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Localization of Lipoxygenase Proteins and mRNA in Pea Nodules: Identification of Lipoxygenase in the Lumen of Infection Threads

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Antibody and nucleic acid probes were used to investigate the localization of lipoxygenase in pea nodules. The antiserum identified several proteins in immunoblots of nodule extracts after polyacrylamide gel electrophoresis. These proteins co-migrated with previously characterized pea seed lipoxygenases. When nodule tissue sections were examined by immunogold labeling, a novel extracytoplasmic location for lipoxygenase antigen was identified in the subapical invasion zone: the antigen was found in the lumen of *Rhizobium*-induced infection threads. Immunogold labeling was also observed in the cytoplasm of cells in the nodule periphery. This labeling was heaviest at the nodule apex, outside the meristematic region. There was also labeling in the vicinity of nodule vascular tissue where phloem companion cells were found to contain lipoxygenase antigen. An RNA probe derived from a genomic clone of a pea lipoxygenase gene was used for in situ hybridization studies on pea nodule sections. The expression pattern observed was broadly consistent with the distribution of the protein antigen as observed by immunogold localization. This multiplicity of localizations suggests that several lipoxygenases are present in pea nodules and that they may have a multiplicity of functions relating to plant-microbe interactions, tissue differentiation, and nitrogen storage.

Additional keywords: immunolocalization, nitrogen fixation, *Pisum sativum*, symbiosis.

Pea root nodules harbor the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum*. The nodules formed have an apical meristem that gives rise to both infected and uninfected cells in the mature organ. Such nodules are said to be indeterminate and the longitudinal axis reveals two parallel processes: cell and tissue invasion by rhizobia, and the differentiation and maturation of cell types from the uninfected meristem through to the senescent zone (Brewin 1991; Brewin 1993). This study reports the localization of lipoxygenase (LOX) in nodules because this class of enzymes is thought to be involved both in plant cell development and in plant-microbe interactions.

LOX catalyzes the peroxidation of lipids and is ubiquitous in eukaryotes (Hildebrand 1989). The enzyme has been identified in many higher plant species and has been shown to exist as a number of isozymes within those species. In soybean, in which LOX has been extensively studied, there are known to be at least three isozymes in mature seed (Hildebrand et al. 1988), two further proteins in the germinating hypocotyl/radicle (Park and Polacco 1989), and another LOX in leaves (Grayburn et al. 1991). Multiple LOXs are also found in pea (Anstis and Friend 1974; Domoney et al. 1990).

The enzyme has been implicated in a range of physiological processes, including responses to wounding (Geerts et al. 1994), water stress (Bell and Mullet 1991), and the production of hexanal, which affects the post-harvest physiology of seeds of commercially important species such as soybean (Hildebrand 1989). A LOX-like enzyme has been linked to abscisic acid biosynthesis (Creelman et al. 1992). The LOX pathway is involved in the production of a number of fatty acid–derived signal molecules such as the wound hormone traumatin and jasmonic acid (Hildebrand 1989). A derivative of the latter, methyl jasmonate, has also been shown to induce LOX expression (Bell and Mullet 1991; Bell and Mullet 1993). It has been suggested that the enzyme plays a part in plant-microbe interactions in a diverse range of species including *Arabidopsis* (Melan et al. 1993), avocado (Prusky et al. 1985), oats (Yamamoto and Tani 1995), *Phaseolus vulgaris* (Croft et al. 1993), rice (Peng et al. 1994), tobacco (Ohta et al. 1990), and tomato (Koch et al. 1992).

Nitrogen metabolism may also involve some LOX isozymes. A 94-kDa storage protein in soybean, which acts as a temporary nitrogen store during vegetative growth, has been identified as a LOX (Grimes et al. 1993; Tranbarger et al. 1991; Kato et al. 1993). The finding that LOX levels are highest in young, rapidly growing tissue (Eiben and Slusarenko 1994; Funk et al. 1985; Matsui et al. 1992; Ohta et al. 1986) hints at a role for the enzyme in plant development. In contrast, the lipid peroxidation mediated by LOX may have a role in senescence (Fobel et al. 1987). LOX could also play an indirect part in cell death through the production of jasmonic acid, a known inducer of senescence.

Nodules may provide a useful model system in which to analyze some of the physiological roles for LOX. Nodules

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have clearly distinguishable regions or structures in which several processes thought to involve LOX could take place. The apex of the nodule shows rapid division and growth during development. Distal to the meristem is a zone in which the infection threads and droplets associated with rhizobial tissue invasion are found. The central part of the nodule is involved in nitrogen metabolism through nitrogen fixation by the endosymbiotic form of rhizobia, bacteroids. Senescence takes place in a definable region at the base of the nodule.

In the present study, we have used probes that recognize LOX protein and mRNA to localize LOX in pea nodules at the cellular and subcellular level. In view of the diversity of LOX genes and proteins this initial work was conducted with "generic" probes that recognized multiple gene transcripts and LOX proteins. These studies have revealed several different tissue localizations, including a new extracellular location for LOX. The results suggest potential physiological roles for several LOX enzymes in nodules.

RESULTS

Anti-lipoxygenase antibodies were used to detect antigens in western blots of nodule extracts. Multiple immunoreactive bands were present in nodule supernatant fractions (Fig. 1). These had an apparent molecular mass of approximately 95 kDa, which is within the molecular mass range of previously described pea LOXs (Domoney et al. 1990). No antigens were detected on filters incubated with preimmune serum (Fig. 1). Dot blots indicated the antigen was absent from free-living rhizobia and purified bacteroids (data not shown).

After the antiserum was shown to detect LOX polypeptides in nodule tissue homogenates, it was then used to localize LOX antigens in longitudinal sections of pea nodules. Following immunogold labeling and silver enhancement, strong labeling was seen in three locations in light micrographs of mature nodules (Fig. 2B). Silver deposition was observed in the outer cortex with labeling heaviest at the nodule apex. This differential staining was most obvious in older nodules (5 weeks postinoculation) where labeling was restricted to the apex. Antigen was also localized in a layer of uninfected cortical cells associated with the nodule vascular tissue that has not previously been described as being biochemically distinct from other regions of the nodule parenchyma (Fig. 2D). The third site of immunoreactivity was in the invasion zone of the nodule where infection threads were heavily labeled (Fig. 2C). The antigen appeared to be present in the lumen of infection threads, which contains, at least in part, a plant-derived matrix equivalent to that found in intercellular spaces (VandenBosch et al. 1989). Infection threads are transcellular tubes formed by plant cell wall deposition, through which rhizobia invade host tissue.

Immunogold-labeled electron micrographs were used to confirm these localizations at the subcellular level. In transverse sections of infection threads, gold label was observed in the matrix but not in the cytoplasm of surrounding cells nor directly associated with the invading bacteria (Fig. 3C). In contrast, in peripheral apical cells, antigen was localized in the cytoplasm but was not seen in extracytoplasmic regions (Fig. 3A). In tissue associated with the nodule vascular bundle, gold was observed in parenchyma adjacent to phloem sieve elements (Fig. 3B). These cells had dense cytoplasm and plasmodesmatal connections to the phloem, features characteristic of phloem companion cells.

In addition to protein localization by antiserum, a LOX clone was used to investigate the distribution of LOX mRNA in nodules. A radiolabeled DNA probe derived from LOX 8-1 kb identified a single band of approximately 3 kb in Northern blots of nodule RNA (Fig. 4). This size is consistent with reported sizes for LOX mRNA in other tissues (Domoney et al. 1990). The probe also identified a message of the same size in root RNA but failed to detect any differences in the strength of hybridization to the RNAs of the two tissues. LOX 8-1kb was used to generate digoxygenin-labeled riboprobes for in situ hybridization studies using longitudinal sections of wax-embedded pea nodules. While a very low level labeling was seen throughout the nodule, heavier staining was observed in the nodule apex outside the meristem, in the invasion zone, and associated with vascular tissue (Fig. 5). This RNA distribution was broadly consistent with the observed protein localizations.

DISCUSSION

Multiple LOXs are known to exist in pea (Domoney et al. 1990). Western blots of pea nodule proteins showed that a number of forms are present, corresponding to at least two mobility groups (Fig. 1). The bands detected by anti-LOX antiserum after one-dimensional gel electrophoresis are of a size consistent with LOXs from other organs in pea. This, coupled with the fact that the antiserum raised against pea seed fractions shows homology to LOX (Domoney et al. 1990), suggests that the antigenic bands do represent LOX polypeptides. Dot blots of nodule supernatant, bacteroids, and free-living rhizobia probed with anti-LOX antiserum indicated the antigen was of plant origin.

Anti-LOX antiserum, when used in immunogold-labeling experiments, revealed three major locations for the antigen observable in light micrographs (Fig. 2): the nodule periphery, particularly at the apex; in infection threads; and in phloem parenchyma. Several subcellular localizations have been previously reported for LOX, including vacuole (Tranbarger et al. 1991), membrane (Fobel et al. 1987; Todd et al. 1990), lipid bodies (Ferrie et al. 1994), cytoplasm and protein

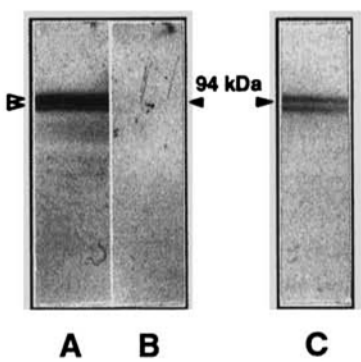

Fig. 1. Western blot analysis of lipoxygenase (LOX) antigen. **A,** Nodule homogenate probed with anti-B antiserum that identifies LOX antigen. **B,** Nodule homogenate incubated with preimmune serum. **C,** Pea seed homogenate probed with anti-B. Open arrowheads indicate the location of multiple immunoreactive bands visible in nodule homogenate lane. Arrowhead shows position of 94-kDa marker.

bodies (Vernooy-Gerritsen et al. 1983, 1984). In addition, putative chloroplast-directing transit peptides have been observed in *Arabidopsis* and rice LOX isozymes (Bell and Mullet 1993; Peng et al. 1994). However, we believe the infection thread localization presented here to be the first description of an extracellular plant LOX and we suggest the presence of LOX within infection threads may indicate a role for the enzyme in plant-microbe interactions. LOX expression (Melan et al. 1993; Peng et al. 1994) and activity (Ohta et al. 1991; Todd et al. 1990) increase in response to pathogens in a number of plant species, and it has been suggested that the enzyme has a role in the hypersensitive response (Croft et al. 1993; Keppler and Novacky 1987; Koch et al. 1992). Products of the LOX pathway have been shown to have antimicrobial properties (Croft et al. 1993; Ohta et al. 1990). LOX also plays a part in the biosynthesis of pathogen- and wound-related fatty acid signaling molecules (Geerts et al. 1994; Hildebrand 1989). It is impossible to suggest which of

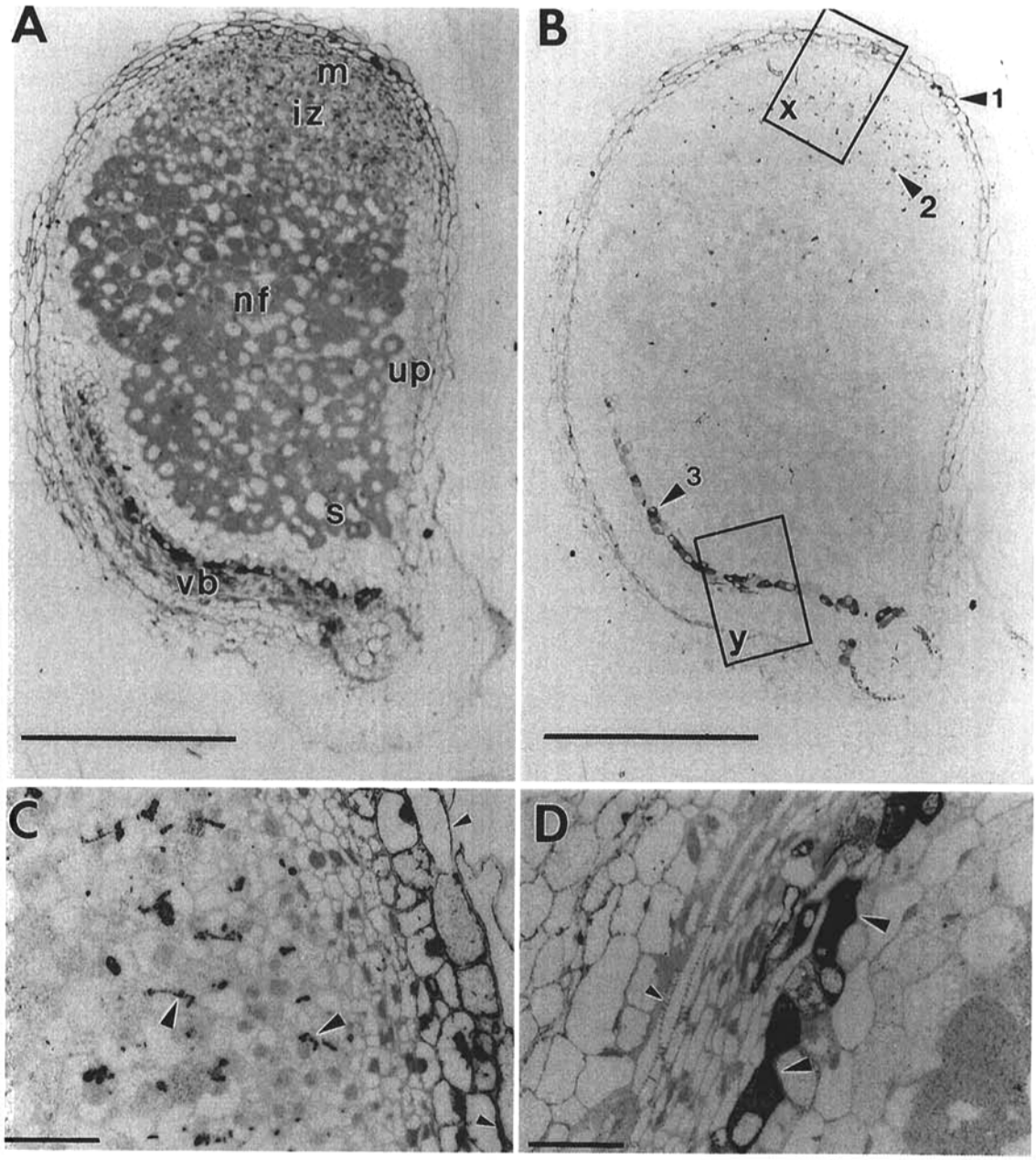

Fig. 2. Light micrographs showing localization of lipoxygenase (LOX) antigen in longitudinal sections of pea nodules following immunogold labeling and silver enhancement. **A, C,** and **D** were counterstained with basic fuschin (no counterstain in **B**). **A,** Pea nodule showing developmental series in longitudinal axis. m, meristem; iz, invasion zone; nf, nitrogen fixing region; s, senescent zone; vb, nodule vascular bundle; up, uninfected parenchyma. **B,** Three major locations for LOX antigen recognized by anti-B antiserum. 1, nodule apex (outer cortical parenchyma); 2, invasion zone (infection threads); 3, parenchyma adjacent to vascular tissue. **C,** Enlargement of box (x) from micrograph **B** (after counterstaining). Silver deposition is seen in infection threads (large arrowheads) and in cytoplasm of vacuolated cells at the periphery of the nodule apex (small arrowheads). **D,** Enlargement of box (y). Antigen is localized in apparently undifferentiated cells adjacent to and inside the vascular tissue (large arrowheads) in which xylem vessels can be distinguished (small arrowheads). Bars represent: 500 μm, **A** and **B**; 100 μm, **C** and **D**.

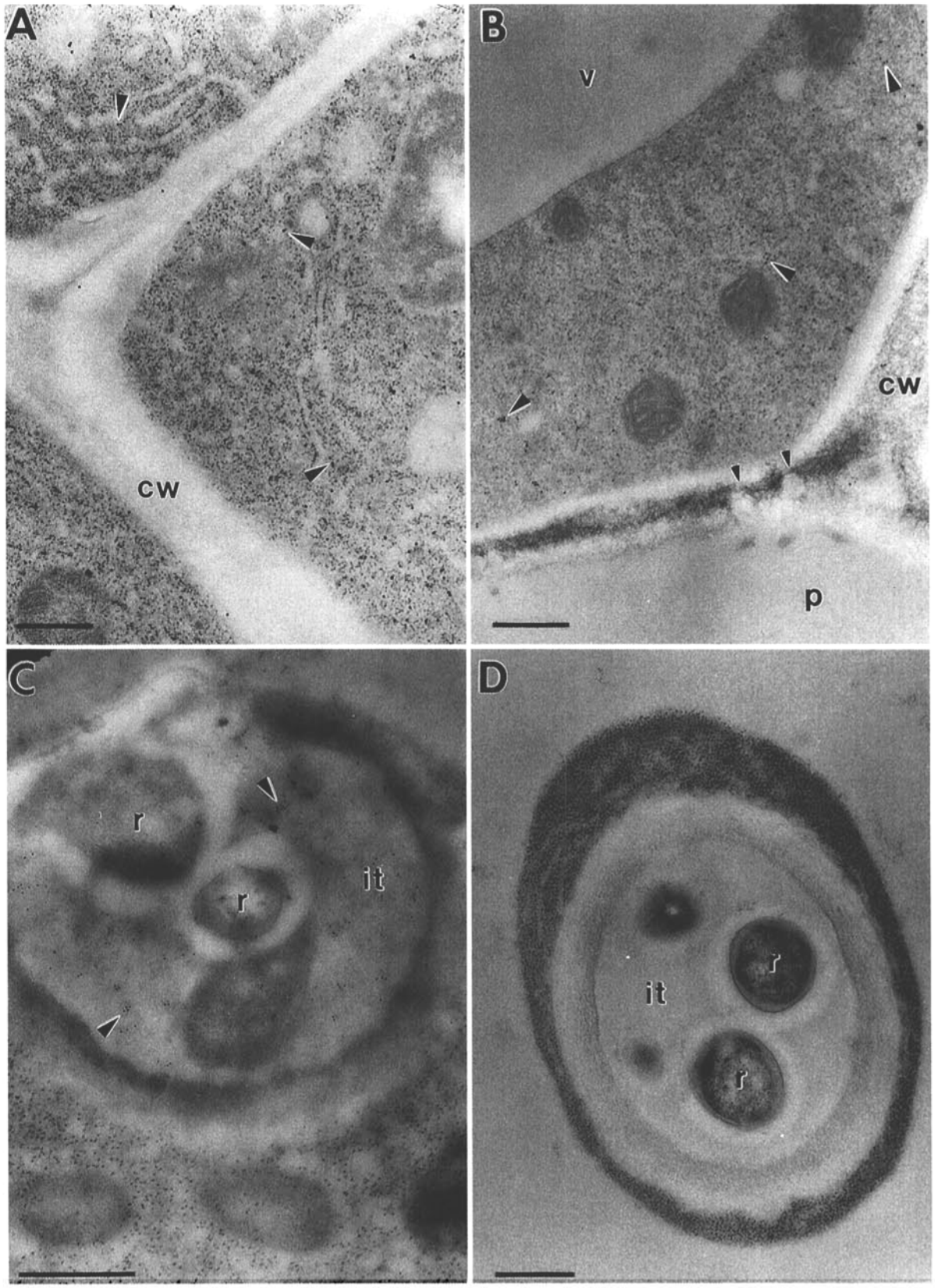

Fig. 3. Electron micrographs showing localization of lipoxygenase (LOX) antigen in ultrathin sections of pea nodules following immunogold labeling. **A,** LOX antigen (arrowheads) recognized by anti-B antiserum in the cytoplasm of vacuolated cells in the peripheral region of the nodule apex. cw, cell wall. **B,** Gold particles (arrowheads) in the cytoplasm of phloem companion cells, characterized by plasmodesmatal connections (small arrowheads) to phloem sieve elements. p, phloem sieve element: v, vacuole. **C,** LOX antigen (arrowheads) is localized in the matrix material in the lumen of an infection thread. r, rhizobia; it, infection thread. **D,** Preimmune serum control showing absence of gold in the infection thread lumen. Bars represent 500 nm.

these previously described functions may be relevant to the infection thread localization. No increase in LOX expression was seen in Northern blots of mRNA from pea roots 5 days after inoculation with wild-type rhizobia (data not shown). However, unlike other infection thread matrix components, which are also found in intercellular spaces in uninfected tissue (Rae et al. 1991; VandenBosch et al. 1989), extracellular LOX antigen was seen only in infection threads and thus only in the immediate vicinity of rhizobia. Assigning a candidate substrate for LOX in infection threads is difficult. Nod factor, a lipochito-oligosaccharide bacterial signal molecule involved in establishing the legume symbiosis, bears fatty acids but these do not contain *cis,cis-1,4*-pentadiene moieties, and thus cannot act as direct subtrates for LOX. The same is also true for *Rhizobium* lipopolysaccharide (LPS). Nod factor or LPS could, however, be subject to LOX-mediated co-oxidation in the same way that carotenoids, which also lack *cis,cis-1,4*-pentadiene moieties, have been shown to be subject to bleaching in the presence of LOX and the substrate linoleic acid (Ben Aziz et al. 1971). This indirect LOX activity is thought to play a part in the biosynthesis of abscisic acid from carotenoid precursors (Creelman et al. 1992; Parry and Horgan 1991).

A second area of strong silver deposition in immunolabeling experiments was in a layer of parenchymal cells associated with the nodule vascular tissue, the phloem companion cells (Fig. 2). These have been studied in the nodules of a number of plant species (Joshi et al. 1993; Newcomb and Peterson 1979; Pate et al. 1969) and are characterized by certain morphological features including dense cytoplasm and cell wall ingrowths (not seen in this study). Companion cells are linked by plasmodesmata to sieve elements and are thought to facilitate phloem-mediated transport. An association with vascular tissue has been reported for LOX in hypocotyls, epicotyls, and petioles of *Phaseolus vulgaris* (Geerts et al. 1994). It was suggested that this localization was analogous to the paraveinal mesophyll cells of soybean leaves where a form of LOX involved in nitrogen storage is located (Tranbarger et al. 1991). The paraveinal mesophyll is thought to have a major impact on the regulation, partitioning, and transport of nitrogenous assimilates (Tranbarger et al. 1991). Phloem companion cells could fulfill a similar function in root nodules. However, the LOX associated with vascular tissue in pea nodules is primarily cytoplasmic whereas the paraveinal mesophyll LOX in soybean is most prevalent in electron-dense flocculent material in vacuoles. It has also been assumed that the transport of nitrogenous compounds from nodules is via the xylem with the phloem being involved in the import of carbon as sugars (Joshi et al. 1993).

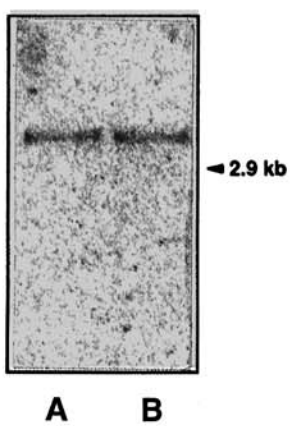

Fig. 4. Northern blot of nodule and root RNA probed with LOX 8-1kb (a pea genomic lipoxygenase subclone). **A,** Nodule. **B,** Root. Arrowhead shows position of 2.9-kb DNA marker.

The cytoplasm of cells at the nodule periphery was also labeled by anti-LOX antiserum, with the protein apparently most abundant at the nodule apex, particularly in older nodules. The prevalence of the antigen at the nodule apex may indicate a role in growth. A similar apical localization for chalcone synthase mRNA was taken to indicate a role for that enzyme in nodule development (Yang et al. 1991) and LOXs are reported to be present in young, rapidly growing tissue (Eiben and Slusarenko 1994; Funk et al. 1985; Matsui et al. 1992; Ohta et al. 1986).

The protein localizations were supported by in situ hybridization experiments with a probe derived from a genomic LOX clone, LOX 8-1kb. Hybridization with LOX mRNA was observed at the apex of the nodule, in the invasion zone, and associated with vascular tissue (Fig. 5). The central tissue of the nodule also appeared to be lightly labeled. Little antigen was detected in this region but this may be due to a rapid turnover of LOX polypeptides or might merely reflect differences in the sensitivity between the nucleic acid and protein probes. A discrepancy between the level of signal with anti-LOX antiserum compared to in situ hybridization is also observed for phloem companion cells. This may indicate that the LOX 8-1kb RNA probe has a low affinity for the companion cell LOX gene, relative to those in the nodule periphery and infection threads, or that the antiserum has higher affinity for the phloem-localized LOX than for the infection thread/periphery cell LOXs.

Surprisingly, no LOX antigen was detected in the nodule senescent zone where the enzyme might be expected to be active in membrane degradation. However, it has been demonstrated that free heme released from leghemoglobin in nodules can catalyze lipid peroxidase activity and thereby contribute to the degradation of peribacteroid membranes in host cells (Herrada et al. 1993).

The data reported here with generic probes show that multiple LOXs are present in pea nodules. LOX antigen was observed in infection threads and in peripheral cells at the nodule apex suggesting roles for the enzyme in plant-microbe interactions and in growth and development. Antigen was also associated with vascular tissue, a localization similar to that of a LOX involved in nitrogen storage in soybean leaves (Tranbarger et al. 1991). These protein localizations were supported by in situ hybridization with a LOX riboprobe. Thus, the use of generic LOX probes has yielded information useful in assigning potential functions for LOX in nodules. However, monospecific probes will be necessary to properly address the questions of LOX function in nodules, and in other plant organs. To this end, we are currently engaged in polymerase chain reaction–based attempts to clone individual LOX cDNAs from pea nodules.

MATERIALS AND METHODS

Biological material.

Pea seeds (*Pisum sativum* L. cv. Wisconsin Perfection) were surface sterilized, and allowed to germinate in the dark

and grow for 7 days in 250-ml conical flasks containing 100 ml of Fahraeus (FP) medium (Fahraeus 1957) in 0.5% agarose. Roots were then inoculated with *Rhizobium leguminosarum* bv. *viciae* strains 3841 (Wang et al. 1982) or B556 (Brewin et al. 1985). After a further 2 days growth, the plumules were pulled through the foam bung of the flask. Plants were then grown in growth cabinets (16-h days, 20°C). Nodules were harvested 3 to 4 weeks after germination as previously described (Brewin et al. 1983). Uninfected root material was prepared by surface sterilizing seeds as above, and harvesting roots into liquid nitrogen after 1 week's growth on FP medium.

Antibodies and conjugates.

Anti-LOX antibodies were a kind gift of C. Domoney (John Innes Centre, UK). The antiserum, named anti-B, was raised in rabbits to preparations of pea seed LOX purified as described in Domoney et al. (1990). Rabbit antiserum reacting to leghemoglobin (Lbc_1) was generously provided by T. Bisseling (University Wageningen, The Netherlands). Secondary antibodies were as follows: for light microscopy, AuroProbe LM GAR (Amersham Int., Amersham, UK); for electron microscopy, 20 nm Super EM Grade Goat Anti-Rabbit (BioCell Research Laboratories, Cardiff, UK); and for immunoblotting, anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate (Sigma, Poole, UK).

PAGE and immunoblotting.

Soluble proteins from pea nodules and uninfected roots were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) using 12% acrylamide mini-gels. Proteins were electroblotted to nitrocellulose sheets in 25 mM sodium phosphate buffer pH 6.8 (Bittner et al. 1980). Immunodetection was carried out using a method based on that of Bradley et al. (1988). The primary antibody was anti-LOX anti-B or preimmune serum diluted to 0.4 µg/ml in blocking solution.

Immunocytochemistry and microscopy.

Pea nodules were fixed overnight in 2.5% (wt/vol) glutaraldehyde in 0.2 M sodium cacodylate buffer pH 7.2, or in 1% glutaraldehyde, 4% formaldehyde in 100 mM sodium phosphate buffer pH 7.2. Samples were dehydrated in a graded ethanol series and embedded in LR White resin (The London Resin Co. Ltd., Basingstoke, UK) containing 0.5% benzoin methyl ether as previously described (Bradley et al. 1988). Immunolabeling and silver enhancement for light microscopy and immunolabeling for electron microscopy were carried out following low temperature embedding (Vanden-Bosch et al. 1989). In control experiments, using preimmune serum, there was no tissue labeling.

RNA extraction and detection.

Total RNA was extracted from pea roots and nodules using the hot phenol method of de Vries et al. (1982). Poly(A)+ RNA was prepared using Dynabeads (Dynal, Oslo, Norway) and separated by gel electrophoresis prior to blotting to Hybond-N membranes (Amersham, UK) using manufacturers' protocols. Filters were hybridized to ^{32}P-labeled DNA probes produced using Boehringer-Mannheim random hexanucleotides according to manufacturer's instructions. Hybridization was carried out overnight at 55°C, followed by low stringency washing in 2× SSC (1× SSC is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 0.1% SDS at 65°C. Filters were exposed to phosphoimaging plates. A LOX clone, LOX 8-1 kb, used for probe preparation, was generously provided by C. Forster (John Innes Centre, UK). LOX 8-1kb contains a 1-kb *Eco*RI fragment from a *Pisum sativum* L. 'Birte' genomic library screened with a seed LOX cDNA, pPE 320 (9B) (Domoney et al. 1991), subcloned into Bluescript KS^+ (C. Forster and R. Casey, unpublished results). The clone shows 77% identity with pPE 320 (Ealing and Casey 1988) and 74% identity with another pea seed cDNA pPE 1036 (Ealing and Casey 1989) at the nucleotide level.

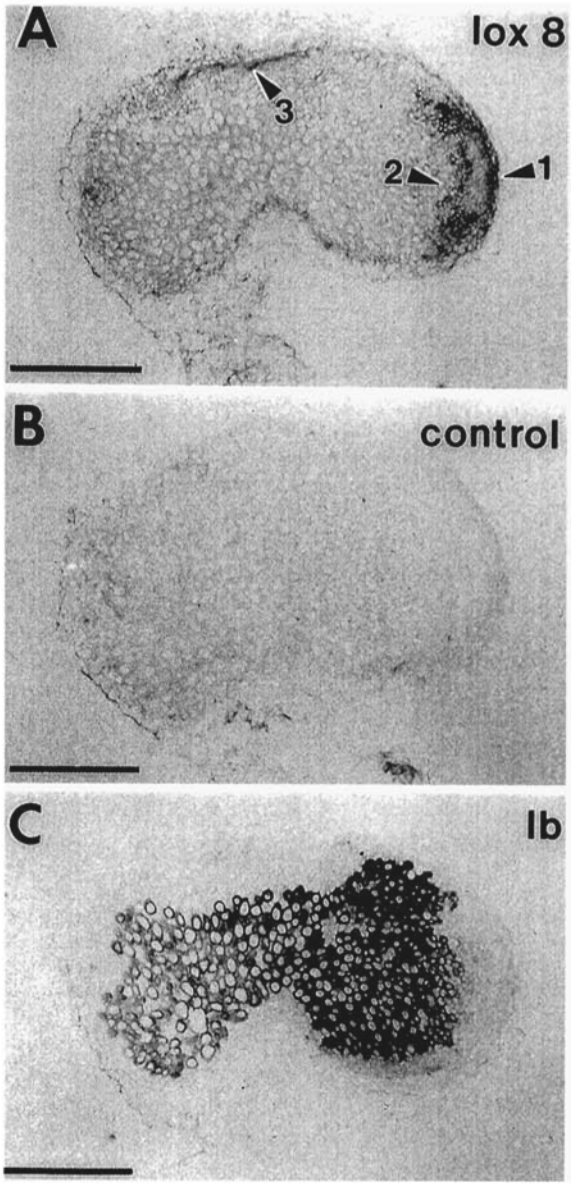

Fig. 5. Lipoxygenase (LOX) mRNA localized in longitudinal sections of pea nodules by in situ hybridization with an RNA probe from genomic clone LOX 8-1kb. **A,** Anti-sense probe identifying LOX mRNA in the nodule apex (1), invasion zone (2) and associated with vascular tissue (3). **B,** Sense control. **C,** Anti-sense probe identifying leghemoglobin in newly infected cells in the early symbiotic zone. Bars represent 500 µm.

In situ hybridization.

Pea nodules (3 weeks postinoculation) were fixed in 4% formaldehyde in phosphate-buffered saline and embedded in paraffin wax as described by Jackson (1991). In situ hybridization was carried out using methods adapted from those of Meyerowitz (1987) and Smith et al. (1987). Tissue sections were dried onto poly-L-lysine coated slides. The sections were deparaffinized in Histolene (CellPath, Hemel Hempstead, UK), rehydrated through a graded ethanol/saline, ethanol series, treated with proteinase K (1 μg/ml) in 50 mM Tris-HCl, 2 mM $CaCl_2$ pH 7.5 at 37°C for 30 min, incubated in 0.5% (vol/vol) acetic anhydride in 0.1 M triethanolamine pH 8 for 10 min, and dehydrated through an ethanol/saline, ethanol series. Probes were produced by in vitro transcription of linearized plasmid DNA using T3 or T7 RNA polymerase (Boehringer-Mannheim, Germany) according to manufacturer's instructions and were subjected to alkaline hydrolysis as in Jackson (Jackson 1991). Hybridization overnight at 50°C was with denatured digoxygenin-labeled RNA probes in 50% formamide, 10% dextran sulphate, 5× Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.3M NaCl, 50 mM $NaPO_4$ pH 7.0, 1 mM EDTA, 0.5 μg/μl tRNA. After hybridization, slides were washed in SSPE (150 mM NaCl, 10 mM $NaPO_4$ pH7.4, 1 mM EDTA) at 60°C, treated with RNAse (20 μg/ml in NTE (0.5M NaCl, 10 mM Tris-HCl pH 7.6, 1 mM EDTA)) at 37°C for 30 min, washed twice in NTE at 37°C for 15 min, then for 1 h in 2× SSPE, 0.1% Tween 20 at 60°C and then for a further 30 min in 0.1× SSPE, 0.05% Tween 20 at 60°C. After rinsing in TBS (200 mM NaCl, 50 mM Tris-HCl pH 7.4), 0.05% Tween 20 for 5 min, slides were blocked for 1 h in 1% blocking reagent (Boehringer-Mannheim), 0.05% Tween 20 in TBS. Sections were incubated for 2 h with 1 in 3,000 dilution of anti-digoxygenin alkaline phosphatase conjugate (Boehringer-Mannheim). Color development was carried out according to Wilson et al. (1995) with the addition of 10% (vol/vol) polyvinyl alcohol (De Block and Debrouwer 1993). The color reaction was stopped by washing in water and the sections were then dehydrated in ethanol, or counterstained with 0.01% Auramine-O (Sigma, UK) in 15% ethanol prior to dehydration, and mounted in Entellan (Merck-BDH, Poole, UK).

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