obtained from Dr. W.E. Fry (Department of Plant Pathology, Cornell University). The fungi were grown on Rye B Agar for 2 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. Leaflets recently expanded from the fourth, fifth, and sixth leaves from the plant apex were placed in Petri dishes that contained water agar. Droplets (10  $\mu$ L) 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, and 24 h, and at 24-h intervals for 7 d after inoculation, frozen in liquid N<sub>2</sub>, 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 cv Superior plants 5 weeks old were used. For inoculum production, cells were harvested from bacterial cultures grown on plate count agar (DIFCO Laboratories, Detroit) at 22°C for 48 h. Cells were suspended in sterile distilled water at a final concentration of  $1.2 \times 10^8$  colony forming units mL<sup>-1</sup>. 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, frozen immediately in liquid N<sub>2</sub>, and stored at -80°C until used for RNA extractions.

### Isolation and Analysis of *POTLX-3* cDNA Clone

A  $\lambda$ -gt11 cDNA library constructed from mRNA extracted from ABA-treated potato leaves (Hildmann et al., 1992), a generous gift from Dr. Salomé Prat (Barcelona), 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 approximately 850 bp, which is an expected size based on known plant LOX sequences. This product was cloned into a pCR1 TA cloning vector (Invitrogen, San Diego), 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% [v/v] 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 plaque forming units 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-11Zf(+) (Promega, 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 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 cv Superior leaves according to the cetyl-trimethyl-ammonium bromide method (Rogers and Bendich, 1985). Ten micrograms of genomic DNA was digested with restriction endonucleases, electrophoretically separated on a 0.9% (v/v) agarose gel, denatured, and transferred to a MagnaGraph nylon membrane (Micron Separations, Westboro, MA). Membranes were hybridized at high stringency in a 50% (v/v) formamide hybridization buffer at 42°C for 48 h. The hybridization buffer was 50% (v/v) formamide, 6× SSC (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate), 3.3× Denhardt's solution (1× Denhardt's: 0.02% [v/v] Ficoll, 0.02% [v/v] polyvinylpyrrolidone, and 0.02% [v/v] bovine serum albumin), 25 mM sodium phosphate buffer (pH 7.0), 0.115 mg/mL salmon sperm DNA. Membranes were washed in 2× SSC, 0.1% (v/v) SDS at 23°C for 10 min, 1× SSC, 0.1% (v/v) SDS at 65°C for 30 min, followed by 0.1× SSC, 0.1% (v/v) SDS at 65°C for 30 min. For autoradiography, membranes were dried and exposed to x-ray film with intensifying screens for 6 d at -80°C. We used a <sup>32</sup>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), which are 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 cv Superior or cv Kennebec plants according to the phenol/chloroform extraction procedure (Dix and Rawson, 1983) or by using an RNA isolation kit (TRI reagent, Molecular Research Center, Cincinnati), according to the manufacturer's manual. Various amounts of RNA were size-fractionated via electrophoresis through a 1% (v/v) agarose gel that contained 5 mM methyl-mercury hydroxide and transferred onto a MagnaGraph nylon membrane (Micron Separations). Equal loading of RNA samples and uniform transfer onto a nylon membrane were 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 1× SSC, 0.1% (v/v) SDS at 23°C for 15 min, in 0.1× SSC, 0.1% (v/v) SDS at 23°C for 30 min, followed by a final wash in 0.1×

SSC, 0.1% (v/v) 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 d. In the wound-induction study, a duplicate blot was hybridized to a <sup>32</sup>P-labeled p755 cDNA fragment that represented a potato proteinase inhibitor II (*Pin2*) gene isolated from a tuber cDNA library (D.J. 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 Figures 4 and 6. Blots presented are representative examples of at least two independent experiments.

### LOX Activity Assays

LOX activity was determined as the consumption of oxygen in  $\mu\text{mol s}^{-1} \text{g}^{-1}$  protein by using linolenic acid as a substrate at pH 6.8 and a Clark electrode for detection (Royo et al., 1996). Optimum pH for the reaction mix was determined by monitoring LOX activity at a range of pH values (Royo et al., 1996). Data presented here were the average of three measurements per sample.

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