Research Note

The *Vicia faba* Lipoxygenase Gene VfLOX1 Is Expressed in the Root Nodule Parenchyma

Andreas M. Perlick, Ulrike Albus, Theodoros Stavridis, Martin Frühling, Helge Küster, and Alfred Pühler

Universität Bielefeld, Lehrstuhl für Genetik, Postfach 100 131, 33501 Bielefeld, Germany

A full-length cDNA encoding the broad bean lipoxygenase VfLOX1 was isolated from a nodule cDNA library. The VfLOX1 gene was strongly expressed in nodules, and only weakly in roots. VfLOX1 transcripts were localized in the nodule parenchyma and in the cells surrounding the root stele.

Additional keywords: differential hybridization; tissue-print hybridization; vegetative lipoxygenase.

A broad bean cDNA library containing root nodule transcript sequences was screened for nodule-specific and nodule-stimulated sequences by differential hybridization (Perlick and Pühler 1993). Preliminary sequence data revealed that clone number 87 contained a partial lipoxygenase (LOX) transcript sequence. Using a 5'-terminal *Eco*RV fragment of 765 bp as a hybridization probe (underlined in Fig. 1), we screened 400,000 clones of the nodule cDNA library for full-length lipoxygenase transcript sequences. Six hybridizing clones contained cDNA inserts of approximately 2,800 bp. From these the cDNA LOX1 was sequenced completely.

The 2,830-bp VfLOX1 cDNA (Fig. 1) consisted of a 2,574-bp open reading frame specifying a gene product of 858 amino acid residues with a calculated molecular mass of 96.5 kDa, which was designated VfLOX1. The open reading frame was flanked by a 19-bp 5'- (untranslated region) UTR and a 237-bp 3'-UTR. Since the initiation codon indicated in Fig. 1 is preceded by an in-frame termination codon, the LOX1 cDNA contained the complete coding region of the broad bean lipoxygenase gene VfLOX1.

Based on sequence homologies, VfLOX1 is most similar to the seed lipoxygenase isoforms LOX3 of *Glycine max* (Yenofski et al. 1988) and of *Pisum sativum* (Ealing and Casey 1989) with identities of 74.9 and 74.5%, respectively (data not shown). Homologies with other legume lipoxygenases were less than 70% and with nonlegume lipoxygenases less than 62%. Since plant lipoxygenase sequences share only limited sequence homologies in their N-terminal parts, a separate comparison was carried out for the first 45 VfLOX1

amino acids. This sequence part aligned best with the *Lens culinaris* LOX1 (59.2% identity; Hilbers et al. 1994) and with the *Glycine max* LOX4 (60.9% identity; Kato et al. 1993), both nonseed lipoxygenases reported to be vegetative LOX isoforms.

In the VfLOX1 amino acid sequence the putative iron ligands His-518, His-523, His-709, and Ile-858 are conserved (shaded boxes in Fig. 1) as are these residues, which are highly conserved in the "histidine-rich" region of most plant and animal lipoxygenases (Nelson and Seitz 1994; open boxes in Fig. 1). Sequence analysis according to von Heijne (1986) did not indicate that VfLOX1 contained a signal or transit peptide sequence (data not shown).

To characterize the expression of this gene, total RNA samples isolated from several broad bean tissues were analyzed by Northern hybridizations. Since hybridization signals turned out to be weak, 30 µg of total RNA was used to obtain signals after 14 days of exposure. The expression pattern obtained is shown in Figure 2. The amount of hybridizing transcripts of 2.9 kb was clearly higher in nodules than in roots or in any other tissue. Additionally, VfLOX1 was expressed strongly in epicotyls and to a much lower extent in stems and flowers. In time course hybridizations a significant increase of VfLOX1 transcripts during 32 days after inoculation in infected roots was not observed, probably because of the expression of the corresponding gene in roots.

As a step towards the identification of possible functions of VfLOX1 in the legume-*Rhizobium* interaction, the distribution of VfLOX1 transcripts in broad bean root nodules was determined by tissue-print hybridizations (Schröder et al., in press). Longitudinal sections of mature nodules were printed onto nylon membranes and hybridized to VfLOX1 sense and antisense probes. The localization of VfLOX1 transcripts in the nodules is shown in Figure 3. Hybridization signals occured exclusively with the antisense probe (data not shown) and were clearly visible as a narrow ring in the nodule periphery and around the root stele (Fig. 3A). Compared to the tissue section used for printing (Fig. 3B), which was stained for starch by iodine to visualize the interzone II/III (Vasse et al. 1990), VfLOX1 transcripts were located outside the infected nodule tissues in the parenchyma. In this peripheral nodule tissue VfENOD2 transcripts and other broad bean transcripts specifying proline-rich nodule proteins were localized in addition to VfLOX1 transcripts (G. Schröder, Universität Biele-

feld, Germany, unpublished data). In contrast to ENOD2 transcripts (van de Wiel et al. 1990), VfLOX1 hybridization signals form a ring closed above the meristem in a number of independent tissue-print hybridizations. Furthermore, signals were most intense in the nodule tissue above the meristem. Recently, Gardner et al. (1996) reported a similar distribution of LOX proteins and transcripts in the outer cortex of pea
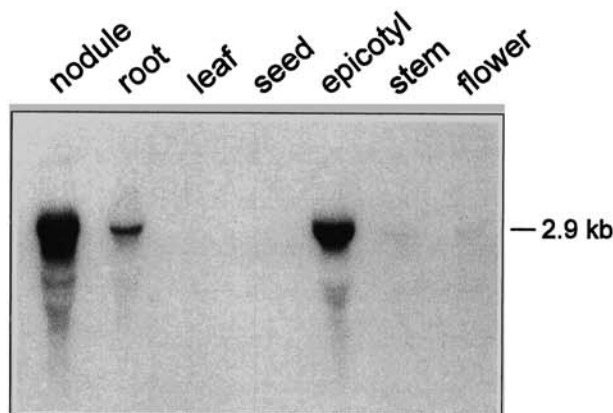
nodules. In contrast to this report we were not able to demonstrate VfLOX1 transcripts in cells associated with vascular bundles or in the prefixation zone II. This is probably due to the limited local resolution of the tissue-print technique. On the other hand our results clearly demonstrate that VfLOX1 transcripts do not occur in the prefixation zone II in a concentration comparable to the results obtained for pea.

```
                M  S  F  I  F  G  G  G  D  K  I  K  G  T  L  V  L  M  Q  K  N  V  L  D    24
GCTTGACTAAAAACCAAAAATGTCCTTCATATTTGGAGGTGGTGACAAGATCAAAGGCACATTAGTGCTTATGCAAAGAATGTGTTGGA  90
     I  N  S  L  T  D  P  A  K  I  L  D  G  A  L  G  F  G  S  I  L  D  T  L  T  S  F  L  G    54
CATAAACAGCTTAACAGATCCTGCAAAGATTCTCGATGGAGCCTTGGGTGGTTTTGGATCCATACTTGATACTCTTACTTCCTTCTTGGG  180
     N  S  V  L  L  Q  L  I  S  S  T  K  P  L  T  S  G  E  G  K  L  G  K  E  T  Y  L  K  Q  A    84
TAACTCGGTTCTTCTTCAGTTAATCAGTTCAACTAAACCTTTGACAAGTGGAGAAGGAAAACTGGGTAAAGAAACTTATTTGAAACAAGC  270
     I  T  N  L  P  T  L  G  D  K  Q  T  A  F  S  I  E  F  E  Y  D  S  S  F  G  I  P  G  A  F    114
AATTACCAATTTACCAACCTTGGGAGACAAGCAAACTGCATTCAGCATTGAATTTGAATATGATAGCAGCTTCGGAATTCCTGGAGCATT  360
     K  I  K  N  Y  M  S  N  E  F  Y  L  V  S  L  T  L  D  D  I  P  N  L  G  T  I  H  F  V  C    144
CAAAATCAAAAACTACATGTCAAATGAGTTCTATCTTGTGAGCTTAACTCTTGATGATATTCCCAATCTTGGAACCATTCACTTTGTTTG  450
     N  S  W  I  Y  N  A  K  N  Y  Q  T  D  R  I  F  F  A  N  N  T  F  V  T  S  E  T  P  P    174
CAACTCGTGGATTTACAATGCAAAAAATTATCAAACCGATCGCATTTTCTTCGCCAACAACACTTTTGTAACAAGTGAAACACCGCCTCC  540
     L  V  Y  Y  R  Q  L  E  L  K  T  L  R  G  N  G  T  G  E  R  Q  E  W  D  R  I  Y  D  Y  D    204
ATTAGTCTACTATAGACAGTTAGAGTTGAAGACTTTACGAGGGAAATGGGTGGAGCGTCAAGAATGGGATAGAATTTATGATTATGA  630
     V  Y  N  D  L  G  E  P  D  K  G  E  S  Y  A  R  P  I  L  G  R  S  S  D  H  P  Y  P  R  R    234
TGTTTACAATGATTTGGGTGAGCCTGATAAGGGTGAAAGTTACGCTCGTCCAATTCTAGGACGATCTAGCGACCATCCTTATCCTCGTAG  720
     G  R  T  G  R  K  P  T  A  T  D  P  N  S  E  S  R  G  N  S  V  Y  I  P  R  D  E  A  F  G    264
GGGAAGAACGGGGAGAAAACCTACCGCAACAGATCCTAACAGTGAGAGTAGGGGAATAGTGTGTATATTCCAAGAGATGAAGCTTTTGG  810
     H  L  K  S  S  D  F  L  V  Y  G  L  K  S  V  S  Q  D  V  I  P  L  L  Q  S  V  F  D  I  N    294
CCATTTGAAATCCTCAGACTTTCTTGTTTATGGACTTAAGTCTGTTTCTCAAGATGTAATTCCATTGCTACAATCTGTGTTCGACATAAA  900
     F  T  P  T  E  F  D  D  V  F  D  L  Y  E  G  G  I  K  L  P  T  D  I  I  S  K  I    324
TTTCACTCCAACCGAATTTGATAGCTTTGATGATGTGTTTGATCTATATGAAGGAGGAATTAAGCTACCTACGGATATAATTAGCAAAT  990
     S  P  L  P  V  I  S  E  I  F  R  T  D  G  E  E  V  L  K  F  P  P  P  P  K  V  I  Q  V  S  K    354
TAGTCCTTTACCAGTGATATCTGAAATCTTTCGAACCGATGGTGAAGAAGTCCTCAAGTTTCCGCCACCTAAAGTAATTCAAGTGAGTAA  1080
     S  A  W  M  T  D  E  E  F  G  R  E  I  L  A  G  V  N  P  G  L  I  R  S  L  Q  E  F  P  P    384
GTCTGCGTGGATGACTGATGAAGAGTTTGGAAGAGAGATCTTGGCTGGCGTAAATCCAGGCTTAATTCGCTCCCTCCAGGAGTTTCCTCC  1170
     K  S  K  L  D  S  A  I  Y  G  D  H  T  S  T  I  T  K  E  Q  I  E  L  N  L  E  G  F  S  T    414
AAAAAGTAAGCTAGATAGTGCAATCTATGGTGATCATACCAGCACCATAACCAAAGAGCAGATAGAGCTTAACTTGGAGGGGTTCAGTAC  1260
     L  D  E  A  I  Q  N  K  K  L  F  L  L  E  H  H  D  T  I  I  P  Y  L  R  L  I  N  S  T  S    444
TCTTGATGAGGCTATACAAAACAAAAAACTGTTCTTGCTAGAACACCATGACACAATAATTCCATATCTAAGGCTAATAAACTCAACTTC  1350
     T  K  A  Y  A  S  R  T  I  L  F  L  K  S  D  G  T  L  K  P  L  A  I  E  L  S  L  P  H  P    474
AACAAAAGCTTATGCTTCAAGAACCATTCTTTTTCTTAAAAGTGATGGAACTTTAAAGCCTCTAGCCATTGAGTTAAGCCTTCCACATCC  1440
     Q  G  D  Q  F  G  V  V  S  N  V  Y  L  P  A  I  E  G  V  E  A  T  I  W  L  L  A  [K  A  Y]  504
TCAAGGAGATCAATTTGGTGTTGTTAGTAATGTCTACTTGCCTGCAATTGAAGGTGTTGAAGCTACAATTTGGCTACTTGCTAAGGCTTA  1530
    [V  I  V  N  [D] S  C  F  [H] Q  L  V  S  [H] W  L  H  T  [H] A  V  V  E  [E] P  F  V  I  [A] T  N   534
TGTTATATAAATGATCCATGCTTTCACCAACTTGTCAGCCACTGGTTGCATACTCATGCAGTTGTTGAACCATTTGTGATAGCAACAAA  1620
     R  Q  L  S  V  L  H  P  I  Y  K  L  L  H  P  H  Y  R  D  T  M  N  I  N  A  L  A  R  Q  S    564
TAGGCAACTTAGTGTGCTTCATCCTATTTATAAGCTGTTACATCCGCATTATCGGGATACCATGAATATAAATGCACTTGCTAGACAATC  1710
     L  V  N  A  D  G  I  I  E  K  T  F  L  W  G  G  Y  A  M  E  I  S  S  K  V  Y  K  D  W  V    594
CTTAGTCAATGCAGATGGTATTATTGAAAAGACATTCTTGTGGGGAGGGTATGCTATGGAGATATCATCAAAAGTTTATAAGGATTGGGT  1800
     F  T  D  Q  A  L  P  A  D  L  I  K  R  G  I  A  V  E  D  S  T  S  P  H  G  L  R  L  V  I    624
TTTCACAGACCAAGCCCTACCCGCTGATCTCATCAAAAGAGGAATAGCAGTTGAGGATTCAACTTCGCCTCACGGTCTTCGTCTAGTGAT  1890
     E  D  Y  P  Y  A  V  D  G  L  D  I  W  D  A  I  K  T  W  V  Q  D  Y  V  S  I  Y  Y  I  T    654
AGAGGACTACCCGTATGCTGTTGATGGGCTCGACATTTGGGATGCTATTAAGACGTGGGTGCAAGACTATGTTTCAATATACTACATAAC  1980
     D  D  K  I  Q  Q  D  S  E  L  Q  S  W  W  K  E  V  V  E  V  G  H  G  D  K  K  G  E  P  W    684
GGATGACAAAATTCAGCAAGACTCAGAACTCCAATCTTGGTGGAAAGAAGTTGTAGAAGTAGGTCATGGTGACAAGAAAGGCGAGCCGTG  2070
     W  P  K  L  Q  T  R  Q  D  L  I  H  V  S  S  I  I  I  W  S  A  S  A  L  [H] A  A  V  N  F    714
GTGGCCTAAATTGCAAACTCGTCAAGATTTGATTCATGTTTCTAGTATTATCATATGGTCTGCCTCAGCTCTTCATGCAGTTGTTAATTT  2160
    [G] [Q] Y  P  Y  G  G  F  I  L  N  R  P  T  L  S  R  R  L  M  P  E  K  G  T  T  E  Y  D  E    744
TGGACAATATCCGTACGGAGGTTTCATATTAAACCGTCCAACTCTCAGTAGGCGATTAATGCCTGAGAAAGGTACTACTGAGTACGATGA  2250
     L  A  T  N  P  Q  K  A  Y  L  K  T  I  T  P  K  L  Q  T  L  I  D  L  S  V  I  E  I  L  S    774
GCTAGCTACAACCCTCAAAAGGCTTACTTGAAAACAATTACACCAAAGCTTCAAACTCTCATTGATCTATCAGTCATAGAAATCTTGTC  2340
     R  H  A  S  D  E  Y  Y  L  G  Q  R  D  S  A  E  Y  W  T  S  D  T  N  A  L  A  A  F  K  K    804
AAGGCATGCTTCTGATGAGTACTACCTTGGACAAAGAGACAGTGCTGAGTATTGGACTTCTGATACAAATGCCTTAGCAGCTTTCAAGAA  2430
     F  G  K  T  L  A  E  I  E  G  Q  L  I  L  R  N  N  N  E  S  L  R  N  R  V  G  P  V  S  M    834
GTTTGGAAAAACACTAGCTGAAATTGAGGGACAACTCATTCTGAGGAACAACAATGAGTCGTTGAGAAACCGAGTTGGGCCAGTTAGCAT  2520
     P  Y  T  L  L  Y  P  S  S  E  E  G  L  T  F  R  G  I  P  N  S  I  S  [I] *    858
GCCTTATACTCTTCTTTATCCTTCAAGTGAAGAAGGACTTACTTTTAGAGGAATTCCCAACAGTATCTCGATCTAAGAGAGTGTTGATAT  2610
AGATTGTGAATGATTGTAGCTGTGGTTTTAAATAAATTGTGTGAGAGAGGGAATTTCGATTCCTTATTACACCTCTCGTGTATAATTATG  2700
TTTGATTGCTTGCTTGTTCGTATGACAATAATGTAAACTATGATGTTTTTAAGGTAAAGTGTTGTATTTGCAGTTTGTAAGGTGTTGCCC  2790
TCAAACTTCTATCTTGTAAAATATTTTACTTCAAGTCAAA  2830
```

Fig. 1. Nucleotide sequence of a full-length VfLOX1 cDNA and deduced amino acid sequence of the VfLOX1 protein. The *Eco*RV fragment used as a hybridization probe is underlined. In the amino acid sequence, the putative iron ligands and the residues conserved in the 'histidine-rich' region of most lipoxygenases (Nelson and Seitz 1994) are emphasized by shaded and open boxes, respectively. The in-frame termination codon preceding the start codon is indicated by an asterisk.

Lipoxygenases (lineolate:oxygen oxidoreductase, EC 1.13.11.12) are nonheme iron proteins which catalyze the dioxygenation of polyunsaturated fatty acids containing a 1,4-(Z,Z) pentadienyl unit. Albeit the distribution of LOX in a wide range of tissues in a large number of plants the function of LOX is still obscure. These enzymes might be involved in plant growth, development, and senescence, in the response to pathogens and wounding (Siedow 1991) and could function as vegetative storage proteins (Kato et al. 1993). LOX genes have been demonstrated to be active in developing tissues (Siedow 1991) where they could be involved in the generation of growth regulators (Vick and Zimmerman 1984; Creelman

Fig. 2. Expression analysis of VfLOX1 in broad bean tissues. For Northern blot analysis 30 µg of total RNA extracted from nodules (32 days after sowing), uninfected roots (32 days after sowing), leaves (32 days after sowing), seeds (90 days after sowing), epicotyls (8 days after sowing), stems (12 days after sowing), and flowers (60 days after sowing) were blotted and hybridized as described against the VfLOX1 probe number 87-5 covering the coding region from position 1321 to 2576 (Perlick and Pühler 1993). The size of hybridizing transcripts was calculated using RNA distance markers.

A.   B.

Fig. 3. Localization of VfLOX1 transcripts in broad bean nodules. **A,** Tissue-print hybridization of a longitudinal broad bean nodule section using a digoxigenin-labeled VfLOX1 antisense riboprobe derived from clone number 87-5. **B,** Nodule section used for tissue-print hybridization after staining with Lugol's solution containing iodine to visualize starch accumulated in the interzone II/III. For a detailed description of the tissue-print technique see Schröder et al. (unpublished). Bar: 500 µm.
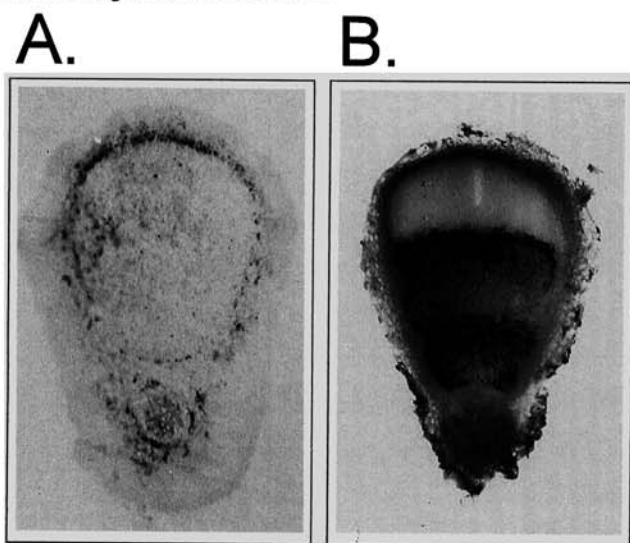
et al. 1992). This probably accounts for the VfLOX expression in broad bean epicotyls, but if VfLOX1 would be involved in the development of the broad bean root nodule, a location in the meristem or in the prefixing zone II had been expected. Since hybridization signals were most prominent in the nodule apex adjacent to the meristem, the broad bean nodule lipoxygenase could be involved in the development of this organ. On the other hand, lipoxygenases are involved in senescence phenomena (Siedow 1991). In this case VfLOX1 should be expressed in the senescence zone IV. Due to the exclusive location of VfLOX transcripts in the non-symbiotic nodule parenchyma tissue, VfLOX1 might participate in the nodule defense against pathogens from outside the plant. This hypothesis is supported by the detection of other proteins in nodule cortex and parenchyma tissues, which could be involved in plant defense mechanisms, e.g., chitinases (Staehelin et al. 1992), extensins, and other proline-rich broad bean proteins (G. Schröder, Universität Bielefeld, Germany, unpublished results). Alternatively, VfLOX1 could encode a vegetative storage protein like the *G. max* LOX4 (Kato et al. 1993). In this case it might be induced by the high level of fixed nitrogen in this plant tissue, but should consequently be expressed in the nitrogen fixing zone III instead of the nodule parenchyma. At the moment we cannot exclude that the hybridization probe number 87-5 covering part of the VfLOX1 coding region hybridized with the transcripts of several LOX genes which may differ in their distribution in broad bean tissues. Further experiments have to be carried out to analyze the specific function of VfLOX1 during root nodule development.

LITERATURE CITED

Creelman, R. A., Tierney, M. L., and Mullet, J. E. 1992. Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. Proc. Natl. Acad. Sci. USA 89:4938-4941

Ealing, P. M., and Casey, R. 1989. The cDNA cloning of a pea (*Pisum sativum*) seed lipoxygenase. Biochem. J. 264:929-932

Gardner, C. D., Sherrier, D. J., Kardailsky, I. V., and Brewin, N. J. 1996. Localization of lipoxygenase proteins and mRNA in pea nodules: Identification of lipoxygenase in the lumen of infection threads. Mol. Plant-Microbe Interact. 9:282-289

Higgins, D. G., and Sharp, P. M. 1988. CLUSTAL: A package for performing multiple sequence alignement on a microcomputer. Gene 73:237-244.

Hilbers, M. P., Rossi, A., Finazzi-Agrò, A., Veldink, G. A., and Vliegenthart, J. F. G. 1994. The primary structure of a lipoxygenase from the shoots of etiolated lentil seedlings derived from ist cDNA. Biochim. Biophys. Acta 1211:239-242

Kato, T., Shirano, Y., Iwamoto, H., and Shibata, D. 1993. Soybean lipoxygenase L-4, a component of the 94-kilodalton storage protein in vegetative tissues: Expression and accumulation in leaves induced by pod removal and by methyl jasmonate. Plant Cell Physiol. 34:1063-1072

Nelson, M. J., and Seitz, S. P. 1994. The structure and function of lipoxygenase. Curr. Opin. Struc. Biol. 4:878-884.

Perlick, A. M., and Pühler, A. 1993. A survey of transcripts expressed specifically in root nodules of broadbean (*Vicia faba* L.). Plant Mol. Biol. 22:957-970

Schröder, G., Frühling, M., Perlick, A. M., and Pühler, A. The temporal and spatial transcription pattern in root nodules of *Vicia faba* nodulin genes encoding glycine-rich proteins. Plant Mol. Biol. (In press.)

Siedow, J. N. 1991. Plant lipoxygenase: Structure and function. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42:145-188

Staehelin, C., Müller, J., Mellor, R. B., Wiemken, A., and Boller, T. 1992. Chitinase and peroxydase in effective (fix+) and ineffective (fix−) soybean nodules. Planta 187:295-300.

van de Wiel, C., Scheres, B., Franssen, H., van Lierop, M.-J., van Lammeren, A., van Kammen, A., and Bisseling, T. 1990. The early nodulin transcript ENOD2 is located in the nodule parenchyma (inner cortex) of pea and soybean root nodules. EMBO J. 9:1-7.

Vasse, J., de Billy, F., Camut, S., and Truchet, G. 1990. Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. J. Bacteriol. 172:4295-4306.

von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. 14:4683-4690.

Vick, B. A., and Zimmerman, D. C. 1984. Biosynthesis of jasmonic acid by several plant species. Plant Physiol. 75:458-461.

Yenofski, R. L., Fine, M., and Liu, C. 1988. Isolation and characterization of a soybean (Glycine max) lipoxygenase-3 gene. Mol. Gen. Genet. 211:215-222.