The Effects of Wounding and Mite Infestation on Soybean Leaf Lipoxygenase Levels

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Numerous studies have demonstrated induction of proteins in plant tissues by wounding and infestations by various pests and pathogens. Lipoxygenase (LOX) is among the proteins that has been found to be induced by pathogens, but detailed information on the induction of LOX has not been reported. We have found a large (up to 10-fold) increase in LOX activity upon wounding of soybean (*Glycine max* L. Merr.) leaves and variable increases due to feeding of the twospotted spider mite. This induction in LOX activity was reflected in increases in amounts of both LOX protein and transcripts suggesting that the induction was at the transcriptional level. LOX activity was also found to be increased in unwounded leaves from plants with wounded leaves lower on the stem indicating that translocatable factors can cause remote induction of LOX activity.

Introduction

A number of proteins have been shown to be induced by pest infestation and/or wounding. The proteins which have been found to be induced in plant tissues of unknown function are known as pathogenesis-related or PR proteins [1–3]. Characterized proteins which are induced by elicitors and/or wounding include β-1,3-glucanase and chitinase [4], cell wall hydroxyproline-rich glycoproteins, phenylalanine ammonia-lyase and chalcone synthase [5] and trypsin inhibitors [6, 7].

Studies have shown that lipoxygenase (linoleate: oxygen oxidoreductase, EC 1.13.11.12) activity of plant tissues and cells increased in response to plant pathogens particularly incompatible races of those pathogens [8–10]. We previously found [11] that lipoxygenase (LOX) and peroxidase activity increased over time in mite infested soybean leaves compared to uninfested controls. Ruzicska *et al.* [12] showed that LOX activity increased along with the hypersensitive reaction in *Nicotiana tabacum* cv. Xanthi nc upon infection with TMV, but not in the TMV susceptible, non-hypersensitive reacting (HR) Xanthi. Yamamoto and Tani [13] also found an increase in LOX activity in crown rust *(Puccinia coronata)* resistant oat lines but not in susceptible lines. Two new LOX isozymes were induced in the resistant but not the susceptible oat lines upon infection with crown rust.

The objective of these studies was to determine the effects of wounding and mite infestation on LOX (and trypsin inhibitor) activity, protein and transcripts in soybean leaves and if translocated signals could induce increases in LOX.

Materials and Methods

Plant material

Soybean plants of cultivar 'Bonus' were grown to stage V_1 [14] under greenhouse conditions [14:10 L:D). At this time, one plant was inoculated with 100 female adults of the twospotted spider mite, *Tetranychus urticae,* which were confined to the first trifoliate leaf with a ring of tanglefoot around the petiole which was first protected with a strip of wax paper. The mites were allowed to feed for 7 days. Another plant received wounding treatment, which consisted of applying pressure with a hemostat pliers once on each of the three leaflets of the first trifoliate leaf. The wounded plant was treated with the hemostat once daily for 7 days. Other plants were treated in

Abbreviations: LOX, lipoxygenase; TMV, tobacco mosaic virus; HR, hypersensitive response; PR, pathogenesis-related; L, light; D, dark; V, vegetative growth stage; CTAB, cetrimethylammonium bromide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; RUBISCO, ribulose-1,5-bisphosphate carboxylase oxygenase; mAP, messenger affinity paper.

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such a manner that at the end of 7 days, there were plants of all the same age that had been wounded daily for 7, 3, and 1 day and plants that had spider mite infestations for 7, 3 and 1 day and a control plant with no treatment. The first trifoliate leaf (treated) from each treated plant and also from the control plant were harvested and frozen immediately on dry ice. The second trifoliate leaf (untreated) from each plant was also harvested and frozen on dry ice. Each leaf was then weighed and ground to a fine powder with a mortar and pestle cooled with liquid nitrogen. Four volumes of extraction buffer (5 mM sodium acetate, pH 4.5 with 1 mM phenylmethylsulfonyl fluoride) was added to each leaf sample, mixed and centrifuged in a microcentrifuge for 10 min at 4 °C (15000 rpm). Supernatants were collected and held on ice for LOX activity measurements and protein assays. Protein content of each leaf extract was determined using the BCA protein assay kit developed by Pierce Chemical Company. This experiment was replicated 4 times within a 3 week period.

Lipoxygenase assays

LOX activity was measured using the standard spectrophotometric assay which involves the measurement of conjugated diene formation at 234 nm [15]. Linoleic acid buffered at pH 6.8 was used as the substrate. Ten µl of leaf extract was used with 1 ml substrate per sample.

Immunoblotting analysis

In order to determine whether LOX activity increased linearly over a 14 day period of wounding, plants at stage V_1 were wounded as described above, but for 4, 7, 10 or 14 days. Plants were harvested and analyzed by immunoblotting. Leaf extracts were diluted 1:1 with SDS loading buffer (50% glycerol, 3% SDS, 190 mM β-mercaptoethanol) and boiled for 5 min. Equal amounts of protein were run on a 7.5 percent SDS-PAGE gel (acrylamide:bis 37.5:1) and proteins were electroblotted to a nylon membrane (Immobilon PVDF, Millipore). The membrane was then incubated with rabbit monospecific LOX 1 antibody followed by alkaline phosphatase linked goat anti-rabbit second antibody and then stained for alkaline phosphatase activity. Stained membranes were scanned with an LKB scanning laser densitometer to quantify band intensities.

Northern blot analysis

In a related experiment, 20 soybean plants were grown to stage V_3 under greenhouse conditions. At this time half of the plants received wounding by pressure with a hemostat pliers on each leaflet of the plant and the other ten plants received no treatment. Wounding was continued daily for 14 days. All leaves on all the plants within a treatment were harvested, pooled, immediately frozen with liquid nitrogen and ground to a fine powder with a mortar and pestle. RNA was extracted by the CTAB (cetyltrimethylammonium bromide) extraction procedure [16].

Approximately 40 g of leaf tissue were ground in liquid nitrogen, added to 80 ml of extraction buffer [2% (w/w) CTAB, 100 mM tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl] containing 2% of 2-mercaptoethanol. The homogenate was warmed to 55 °C, extracted with phenol/chloroform (1:1) and subsequently twice with chloroform. Total nucleic acids were precipitated by the addition of CTAB precipitation buffer [1% (w/w) CTAB, 50 mM tris-HCl, pH 7.6, 5 mM EDTA) and finally precipitated with 2.5 vol of ethanol. After being redissolved in 10 mM tris-HCl, pH 7.6 containing 1 mM EDTA total RNA was precipitated in the presence of 2 M LiCl overnight at −20 °C, centrifuged, redissolved in 10 mM tris-HCl, pH 7.6 containing 1 mM EDTA and again precipitated by the addition of 2.5 volume ethanol. The precipitated RNA was redissolved in 0.5 M NaCl and used for the isolation of poly (A) enriched RNA with mAP paper from Amersham according to the manufacturer's instructions. Poly A enriched RNA was separated by electrophoresis in a 1% agarose/formaldehyde gel, transferred to Zetabind (CUNO Inc.) filters by standard Northern blot techniques [17] and hybridized with the cDNA probe of LOX 3 [18; kindly provided by Dr. Yenofsky], the actin gene [19; kindly provided by Dr. Meagher] and the gene for the small subunit of RUBISCO from pea [20; kindly provided by Dr. Hunt]. All probes were labelled with [^{32}P]dCTP by the oligo-nucleotide labelling procedure [21].

Results

Wounded leaves showed a steady increase in LOX activity up to 7 days of daily wounding with a further increase up to 14 days of wounding after which the experiment was discontinued due to extensive dam-

age to the wounded leaves. The results with mite infestation were quite variable with respect to increases in LOX activity. Sometimes increased LOX activity was observed in the mite infested leaves while in other cases no increases were seen resulting in no statistically significant increase in LOX being attributable to mite feeding in these experiments. Both the wounded leaf (which was the first trifoliate) and the next leaf up the plant (which was the unwounded, second trifoliate) showed the increase in LOX activity with the increase seen ranging from 3- to 10-fold after seven days (Fig. 1). The stimulation of trypsin inhibitor activity [6, 7] was also examined, but only small and variable and therefore non significant changes were seen (data not shown).

The increase in LOX activity was paralleled by increases in the LOX protein as evidenced by immunoblots probed with LOX 1 antibodies (a representative gel is shown in Fig. 2). This increase was again seen both in the wounded leaves and in the unwounded leaves from wounded plants. The correlation between the LOX protein band on immunoblots and LOX activity was $r = 0.93$.

Northern blot analysis of poly (A) enriched RNA from wounded and unwounded leaves (from unwounded plants) indicated that the LOX transcript levels also increased due to wounding (Fig. 3a). The increase in LOX transcripts seen in wounded compared to unwounded soybean leaves was about five fold as determined by densitometry. The levels of actin transcripts were determined from the same RNA as an internal control and surprisingly were found to increase about three fold due to wounding (Fig. 3b). The levels of RUBISCO small subunit (ss) transcripts were therefore also determined from the same RNA preparations and were found to be identical (Fig. 3c).

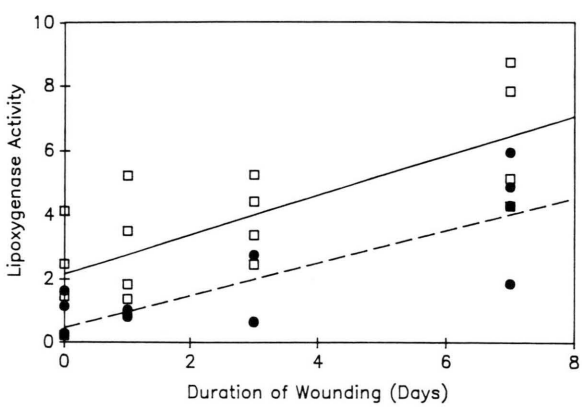

Fig. 1. Increase in lipoxygenase activity due to wounding (see experimental section for details) in the wounded leaves per se (□) ($y = 2.159 + .615x$) and in the next unwounded leaves from the wounded plants (●) ($y = .466 + .507x$). LOX activity was measured as ΔA_{234} min^{-1} mg $protein^{-1}$. The data are presented as regressions *versus* unwounded controls which were the same age. Intercepts were significantly different ($p < 0.05$), but slopes were not significantly different ($p > 0.05$).

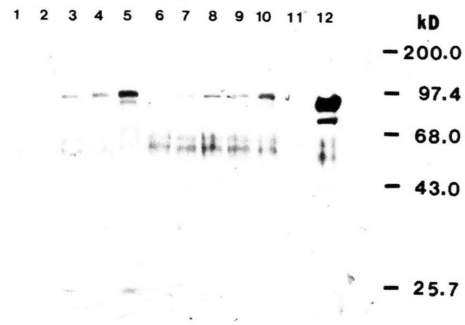

Fig. 2. Immunoblot of an SDS-PAGE gel of the wounded trifoliates (lanes 1–5) and the next trifoliates which were unwounded from plants with wounded leaves (lanes 6–10) and purified lipoxygenase from Sigma Chem. Co. (lane 12). Wounding for 0 days (lanes 1 and 6), four days (lanes 2 and 7), seven days (lanes 3 and 8), ten days (lanes 4 and 9) and fourteen days (lanes 5 and 10).

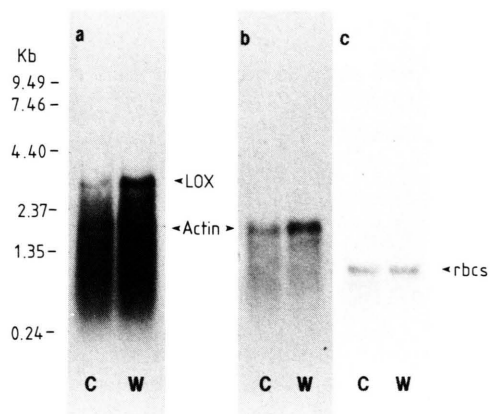

Fig. 3. Northern blot analysis of poly (A) enriched RNA from unwounded (control; C) and wounded (W) soybean leaves. a. A blot that was hybridized with the LOX 3 cDNA probe and with the actin probe; b. A blot that was hybridized with the actin probe only and c. A blot that was hybridized with the pea rbcs probe. Based on the absorbance at 260 nm 12 and 9 μg of poly A enriched RNA were electrophoresed in lanes C and W respectively.

Discussion

These studies demonstrate that LOX in soybean leaves can be induced by wounding and that this induction is at least partly at the transcriptional level. The results also show that wounding can indirectly increase LOX activity presumably by signals that are translocated to the unwounded leaves in wounded plants. The variable effects of mite infestations are probably due to different feeding activity (and therefore different levels of wounding) of the mites applied. However, the mites which were applied were confined to the same leaf and feeding surely took place as evidenced by chlorotic spots on the leaves. It is also possible that the mites feed in such a way that the plant does not recognize their feeding as the type of wounding to elicit increased LOX or maybe mites inject something into the plant that blocks the induction of LOX. The increased LOX activity due to wounding and the fact that continued wounding up to 14 days resulted in a steady increase in LOX indicates that LOX induction is more quantitative than that of many other so-called PR proteins [1, 2]. Some of the PR proteins which are induced for which functions can be readily postulated include chitinase, trypsin inhibitor, and β-glucanase [4–6]. In genotypes of some plant species these proteins can be very readily induced and may be final targets of signals in the induced resistance process. LOX changes are more consistent with it playing a role in enhancing signal production. It is possible that some of the products of LOX reactions (such as hexenal, nonenal or 12-oxo-dodecenoic acid) or cooxidation reactions (such as β-ionone) act as signals or elicit signal production [22]. If this is the case, then the increase seen in LOX upon wounding could enhance the capacity of plant tissues to produce antipest metabolites. It is also possible that LOXs show a general quantitative increase but some isozymes show large qualitative increases analogous to what is seen for peroxidases [23]. This is apparently the case for LOXs in oats [13].

The increase in LOX may have direct effects in increasing the pest resistance of plant tissues. For instance the increased LOX may stimulate the hypersensitive response (host cell necrosis) which is a major form of pest resistance effective in many crop plants to viral, bacterial and fungal pathogens [24–27]. Often associated with the HR is the loss of chlorophyll followed by browning and localized, rapid death of cells at the infection site [9, 28–31]. The accumulation of phytoalexins, deposition of lignin-like materials and the oxidation of phenols are often associated with HR [28]. The oxidation of membrane lipids appears to be a key component of the hypersensitive response [10]. The increased LOX activity may enhance the peroxidizing conditions, which in turn could promote the elicitation of HR. Also, some LOX products have direct antipest activity as previously described [22, 32–36].

Karban [37] found that mite resistance in cotton was inducible both by prior mite infestation, wounding or infection with a fungal pathogen [38]. Previously, we showed increased LOX activity in mite infested [11], and in this study, wounded soybean leaves. Further studies are needed to determine the biological significance of the wound and pest induced LOX activity. One way in which we are approaching this is by altering levels of LOX in transgenic plants in order to provide useful materials for *in vivo* studies.

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[1] J. G. Parent and A. Asselin, Phytopathology **77,** 1122 (1987).
[2] L. C. van Loon, Y. A. M. Gerritsen, and C. E. Ritter, Plant Molec. Biol. **9,** 593 (1987).
[3] J. Logemann, J. E. Mayer, J. Schell, and L. Willmitzer, PNAS **85,** 1136 (1988).
[4] R. Vogeli-Lange, A. Hansen-Gehri, T. Boller, and F. Meins Jr, Plant Science **54,** 171 (1988).
[5] M. A. Lawton and C. J. Lamb, Molec. Cell. Biol. **7,** 335 (1987).
[6] W. E. Brown and C. A. Ryan, Biochemistry **23,** 3418 (1984).
[7] M. E. Kraemer, M. Rangappa, W. Gade, and P. S. Benepal, J. Econ. Entomol. **80,** 237 (1987).
[8] J. Fournier, B. Pelissier, and Esquerre-Tugaye, C. R. Acad. Sc. Paris **303,** 651 (1986).
[9] C. A. Ocampo, B. Moerschbacher, and H. J. Grambow, Z. Naturforsch. **41,** 559 (1986).
[10] L. D. Keppler and A. Novacky, Physiol. Molecul. Plant Pathol. **30,** 233 (1987).
[11] D. F. Hildebrand, J. G. Rodriquez, G. C. Brown, K. T. Luu, and C. S. Volden, J. Econ. Entomol. **79,** 1459 (1986).
[12] P. Ruzicska, Z. Gombos, and G. Farkas, Virology **128,** 60 (1983).
[13] H. Yamamoto and T. Tani, Phytopathology **116,** 329 (1986).
[14] W. R. Fehr and C. E. Caviness, Iowa State Univ. Agri. Expt. Sta., Special Report **80,** 1 (1977).
[15] D. F. Hildebrand and T. Hymotwitz, J. Am. Oil Chem. Soc. **58,** 583 (1981).
[16] B. Taylor and A. Powell, Bethesda Research Labs. Focus **4,** 6 (1982).
[17] T. Maniatis, E. F. Fritsch, and J. Sambrook, Molecular cloning, a laboratory manual. Cold Spring Harbor Press 1982.
[18] R. L. Yenofsky, M. Fine, and C. Liu, Mol. Gen. Genet. **211,** 215 (1988).
[19] D. M. Shah, R. C. Hightower, and R. B. Meagher. PNAS **79,** 1022 (1982).
[20] G. Coruzzi, R. Broglie, E. C. Edwards, and N.-H. Chua, EMBO J. **3,** 1671 (1984).
[21] A. P. Feinberg and B. Vogelstein, Analyt. Biochem. **132,** 6 (1983).
[22] S. D. Salt, S. Tuzun, and J. Kuc, Physiol. Molecul. Plant Pathol. **28,** 287 (1986).
[23] E. Roberts, T. Kutchan, and P. E. Kolattukudy, Plant Molec. Biol. **11,** 15 (1988).
[24] R. N. Goodman, Z. Kiraly, and K. R. Wood, in: The biochemistry and physiology of plant disease, 433 p, University of Missouri Press, Columbia, Missouri 1986.
[25] Z. Klement, in: Phytopathogenic Prokaryotes (M. S. Mocurt and G. H. Lacy, eds.), **Vol. 2,** p. 149, Academic Press, New York 1982.
[26] F. Ponz and G. Bruening, Annu. Rev. Phytopathol. **24,** 355 (1986).
[27] M. Zaitland and R. Hull, Annu. Rev. Plant Physiol. **38,** 291 (1987).
[28] R. M. Bostock and D. A. Schaeffer, Physiol. Molecul. Plant Pathol. **29,** 349 (1986).
[29] M. Zook and J. Kuc, in: The metabolism, structure, and function of plant lipids (P. K. Stumpf and J. B. Mudd, eds.), p. 75, 1987.
[30] Y. Cohen and H. Eyal, Phytopathology **78,** 144 (1988).
[31] Y. Cohen, S. Peer, O. Balass, and M. D. Coffey, Phytopathology **77,** 201 (1987).
[32] I. S. Barimalaa and M. H. Gordon, J. Agric. Food Chem. **36,** 685 (1988).
[33] R. C. French, C. L. Graham, A. W. Gale, and R. K. Long, J. Agric. Food Chem. **25,** 84 (1977).
[34] R. C. French, J. Agric. Food Chem. **31,** 423 (1983).
[35] A. Miglietta, G. Ludovica, and E. Gadoni, Cell Biochem. Function **5,** 189 (1987).
[36] M. Shimura, S. Mase, M. Iwata, A. Suzuki, T. Watanabe, Y. Sekizawa, T. Sasaki, K. Furihata, H. Seto, and N. Otake, Agric. Biol. Chem. **47,** 1983 (1983).
[37] R. Karban, Entomol. Exp. Appl. **37,** 137 (1985).
[38] R. Karban, R. Adamchak, and W. C. Schnathorst, Science **235,** 678 (1987).