A Novel Lipoxygenase in Pea Roots. Its Function in Wounding and Biotic Stress


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The genome of pea (Pisum sativum) contains genes encoding a family of distinct lipoxygenases (LOX). Among these, LOXN2 showed eight exons encoding a 93.7-kD enzyme, harboring two C-terminal deletions and an unusual arginine/threonine-tyrosine motif in the domain considered to control the substrate specificity. LOXN2, when overexpressed in yeast, exhibited normal enzyme activity with an optimum at pH 4.5, and a dual positional specificity by releasing a 3:1 ratio of C-9 and C-13 oxidized products. The predicted LOXN2 structure lacked a loop present in soybean (Glycine max) LOX1, in a position consistent with control of the degree of substrate access to the catalytic site and for LOXN2’s dual positional specificity. The LOXN2 gene was tightly conserved in the Progress 9 and MG103738 genotypes, respectively, susceptible and resistant to the root cyst nematode Heterodera goettingiana. LOXN2 transcription was monitored in roots after mechanical injury and during nematode infection. The message peaked at 3 and 24 h after wounding in both genotypes and was more abundant in the resistant than in the susceptible pea. In nematode-infected roots, transcription of several LOX genes was triggered except LOXN2, which was repressed in both genotypes. In situ hybridization revealed that LOXN2 message was widespread in the cortex and endodermis of healthy roots, but specifically localized at high level in the cells bordering the nematode-induced syncytia of infected roots. However, LOXN2 transcript signal was particularly intense in collapsing syncytia of MG103738 roots, suggesting LOXN2 involvement in late mechanisms of host resistance.

Lipoxygenases (LOX; linoleate:oxygen reductase, E.C. 1.13.11.12) are nonheme iron-containing enzymes that catalyze the addition of molecular oxygen at either the C-9 or C-13 residue of fatty acids with a 1,4-pentadiene structure. Linoleic and linolenic acids are the most abundant fatty acids in the lipid fraction of plant membranes and are the major substrates for LOXs. The oxygenation step leads to a reaction cascade (termed the LOX pathway), in which the hydroperoxides (HPOs), produced by the LOX activity, are substrates of HPO lyases and allene oxide synthases (Vick, 1993). HPOs are highly reactive and rapidly degrade into metabolites that are precursors for jasmonic acid, methyl jasmonate, conjugated dienoic acids, and volatile aldehydes. These products are known to play a role in plant defense (Wasternack et al., 1996). The transcription of each gene member is under tight developmental control, and more than one member is often active at a specific developmental stage, accounting for the occurrence of multiple LOX isoforms. These exhibit distinct features for preference of substrate, kinetic parameters, and positional specificity of substrate oxygenation (Feussner and Wasternack, 2002). In pea (Pisum sativum), LOX genes exhibit tissue specificity and are developmentally regulated (Domoney et al., 1990). A few of them have been proposed to be involved in the development of root nodules (Wisniewski et al., 1999) and to play a defensive role in trophic relationships between plants and pathogenic nematodes (Leone et al., 2001).

Phytoparasitic nematodes are the most widespread and deleterious pests for many crops. Several complex mechanisms, which underlie the interaction between the plant and endoparasitic pathogen, induce the differentiation of host cells into feeding structures necessary for parasite development. In particular, cyst nematodes enter the root and migrate to the vascular cylinder where they select a cell that becomes the initial feeding site. Once the parasite has established, this cell is induced to develop into a syncytium as the result of cell-wall breakdown and subsequent fusion of neighboring cell protoplasm. A particularly serious pest of pea is the cyst nematode Heterodera goettingiana,
which is difficult to control due to its long soil persistence and resistance to chemical control (Thompson et al., 2000). In this context, the isolation of genes involved in conferring host resistance represents a potentially valuable approach to improving crop breeding programs.

In the past decade, a considerable number of pea genes responsible for resistance against a range of pathogens, but not nematodes, have been characterized (Chang et al., 1995; Culley et al., 1995; Pilet-Nayel et al., 2002; Prioul et al., 2004; Timmerman-Vaughan et al., 2004). In addition, sources of resistance to H. goettingiana have been found in the germplasm MG103738 accession of pea (Di Vito and Perrino, 1978). Moreover, it has been reported that the nematode was able to infect MG103738 roots and to induce syncytia, but they degenerated very rapidly (Bleve-Zacheo et al., 1990; Melillo et al., 1990). The degradation of trophic cells, indicative of a hypersensitive response (HR), was accompanied by an increased activity of LOXs (Zacheo et al., 1997). The LOX activity was triggered during nematode infection in the susceptible genotype (Progress 9) and in the resistant MG103738, although the levels were remarkably higher in the resistant than in the susceptible roots. In MG103738, the highest level of LOX activity coincided with the occurrence of collapsed syncytia and necrotizing cells (48 h after nematode infection), whereas in Progress 9 it occurred later during syncytial development and was accompanied by a weak increase of HPOs (Leone et al., 2001).

This study focused on the molecular cloning and characterization of LOXN2, a member of the pea LOX family genes, during plant-nematode interaction. LOXN2 represented a novel isofrom in the pea able to produce both C-9 and C-13 HPOs. The gene transcription was down-regulated upon nematode infection in the resistant genotype, exhibiting an inverse trend to that of other LOX family members. Moreover, the message localization in the resistant and susceptible genotypes leads us to hypothesize a role for LOXN2 in the antinematode defense system.

**RESULTS**

**The LOXN2 cDNA, Encoded Protein, and Protein Model Structure**

The full-length cDNA of LOXN2 (EMBL accession no. AJ749702) harbored an open reading frame (ORF) of 2,481 nt encoding a presumptive translation product of 826 amino acids with a M, of 95,787 and a pI of 5.18 (ProtParam tool: Wilkins et al., 1998). The ORF was flanked by a 21-nucleotide (nt) 5’-untranslated region (UTR) and a 148-nt 3’-UTR containing a canonical polyadenylation signal.

The predicted protein product LOXN2 showed 57.4%, 57.1%, and 58.2% identity and 84.4%, 85.6%, and 84.9% similarity to pea LOX1 (EMBL accession no. AAB71759), LOXN4 (EMBL accession no. CAC04380), and LOXN5 (EMBL accession no. CAA75609), respectively. LOXN2 contained all the functional domains typical of LOXs (Fig. 1): an N-terminal region forming a β-barrel structure (Boyington et al., 1997), shown to target lipid body LOX to liposomes and lipid bodies (May et al., 2000), and the catalytic site in the C-terminal domain. Moreover, a search for intracellular sorting and processing peptides in the predicted amino acid sequence of LOXN2 (pSORT program; http://psort.im.s.u-tokyo.ac.jp/) suggested that LOXN2 is probably confined to the cytosol because it contained neither consensus targeting nor retention signals for any organelles.

A stretch of approximately 50 amino acids (positions 1–52) in the N-terminal region differed from those of other pea LOXs. However, a high degree of similarity was observed in the following region, spanning over 100 residues, which is predicted to be a β-barrel structure by standard secondary structure analysis programs (e.g. National Center for Biotechnology Information conserved domain search) and three-dimensional molecular modeling (see below). In the C-terminal region, LOXN2 has two deletions of six and 27 amino acids as compared to the stretches 277 to 281 and 296 to 322 of the soybean (Glycine max) LOX1 (Swiss-Prot accession no. P08170), which is one of the best characterized among LOXs. A molecular model of LOXN2 was produced by homology modeling using five templates for crystal structures of LOX proteins from another legume, soybean (Fig. 2).

The deletions located in the domain III (Fig. 1) lead to the absence of an external loop, but did not change the position and conformation of the β-barrel and the catalytic site of LOXN2 (Fig. 2). The residues involved in iron binding (His-486, His-491, His-677, Asn-688, and Ile-826) were conserved in domain V. The positional specificity of plant LOXs is usually determined by the motifs R/TH or R/TF for 13-LOX and R/TV for 9-LOX in the active site (Feussner and Wasternack, 2002). Unusually, LOXN2 contained a Thr-Tyr motif (R/TY; Fig. 1), which has not been described previously to our knowledge.

A phylogenetic tree (Fig. 3) was constructed based on gap-free multi-alignments of dicot and monocot LOX sequences currently available. Pea LOXN2 fell into the highly supported monophyletic group of leguminous species. However, its closest relative was not the other previously identified pea LOX (LOXN5) but lentil (Lens culinaris) LOX (0.22 substitutions/site). Indeed, within leguminous species, two well-supported clusters were found, each containing a different pea LOX sequence. Interestingly, none of the closely related LOX showed the deletions described for the pea LOXN2.

**Biochemical Activity of Recombinant Yeast LOXN2**

To study the biochemical function and to test whether the lack of a loop in the C terminus affected its activity, LOXN2 was cloned in the expression vector
pPIC9 and over-expressed in Pichia pastoris (strain GS115). In induced strains (see “Materials and Methods”), LOXN2 was represented by a strong band of expected size (approximately 94 kD) in both silver-stained polyacrylamide gels and western blots, whereas no signal was revealed in control strains harboring the empty vector (Fig. 4).

The biochemical activity of recombinant LOXN2 was investigated in extracts of yeast expressing the protein (Table I) by measuring the increase of A

        234

using linoleic, linolenic, and arachidonic acids as substrates. The optimum pH was determined for each substrate by varying the pH of the reaction buffers. The activity of LOXN2 was detected with all the substrates in a range of pH 4.5 to 8.0. In particular, at pH 4.5, the maximum activity of 1.35 units/mg protein occurred with linoleic acid, whereas it fell to 0.23 units/mg protein and 0.18 units/mg protein with linolenic and arachidonic acids, respectively. However, at pH 6.0, the maximum activity was 0.45 units/mg protein with arachidonic acid, followed by 0.18 units/mg protein and 0.07 units/mg protein with linolenic and linoleic acids, respectively. The extracts from controls did not show any activity.

Plant LOXs are usually classified as 9-LOXs and 13-LOXs on the basis of their product specificities. As pea LOXN2 was found to contain the unusual R/TY motif in the active site, its positional specificity was tested. Linoleic acid was selected as substrate in 0.1 M sodium acetate buffer at pH 4.5 and 0.1 M sodium phosphate buffer at pH 6.0. The reaction products were reduced with sodium borohydride and separated by reverse-phase HPLC. The peak absorbing at 234 nm and containing the HPOs was collected and subjected to straight-phase HPLC to separate 13- and 9-hydroxy octadecadienoic acid (HODE). The retention times of LOXN2 reaction products were consistent with both authentic standards of 9- and 13-HODE and those of soybean LOX1. In the presence of both buffers, LOXN2 produced 9- and 13-HODE in a 3:1 ratio (Table I).

Genomic Organization

Genomic DNA of MG103738 and Progress 9 was endonuclease restricted, size fractionated, transferred onto a nylon membrane, and hybridized with a digoxigenin (DIG)-labeled genomic DNA probe spanning...
LOXN2 transcript detected by semiquantitative reverse transcription (RT)-PCR (Fig. 6, A and B). In unwounded plants, the variation of LOXN2 expression appeared to be constant and the message abundance at 1 h was recorded as control. In both genotypes the transcript showed a peak level within 3 h, followed by a decrease at 6 h and a further growth at 24 h after injury. Within 3 h, the transcript in Progress 9 was 9-fold higher, whereas in MG103738 it was 15-fold higher than in the respective controls. The response to wounding was therefore more intense in MG103738 than in Progress 9, suggesting a different reactivity between the two genotypes. Moreover, during the

the two- to six-exon region (Fig. 5A). This probe, which lacked 105 bp compared to other pea LOX members, was designed for LOXN2 specificity. The resulting pattern of hybridization (Fig. 5B) consisted of a single band with all the restriction enzymes used (which did not cut in the probe) and was identical in both genotypes. The sizes of DNA fragments detected were consistent with those predicted from the restriction map of genomic sequences. These results suggest the occurrence of one LOXN2 copy and a very high degree of identity in both genotypes.

To search for introns, PCR experiments were performed on genomic DNA with primer combinations designed along the full-length transcript. The sequence comparison confirmed that LOXN2 was identical in both genotypes and contained eight exons and seven introns (Fig. 5A). The exon positions were conserved with respect to pea LOXN5 (GenBank accession no. Y15410), in which the exons 6 and 7 were separated by an intron that is absent in LOXN2 (Fig. 5A). Moreover, LOXN2 introns were rich in A/T nucleotides (73%) and harbored the canonical splicing GT/AG motifs.

LOXN2 Expression in Response to Mechanical Injury

Pea LOXs represent a gene family with a discrete polymorphism in the 3’ ends among the members. Therefore, a specific probe was designed in the 3’-UTR (Fig. 5A) to monitor LOXN2 expression in roots under abiotic and biotic stresses. Because nematodes invade host roots by cell perforation using their stylet, changes in LOXN2 gene transcription following mechanical injuries were examined.

The root elongation zone and apices of 1-week-old MG103738 and Progress 9 seedlings were needle punctured, collected at different time intervals, and

Figure 2. Model of LOXN2 structure. Shown are a homology model of pea LOXN2 (right) created from high-resolution crystal structures of similar proteins from legumes, as described in “Materials and Methods,” and the crystal structure of soybean LOX1 for comparison (left). The distinctive β-rich region is highlighted at the top left of each structure, and the external loop that is present in soybean LOX1 but absent in pea LOXN2 is shown space-filled.

Figure 3. Phylogenetic tree of LOXN2. Pea LOXN2 amino acid sequence was compared to other monocot and dicot LOXs available in the EMBL database (accession nos. are shown in parentheses). Amino acid sequences were multialigned with ClustalW and edited within PILEUP program where necessary (1994 GCG program manual). A nucleotide multialignment was also created based on the result of the optimized amino acid multialignment. The phylogenetic relationship between sequences was established using the following procedures. Genetic distances were obtained from the multaligned nucleotide sequences using the GTR method implemented in PAUP (Swofford, 1998). The resulting distance matrix was used for tree reconstruction using both the neighbor-joining (NJ) and minimum evolution (ME) approaches. In addition to distance methods, we have also applied a discrete method, Maximum Parsimony (MP), to the multialigned protein sequences using PAUP (Swofford, 1998). In all cases, monocot species were used as outgroups and bootstraps values (listed at the branching point) were based on 1,000 repetitions. The lengths of the branches in the tree are proportional to the genetic distance, according to the scale at the bottom.
decrease phase, LOXN2 transcript abundance in MG103738 was 10 to 8 times higher than in the control, while in Progress 9 it fell down to the level of its control. LOXN2 message levels in MG10378 at 24 h were 16-fold higher than in the control, whereas the message abundance in Progress 9 was 8 times higher than in the unwounded control samples. Taken together, these data suggest that LOXN2 maintains a higher grade of expression in MG103738 than in Progress 9.

Global LOX Transcription Is Triggered in the Infected Resistant Genotype, But LOXN2 Is Repressed

Transcript abundance was monitored in nematode-challenged roots of MG103738 and Progress 9 genotypes and compared to uninfected root tissues. Northern analyses were performed at 24 and 48 h after H. goettingiana infection using two distinct probes (Fig. 5; see also “Materials and Methods”): one contained a highly conserved stretch in the ORF and was used to detect a message pool from LOX members (Fig. 7A), and the other was specific for LOXN2 (Fig. 7B). As for plants left uninfected, LOX levels were observed to vary with time, suggesting a regulation during root development. Hence, it was considered that the comparison of transcript levels between infected and uninfected roots, at a specific time point, had a biological meaning in the responses to nematode infection elicited in the different host genotypes. The global expression level of LOXs (Fig. 7A), which did not vary significantly between resistant and susceptible peas at 24 h, dropped in the susceptible and increased in the resistant genotype 48 h after nematode infection compared to uninfected roots. On the contrary, LOXN2 transcript (Fig. 7B), 24 and 48 h following nematode infection, was down-regulated in both infected resistant (0.6- and 0.8-fold versus control) and susceptible roots (0.79- and 0.8-fold versus control).

Localization of LOXN2 Message upon Nematode Infection

To determine the spatial expression pattern of LOXN2 during nematode infection, in situ experiments were conducted on serial cross sections of uninfected and 48 h infected roots. Sense and antisense riboprobes spanning the LOXN2 3′-UTR were used (Fig. 5A). In uninfected roots, the transcript was visualized in cortical and endodermal cells as a purple staining, which appeared more intense in the resistant than in the susceptible genotype (Fig. 8, A and B). In resistant infected roots, faint and infrequent signal spots were observed in the cortex, but LOXN2 message was abundant in the outer cells surrounding the vascular cylinder, in those cells injured by nematode penetration and those flanking the induced syncytia (Fig. 8, C and E). In susceptible infected roots, the transcript was detected in cortical cells damaged by nematode invasion and undergoing necrosis, and in parenchymatous cells of the vascular cylinder strictly related to syncytia development (Fig. 8D). In MG103738 roots, syncytia rapidly degenerated and were highly reactive to the probe due to the condensed cytoplasm, which is a feature of collapse (Fig. 8, E and D). No LOXN2 mRNA was detected in infected and uninfected root sections when hybridized with a sense probe (Fig. 8, G and H).

Table 1. Substrate and product specificity of pea 9-/13-LOXN2

All activities were determined in 0.1 M sodium acetate buffer, pH 4.5, at 25°C with 0.3 mM substrate measuring the increase of A234. The relative rate (%) of hydroperoxidation with respect to linoleic acid hydroperoxidation is indicated in parentheses.

<table>
<thead>
<tr>
<th>Property</th>
<th>Substrates</th>
<th>Linoleic Acid (18:2)</th>
<th>Linolenic Acid (18:3)</th>
<th>Arachidonic Acid (20:4)</th>
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<tbody>
<tr>
<td>Substrate specificity</td>
<td>Specific activity (μmol/min per mg of protein)*</td>
<td>1.35 ± 0.02 (100%)</td>
<td>0.23 ± 0.04 (17%)</td>
<td>0.18 ± 0.03 (13%)</td>
</tr>
<tr>
<td>Product specificity</td>
<td>Ratio of positional isomers of HPOs (13-HPOD:9-HPOD)</td>
<td>1:3</td>
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*Data are mean ± se from three independent experiments.

Figure 4. Protein-blot analysis and SDS-PAGE of LOXN2. A, Crude protein extracts (10 μg) from P. pastoris GS115 transformed either with pPIC9 (control plasmid) or with pPIC9/LOXN2 were separated on SDS-PAGE gels, blotted, and probed with anti-LOX antibody. B, Silver-stained SDS-PAGE gel of pPIC9 and pPIC9/LOXN2 extracted from yeast cultures. Positions of Mr marker proteins are as indicated (kD).
DISCUSSION

In the genome of the pea, the LOX genes encode a family of enzymes, and those expressed in roots have been proposed to play a role in defense mechanisms against cyst nematodes (Leone et al., 2001). In this report, we focused on the structure, the transcription pattern, and localization of LOXN2 in response to the cyst nematode H. goettingiana and the biochemical properties of the enzyme. The unusual structural features of LOXN2 are, surprisingly, not reflected in an alteration of the biochemical activity of the enzyme.

The Unusual Structure of LOXN2 Does Not Affect the Enzyme Function and Confers Dual Positional Specificity

Analysis of the LOXN2 coding sequence showed that the predicted protein contains all of the domains typical of LOX enzymes. However, a few peculiarities distinguished LOXN2 from most of the plant LOXs, which are usually organized into a small N-terminal β-barrel and a large C-terminal catalytic domain (Boyington et al., 1993). In contrast, Minor et al. (1996)
proposed to configure LOXs into four small domains that associate on the surface of a large C-terminal domain. The latter harbors the catalytic site, whereas the other domains are proposed to facilitate binding, transport, and release of both substrates and products. Modeling (Swissmodel) of pea LOXN2 against the crystal structure of soybean LOX1 (Protein Data Bank [PDB] accession no. 1FGM) indicated that LOXN2 lacked an external loop, lost due to two deletions of six and 27 amino acids in the domain III. This loss did not cause any conformational change in the β-barrel domain and the catalytic site of the protein. LOXN2 maintained an intact LOX-like activity after being expressed in *P. pastoris*, and the optimal pH of activity...
had a much lower value (4.5) than that of other pea LOXs (Hughes et al., 1998). Other LOXs with acidic pH optima have been described in rice (Oryza sativa; Ida et al., 1983), soybean (Baracat-Pereira et al., 2001), carnation (Dianthus caryophyllus; Rouet-Mayer et al., 1992), and rose (Rosa hybrida; Fukuchi-Mizutani et al., 2000). A role of acidic LOXs in the senescence of carnation and rose flowers has been suggested to be the result of membrane disruption by the HPO and free radical action (Rouet-Mayer et al., 1992; Fukuchi-Mizutani et al., 2000).

Plant LOXs are classified into 9- and 13-LOXs according to the position at which the oxygenation of linoleic acid occurs. Most of the plant LOXs harbor a tightly conserved Arg in the substrate-binding pocket. In 13-LOXs of several plants, the occurrence of His or Phe has been proposed to mask the positive charge of Arg and to hamper the interaction with the linoleate carboxylate. This should favor the entry of the linoleate methyl terminus and the C-13 oxidation (Hornung et al., 1999). In contrast, in 9-LOXs the His and Phe are replaced by a Val, which is thought thereby to allow the inverse orientation of linoleate (Feussner and Wasternack, 2002). However, the positional specificities of some plant LOXs (e.g., those of monocots) do not fit this predictive model and a class of nonconventional LOXs was therefore proposed (Feussner and Wasternack, 2002). Moreover, Hughes et al. (2001) reported that the mechanisms controlling dual positional specificity (DPS; Hughes et al., 1998) in pea 9-/13-LOX and cucumber (Cucumis sativus) 13-/9-LOX were different. DPS of pea LOX was more likely to be determined by the degree of penetration of the methyl terminus and the volume of the linoleate binding pocket rather than substrate orientation. As for pea LOXN2, the common His, Phe, or Val residues were replaced by a Tyr, which is without precedent among plant LOXs. Tyr is of a similar size to His and Phe, and this might lead to a 13-LOX activity. Surprisingly, LOXN2 showed a DPS by releasing 9- and 13-HPOs in a 3:1 ratio. DPS was also observed in pea LOX-2 and LOX-3 (Hughes et al., 1998), but they retain the Phe residues. Consequently, our findings are consistent with the hypothesis that the conformation of domain III of LOXN2 may have a role in determining DPS by controlling the degree of linoleate access into the catalytic site, in agreement with Hughes et al. (2001). Finally, the LOX specificity has implications for HPO metabolism; 13-HPO of linolenic acid is converted into jasmonate by allene oxide synthase, whereas the HPO lyase acts on 9- and 13-HPOs to form volatile aldehydes. In this regard, the DPS of LOXN2 suggests that it is able to provide compounds that play a role in both developmental process and defense response (Siedow, 1991).

**LOXN2 Response to Wounding and Nematode Infection**

Accumulation of LOXN2 transcripts over their normal basal level was induced by wounding in both resistant and susceptible genotypes. The LOXN2 response to wounding was remarkably rapid; maximum transcript levels were reached within 3 h after treatment and they were transient and biphasic. The resistant genotype reacted more strongly than the susceptible genotype in increasing LOXN2 expression to wounding. A similar kinetic expression of LOX with DPS has been reported in maize (Zea mays) in response to exogenous methyl jasmonic acid treatment (Kim et al., 2003). These authors suggested that the activation of LOX expression in the early phase of a wound response involved 13-LOX activity that was in turn followed by its 9-LOX activity at the late phase. Together with these previous findings, our results suggest that LOXN2 expression in pea genotypes might exhibit a similar behavior to nonconventional maize LOX. LOXN2 seems to be rapidly involved in the oxidative burst elicited by mechanical cell damage, thereafter coming back to its normal function in tissue development. In pea, LOXN2 transcript levels were higher in resistant than in susceptible genotypes at all time points tested. The exon-intron structure, the sequence, and the copy number (one) of LOXN2 were identical in both genotypes. Therefore, the difference of expression levels is likely due to divergences in transcription control mechanisms (e.g., distinct epigenetic factors, promoter/enhancer sequences, repressors, signal transducers, etc.).

Following nematode infection, the abundance of mRNA representing root LOXs increased in the resistant (but not in the susceptible) genotype while mRNA of LOXN2 decreased, indicating that LOX family members responded differentially to the infection. Triggering of plant LOX gene transcription is the most frequently observed response to pathogen attacks (Porta and Rocha-Sosa, 2002). However, there is a body of evidence that some LOXs, together with other defense genes, decrease their expression during infection (Moy et al., 2004). Down-regulation of LOXN2 occurred at 24 h after nematode inoculation and maintained the same trend until 48 h, when the parasite induces the formation of syncytial cells. Interestingly, LOXN2 mRNA levels dropped sharply in the resistant genotype, suggesting again that distinct mechanisms of transcriptional regulation occur for the two LOXN2 paralogs.

In uninfected peas, the occurrence of LOXN2 transcript in cortical and endodermal cells suggests a central function for this enzyme in root development. In light of these results, it can be hypothesized that LOXN2 with acidic pH optimum, as for other proteins like expansins, could mediate relaxation of cell walls during acid-induced growth (McQueen-Mason et al., 1992). In infected roots, LOXN2 message was mostly detected in the cells bordering the syncytia. The abrupt loss of signal in the cortical tissue, accompanied by its confinement to the few cells delimiting the syncytium, was consistent with the expression dropping, as revealed by northern analyses in infected roots. It is worth noting that resistant and susceptible genotypes shared a similar pattern of transcript localization, though staining signal dropped more
in resistant than in susceptible tissues. These findings seem to confirm the occurrence of a different degree or mechanism of transcriptional regulation between the two genotypes. The difference in stain distribution between uninfected and infected roots suggests that the LOXN2 might work in concert with other enzymes (e.g. anionic peroxidases) in limiting syncytium development by strengthening the cell walls close to nematode feeding sites (Ros Barceló et al., 1989; Zacheo et al., 1997).

Likewise, LOX action leads to membrane degradation and related cell death in oxidative conditions (Melillo et al., 1990) as a result of the synthesis of jasmonic acid, known to be involved in senescence, necrosis, and HR (Creelman and Mullet, 1997; Rusterucci et al., 1999). Indeed, there is evidence that LOXs play an important role in plant-pathogen interactions by initiating membrane damage during the HR through either direct or indirect promotion of lipid peroxidation (Brash, 1999; Maccarone et al., 2000). Our data provide evidence that a newly identified LOX gene in pea is regulated by wounding, and, additionally, they suggest that LOXN2 expression in infected roots, at the level of injured cells and induced synctium, seems to be a direct response to signals from the nematode.

MATERIALS AND METHODS

Plant Material, Nematodes, and Treatments

All experiments were performed using a germplasm from pea (Pisum sativum subsp. transcaucasicum Goversov; Gatersleben collection; accession no. MG103738) and a commercial cultivar Progress 9, resistant and susceptible to the cyst nematode Heterodera goettingiana, respectively. Seeds of both genotypes were surface sterilized, germinated on filter paper, transferred in clay pots containing 10 mL of sterilized sand, and maintained in a growth chamber with a light intensity of 200 μmol m–2 s–1 at 19°C with a 16:8 h light/dark cycle. Ten-day-old seedlings were inoculated with batches of 50 freshly hatched sterile second-stage juveniles of H. goettingiana, obtained from cysts collected from a culture maintained on pea. Pools of 0.3-cm infected roots were sampled at time intervals of 24 and 48 h following nematode infection, immediately frozen in liquid nitrogen, and stored at −70°C until required. Noninfected roots were used as controls.

For wounding experiments, seeds of both pea genotypes were surface sterilized, germinated on filter paper, soaked in tap water, transferred in Hoagland solution, and maintained in growth chambers at the same conditions as reported above. The roots of 1-week-old seedlings were injured by a needle at three different points (from the apices to elongation zones). Punctured seedlings were laid on filter paper and the roots covered with filter paper soaked in Hoagland solution. Samplings (1 cm) of injured roots and controls were collected at 3, 6, 12, and 24 h after wounding.

Cloning of Full-Length LOXN2 cDNA and Genomic Fragments

A PCR-based strategy was used to isolate LOX cDNA fragments from pea roots using degenerate primers, which were designed from conserved functional domains of plant LOX sequences. Total RNA (2 μg) of resistant and susceptible genotypes was reverse transcribed using an oligo(dT) and single-strand cDNA used in PCR reactions (see details in the RT-PCR paragraph). The primer combinations 2FW (5′-GKGRCACTTACATGACAGAC-3′)/2BW (5′-GACTCTTCTGTATCGAGAAG-3′), 3FW (5′-GCGATGAGCAGTGARGAA-3′)/3BW (5′-ATCCAAATGCCGGATCGACCC-5′), 4FW (5′-GGCAGWCKRTGTGTTGACGC-3′)/4BW (5′-CCATAGAAGCTGCATTACACC3′); K = G, T, R = A, G; Y = C, T, S, C; G, W = A, T) produced single amplicons of 658, 534, and 1,072 bp, respectively, and the sequencing of cloned fragments revealed that amplified products shared homology with LOX sequences. The full-length cDNA of LOXN2 (EMBL accession no. AJ749702) was obtained by 5′- and 3′-RACE approaches. The combination of primer 1FW (5′-CCCTGAAGATAAGACCCCTTGGAAG-3′) and an oligo(dT) allowed amplings containing the 3′ end of 530 bp to be obtained. In 5′-RACE, the manufacturer’s instructions were followed (Invitrogen). The RNA (1 μg) was reverse transcribed using primer 4BW; the 5′-modified single-strand cDNA was amplified using the LOXN2 nested reverse primer 5B (5′-CATTTCATATCTTGGGAAG-3′) and the adapter oligonucleotide provided in the kit as forward primer. A product of 267 bp was achieved, which harbored the 5′-UTR.

To search for introns, a set of PCR experiments was performed on genomic DNA (100 ng) of Progress 9 and MG103738. The primer combinations covered the full-length LOXN2 cDNA and were 6FW (5′-ATGACTCTTACATTGAAAGG-3′)/6BW (5′-TCTAGTTTGTATCTTGGACG-3′), 5FW (5′-CCAAAACATGAACTTAAACC-3′)/4FW (5′-CAGACAGATAGATTTTTCAG-3′). The sequences of products from both genomic DNA and cDNAs were aligned, and seven introns were located in LOXN2 (EMBL accession no. AJ749704). All PCR products of interest were cloned into pGEM-T Easy Vector (Promega) and propagated in Escherichia coli XL1 blue cells. Sequencing was performed by the CRIBI service (Università di Padova, Italy).

Southern Blot

Genomic DNA of both genotypes was extracted from 1 g of pea roots (Dellaporta et al., 1983), and 8 μg were digested with EcoRI, HindIII, and SacI, fractionated in a 0.8% (w/v) agarose gel, and transferred onto a nylon membrane (Hybond N+; Amersham Biosciences) following standardized procedures (Sambrook et al., 1989). A 1.1-kb LOXN2-specific probe from exon 2 to exon 6 (bases 968–2,045, referring to AJ749704) was chosen and labeled with DIG, using a PCR-DIG probe synthesis system (Roche Diagnostics GmbH). The genomic fragment was amplified using the couple of primers 5FW (5′-CCAAATAGACAATCAAACC-3′) and 4BW (5′-CCATAGTGCCTACTCAACC-3′). High-stringency hybridization was performed at 42°C in DIG Easy Hyb solution (Roche Diagnostics GmbH) overnight, and the filter was washed twice at room temperature in 2× SSC/0.1% (w/v) SDS for 5 min, followed by two washes for 15 min at 65°C in 0.5× SSC/0.1% (w/v) SDS. The signals were detected by alkaline phosphatase-conjugated anti-DIG antibody and CDP Star (Roche Diagnostics GmbH) chemiluminescent substrate reaction. The membrane was exposed to high performance chemiluminescence films (Amersham Biosciences UK) at room temperature for 30 min.

Northern Blot and RT-PCR Analysis

Total RNA was extracted according to the manufacturer’s instructions (Trizol; Invitrogen) and used in northern and RT-PCR analyses. RNA (5 μg/lane) was separated on a 1.2% (w/v) agarose-formaldehyde gel and transferred to Hybond N+ membrane (Amersham Biosciences UK). Hybridization was carried out overnight at 42°C with Ultrasub buffer (Ambion) containing formamide, followed by two washes in 2× SSC/0.1% (w/v) SDS at 60°C for 10 min and one wash in 0.1× SSC/0.1% (w/v) SDS. Filters were exposed to Kodak BIOMAX films (Amersham Biosciences UK) for at least 4 h. A LOXN2-specific probe spanned the 5′-UTR region (bases 2,475–2,650, referring to AJ749702), whereas the probe to detect the abundance of other LOX members included the conserved ORF region (bases 994–2,160, referring to AJ749702). The probes were radiolabeled with 32P-DCTP using the Ready Primer kit (Amersham Biosciences UK). The optical density (OD) of the signal bands was determined by the ID Image Analysis software (Kodak Digital Science), and the relative OD was graphed as histograms (Microsoft Excel) representing the ratio between OD of LOX genes and 26S rRNA (checking for the equal loading of RNA).

Semi-quantitative RT-PCR was performed on single-strand cDNAs derived from anion-treated RNA (AMV) and reverse transcribed (AMV) by oligo(dT) primers, according to the provider (Roche Diagnostics). The PCR reaction was conducted in 50 μL total volume containing cDNA (2 μL), 5 mM MgCl2, 200 μM of each dNTP, 10 pmol of each specific primer, and one unit of Taq DNA polymerase (Roche Diagnostics). The cycle parameters were as follows: denaturation at 94°C for 2 min; 30 cycles at 94°C 30 s, 60°C 30 s, and 72°C 30 s; and final extension at 72°C for 7 min. LOXN2 message was amplified by primer 1 (5′-CTCCGTAAAGGACCCCTTGGAAG-5′) and a anchor primer (5′-GCCACCGTGATATCGATGCG-3′), whereas the probe to detect the abundance of other LOX members included the conserved ORF region (bases 994–2,160, referring to AJ749702). The probes were radiolabeled with 32P-DCTP using the Ready Primer kit (Amersham Biosciences UK). The optical density (OD) of the signal bands was determined by the ID Image Analysis software (Kodak Digital Science), and the relative OD was graphed as histograms (Microsoft Excel) representing the ratio between OD of LOX genes and 26S rRNA (checking for the equal loading of RNA).

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equal amounts of cDNA template. A mock reaction was also performed to check for DNA contamination in RNA samples. Diagrams were designed by measuring band signal intensity normalized with respect to 26S signals. Northern-blot and RT-PCR experiments were carried out several times with independent RNA extracts. Standard errors were calculated and indicated as size bars. All data sets were subjected to the Student’s t test, and those presented in this work have a P < 0.05.

In Situ Hybridization

The roots were collected 48 h after nematode infection and fixation (4% [w/v] formaldehyde in phosphate-buffered saline), embedding in paraffin, and in situ hybridization carried out as described by Jackson (1991). The LOXN2-specific probe spanned the 3'-UTR region from nt 2,475 to nt 2,650 (with reference to A749702). Sense and antisense DIG-labeled riboprobes were produced by in vitro transcription of linearized plasmid DNA (cut with either Spel or NcoI) using 17 and 36 RNA polymerase, respectively (Roche Diagnostics). Hybridization was performed at 54°C overnight using 50 ng of riboprobe. The slides were soaked twice in prewarmed (54°C) 0.2 × SSC for 30 min, rinsed twice for 5 min with NTE solution (500 mM NaCl, 10 mM Tris-HCl, pH 8, 1 mM EDTA) at 37°C, and incubated at the same temperature for 30 min in prewarmed NTE solution containing RNase A (20 μg/mL). The slides were then rinsed twice for 5 min in NTE solution at 37°C, and washed 1 h in 0.2 × SSC at 54°C and 5 min in phosphate-buffered saline at room temperature. The signals were detected using an alkaline phosphatase-conjugated antibody (1:1,000), and the reaction was stopped by adding Tris-EDTA. Sections were viewed under a bright-field microscope. Controls to check for signal back-­

Sequence Analyses, Alignment, and Phylogenetics

LOXN2 full-length cDNA and its deduced protein were first aligned with other LOX sequences by means of ClustalW (http://www.ebi.ac.uk/clustalw). ClustalW and visual inspection (PILEUP program) were used to optimize and produce the final alignments. Phylogenetic trees were constructed based on either a nucleotide or amino acid sequences and reconstructed using different approaches were obtained using PAUP 4.0 b10 package (Swofford, 1998), and bootstrap values were based on 1,000 replicates. Bootstrap values assess the degree of support for each branch on the trees, and the 80% value was accepted as an indicative of a well-supported branch. The sequences of the monocot plant species (barley [Hordeum vulgare], maize [Zea mays], and rice [Oryza sativa]) were used as an outgroup in the phylogenetic reconstructions.

Molecular Modeling

The pea LOXN2 protein structure was modeled using Swissmodel set automatically to seek appropriate known protein crystal structures as templates based on sequence similarity. The selected five template structures (protein structure data bank accession codes 1RRL, 1JNQ, 1NQI, 1RRH, and 1RRK) were all from another legume, soybean (Glycine max) LOX (LOX3), all solved at 2.1 Å resolution or better, and two of which were solved with ligands (epigallocatechin or protocatechuic acid) in the active site adjacent to the bound iron ion, and energy minimization was carried out with the GROMOS96 program. When this was repeated but with the program set to automatically to seek appropriate known protein crystal structures as templates based on sequence similarity, PAUP 4.0 b10 package was used to optimize and produce the final alignments. Phylogenetic trees were constructed based on either a nucleotide or amino acid sequences and reconstructed using different approaches were obtained using PAUP 4.0 b10 package (Swofford, 1998), and bootstrap values were based on 1,000 replicates. Bootstrap values assess the degree of support for each branch on the trees, and the 80% value was accepted as an indicative of a well-supported branch. The sequences of the monocot plant species (barley [Hordeum vulgare], maize [Zea mays], and rice [Oryza sativa]) were used as an outgroup in the phylogenetic reconstructions.

Synthesis of LOXN2 in Yeast

The ends of the LOXN2 coding region (2.6 kb) were modified to include an AvrII site upstream the ATG start codon and a Nol site at the 3’ end using the sense primer (5′-TCATCTTACATGATCATCATTATCTGAA-3′) and the antisense primer (5′-ATAAGATCCGGCCGCTTATGATAGACTGT-3′), respectively. This LOXN2 was cloned in the AvrII-Nol-ori-oriented direction into the expression vector pPIC9 for Pichia pastoris (Invitrogen). The recombinant plasmids harboring the yeast α-factor signal in-frame with LOXN2 coding sequence were selected by PCR control and sequence analysis. For yeast transformation, 10 μg of Sall-linearized recombinant vector was transferred into GS115 Pichia host strain using a GenePulser electroporator (Bio-Rad Laboratories) and subsequently plated on minimal methanol agar substrate. Negative controls included clones containing empty, unmodified vectors.

The recombinant colonies were first grown in 1 mL of buffered glycerol (0.1 M potassium phosphate, pH 6.0, 13.4 g/L of yeast nitrogen base without amino acids, 400 μg/L, and 1% [v/v] glycerol). The cells (1 OD600) were recovered by centrifugation, suspended in 1 mL BMM (0.1 M potassium phosphate, pH 6.0, 13.4 g/L of yeast nitrogen base without amino acids, 400 μg/L, and 0.5% [v/v] methanol), and grown in 15-mL tubes at 30°C. Methanol (0.5% [v/v]) was added daily. After 3 d the cells were precipitated by centrifugation at 13,000 × g and the supernatant used for western-blot analysis. For large-scale biosynthesis, one Pichia transformant was grown in 50 mL of buffered minimal glycerol to generate sufficient biomass, and the cells recovered by centrifugation and suspended in 0.2 L of BMM.

LOXN2 biosynthesis was induced by adding 0.5% (v/v) methanol in BMM for 3 d. The cells were then precipitated by centrifugation, and ammonium sulfate up to 70% was added to the supernatant. The precipitated proteins were centrifuged at 10,000 × g for 30 min, resuspended in 5 mL of 0.1 M sodium phosphate buffer, pH 6.5, and dialyzed against 4 L of 0.1 M sodium phosphate buffer, pH 6.5, overnight at 4°C. An aliquot of proteins was assayed for LOX activity.

Western Blot

Total proteins, extracted from recombinant Pichia for LOXN2 were separated by SDS-PAGE (12% [w/v] acrylamide). Samples were heated at 95°C for 5 min prior to loading. The gel was calibrated for molecular mass with precasted MultiMark multicolored standard (Invitrogen). After electrophoresis, proteins were transferred onto nitrocellulose membrane by semidry blotting according to Bio-Rad Laboratories’ manual instruction. Blots were probed with peroxidase-conjugated antibodies (Donnoney et al., 1990), incubated 1:5,000 in blocking solution (BLOTTO: 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1% [v/v] Triton X-100, containing 5% [w/v] nonfat dry milk) overnight at 4°C. The membrane was washed with the same blocking solution and incubated for 1 h with an anti-rabbit IgG conjugated to horseradish peroxidase diluted 1:5,000 in blocking solution. Blots were revealed for peroxidase activity by enhanced chemiluminescence (ECL Plus kit; Amersham Biosciences UK).

LOXN2 Activity

One unit of LOX activity is the amount of enzyme required to produce 1 μmol of HPO and hydroxy acid per minute at 25°C (Hughes et al., 1998). LOXN2 activity was determined spectrophotometrically by monitoring the increase in absorbance at 436 nm resulting from the formation of conjugated diene structures from exogenously supplied polyunsaturated fatty acids as substrates. To determine the optimum pH of the reaction, the assays of LOX activity were carried out with linoleic, linolenic, and arachidonic acids (0.3 mM) testing the following buffers for each substrate: 0.1 M sodium acetate buffer, pH 4.5; 0.1 M sodium phosphate buffer, pH 5.5, 6.0, and 6.5; and 100 mM Tris-HCl, pH 8.0, at 25°C.

Analysis of LOXN2 Products

Crude extracts in 0.1 M sodium phosphate buffer, pH 6.5, from recombinant and control yeasts were incubated for 30 min in 1 mL of 0.1 M sodium phosphate buffer, pH 6.0, or 0.1 M sodium acetate buffer, pH 4.5, containing 0.3 mM linoleic acid. Reaction products were reduced with sodium borohydride, extracted with chloroform/methanol (2:1, v/v), and dried. The reaction products were respunseined in methanol/water/acetic acid (85:15:0.1, v/v/v) and separated by reverse-phase HPLC using a C18 Ultrasphere column (Beckmann; 0.46 × 25 cm) as described previously (Santino et al., 2003). The pool absorbing at 234 nm containing the HODE was collected, dried, and respunseined in n-hexane/propan-2-ol/acetic acid (100:2:0.1, v/v/v). The 9-HODE and 13-HODE were separated by straight-phase HPLC with a silica Ultrasphere column (Phenomenex; 250 × 4.60 mm, 5 μm). Authentic standards of 9- and 13-HODE were purchased from ICN. HPLC analyses were
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McQueen-Mason S, Durachko DM, Cosgrove DJ (1992) Two endogenous proteins that induce cell wall extension in plants. Plant Cell 4: 1425–1433


Wisniewski JP, Gardner CD, Brewin NJ (1999) Isoexpression of lipoygenases cDNA clones from pea nodule mRNA. Plant Mol Biol 36: 775–787


LITERATURE CITED


Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AJ769702 and AJ769704.

ACKNOWLEDGMENTS

The excellent assistance with figures from Dr. Alberto Troccoli is gratefully acknowledged. We thank Mr. Roberto Lerario for his technical assistance.

Received April 7, 2006; revised April 7, 2006; accepted April 26, 2006; published May 5, 2006.