

Characterization of an *Arabidopsis* Lipoxygenase Gene Responsive to Methyl Jasmonate and Wounding¹

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A cDNA corresponding to the gene *AtLox2* was isolated from an *Arabidopsis thaliana* library using a lipoxygenase (LOX) probe from soybean. *AtLox2* encodes a 102-kD protein, AtLOX2, which has 42 to 45% amino acid sequence identity with other plant LOX sequences. The AtLOX2 sequence is more than 30 amino acids longer at the amino terminus than other plant LOX sequences, and this extension has features reminiscent of chloroplast transit peptides, suggesting that AtLOX2 may be chloroplast localized. *AtLox2* mRNA levels are high in leaves and inflorescences but very low in seeds, roots, and stems. *AtLox2* mRNA accumulation is rapidly induced in leaves in response to methyl jasmonate. Leaves that have been wounded and adjacent leaves on the same plant also accumulate *AtLox2* mRNA.

LOXs catalyze the hydroperoxidation of specific unsaturated fatty acids. During the past several years genes encoding these enzymes have been characterized from both plants and mammals. In mammals, LOXs catalyze an initial step in the production of biologically active compounds including leukotrienes and lipoxins. In plants, the growth regulator JA is produced from a LOX metabolite (Vick and Zimmerman, 1983), and there is evidence that LOX or a related enzyme is involved in ABA biosynthesis (Creelman et al., 1992a). LOX mRNA accumulates in response to water deficit or wounding (Bell and Mullet, 1991), and a differential expression of LOX mRNA or activity in response to sensitive or resistant pathogen interactions has also been reported (Ocampo et al., 1986; Melan et al., 1993). Thus, there is evidence for important roles for LOX in several plant processes, either through the production of growth regulators such as JA or by some other activity. So far, however, it has been difficult to identify specific functions that require the changes in LOX expression that are seen in different growth conditions.

As a first step toward examining the physiological significance of regulated LOX expression, we set out to characterize the LOX gene family in *Arabidopsis thaliana*, with the hope that it would be relatively noncomplex. We report here the isolation and characterization of an *Arabidopsis* cDNA representing the gene *AtLox2*. Although *AtLox2* encodes a protein with similarity to other plant LOXs, the degree of identity seen suggests that the AtLOX2 protein may belong to a previously unidentified LOX class. *AtLox2* mRNA shows tissue-specific distribution within the plant and accumulates

in response to wounding or meJA treatment. The expression pattern of *AtLox2* is different from that of another recently characterized *Arabidopsis* LOX gene, *AtLox1* (Melan et al., 1993), supporting the possibility that the corresponding proteins have separate roles within the plant.

MATERIALS AND METHODS

cDNA Cloning

Arabidopsis thaliana (Columbia) was grown (8-h day) for several weeks. Rosettes were excised, dehydrated to 85% of their fresh weight, incubated for 4 h, and then harvested. The resulting mRNA was used for cDNA library construction in λ gt10.

As described by Bell and Mullet (1991), primers to conserved regions of soybean LOX sequences were used to amplify soybean cDNA sequences by the PCR, and the amplification products were used as a probe. This soybean probe was used for screening the *Arabidopsis* cDNA library described above. For plaque screening, filters were hybridized as described previously (Bell and Mullet, 1991) and washed in 2 \times SSC, 0.1% SDS at 23°C, followed by a 37°C wash in the same solution. *EcoRI* inserts from positive isolates were cloned into the plasmid pTZ19R (United States Biochemical) at the *EcoRI* site for further analysis.

Growth Conditions

Plants grown in soil under continuous light for 5 weeks were harvested for different above-ground tissues. Roots were obtained by growing plants in culture. For cultured plants, 30 to 40 sterilized seeds were added to 20 mL of liquid germination medium (Valvekens et al., 1988) in a Magenta (Chicago, IL) GA7 vessel and incubated (14-h day) for 18 to 24 d. Experiments requiring the treatment of plants with different compounds were performed on cultured plants, with the compound of interest added directly to the liquid medium, followed by brief agitation to distribute it. ABA and meJA were added as 1000 \times stocks (20 mM, to give a final concentration of 20 μ M) in ethanol, and an equal amount of ethanol was added to an ethanol control sample. Sodium salicylate was added as a 1000 \times stock (30 mM, to give a final concentration of 30 μ M) in water.

For the time course sterilized seeds were sown in rows on germination medium agar plates (day 0), incubated at 4°C

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Abbreviations: JA, jasmonic acid; LOX, lipoxygenase; meJA, methyl jasmonate; PCR, polymerase chain reaction.

overnight, and then oriented vertically in a growth chamber (14-h day) until harvest.

Plants for wounding were grown for 8-h days for several weeks and then transferred to 14-h days for 10 to 12 d. Each leaf was wounded once with a hemostat on the upper third of the leaf in line with the midvein. In each rosette half of the leaves were wounded and half were not. As controls some plants were touched to approximate the handling wounded plants received, and others were not intentionally touched.

RNA Analysis

Nucleic acid was isolated and analyzed as described by Bell and Mullet (1991). The *AtLox2* probe was the 2100-bp *SacI* fragment of the plasmid clone, which contains all but the 5' end of the gene. The *AtLox1* probe was the 2640-bp *EcoRI* fragment of that plasmid clone (Melan et al., 1993). For each blot a duplicate was hybridized with an rRNA gene fragment to verify equal loading of different RNA samples (data not shown).

Sequence Comparison

Sequence analysis and comparison was done using the GAP and PILEUP programs of the Genetics Computer Group (Madison, WI) sequence analysis software.

RESULTS

Gene Isolation and Analysis

In an attempt to isolate LOX genes from *Arabidopsis* we used a PCR strategy that had been successful in obtaining LOX genes from soybean (Bell and Mullet, 1991). Oligonucleotide primers to conserved regions of LOX sequences were used to amplify DNA, and amplification products were used as a probe. Screening of our *Arabidopsis* cDNA library with this probe did not yield any clones that showed LOX homology when sequenced. Therefore, the original soybean LOX PCR products were used as a heterologous probe. Of the positive clones obtained, one, *AtLox2*, was analyzed most extensively. Exhaustive screening of the cDNA library with *AtLox2* did not yield any isolates with a sequence that differed from it, and analysis of genomic DNA gave a pattern suggesting the presence of one or possibly two genes that hybridize to *AtLox2* (data not shown).

The nucleotide and deduced amino acid sequences of *AtLox2*, which encodes the 102-kD protein AtLOX2, are shown in Figure 1. This amino acid sequence aligns well with other LOX sequences, including that of the *Arabidopsis* LOX, AtLOX1, recently characterized by Melan et al. (1993). Somewhat surprisingly, AtLOX2 has only 45% amino acid sequence identity with AtLOX1, comparable to the 42 to 44% identity seen between AtLOX2 and other available plant LOX amino acid sequences. Alignment of AtLOX2 with AtLOX1 occurs throughout the sequences, with the least identity seen at the extreme amino terminus.

As shown in Figure 2, AtLOX2 contains the six conserved His residues (five clustered, one distant) that have been postulated to be important for enzyme activity of both plant

and mammalian LOXs. Mutational analysis of human 5-LOX and soy LOX1 has shown that three of these His's, corresponding to residues 554, 559, and 746 of AtLOX2, are essential for enzyme activity (Steczek et al., 1992; Zhang et al., 1992). When sequence comparison is done between the two *Arabidopsis* LOXs, a legume LOX (soy LOX3), rice LOX, and a mammalian LOX (human 5-LOX), the clustered His residues are in a region where 23 of 60 residues show complete identity. The other conserved His is at the start of a nine-amino acid block that is identical in all five sequences. Mutation of the Gln in this block reduces the activity of human 5-LOX (Zhang et al., 1992).

Gene Expression

To characterize the expression pattern of the *AtLox2* gene, RNA was isolated from different tissues of *Arabidopsis*. All mature above-ground tissues were obtained from plants grown in soil, whereas seedlings and roots were obtained from plants grown in culture. As shown in Figure 3, substantial levels of *AtLox2* mRNA were present in leaves and inflorescences, whereas this mRNA was almost undetectable in green siliques, stems, and roots. *AtLox2* mRNA was not detected in dry seeds but increased incrementally as seedling development occurred. Levels of this mRNA in 7-d-old seedlings were lower than those seen in mature leaves. This pattern of expression is different from that reported for *AtLox1*, whose mRNA is 2- to 2.5-fold more abundant in roots and in 3-d-old seedlings than in leaves (Melan et al., 1993).

Three compounds, ABA, meJA, and salicylate, which have been proposed as modulators of stress responses in plants (Enyedi et al., 1992; Hildmann et al., 1992), were tested for the ability to induce *AtLox2* mRNA accumulation (Fig. 4A). Of these, only meJA treatment resulted in increased *AtLox2* mRNA levels, whereas ABA and salicylate caused some reduction in *AtLox2* mRNA. Ethanol also appeared to slightly induce *AtLox2* expression. The meJA-induced mRNA accumulation was both rapid, reaching near-maximal levels within 2 h, and tissue specific, occurring in the leaves but not the roots. Additional analysis showed no induction in roots even at an earlier time (8 h) when maximal levels were still present in leaves (data not shown). In contrast, the reported induction of *AtLox1* by meJA (Melan et al., 1993) appeared to be root specific, with no induction over ethanol control levels detected in leaves (Fig. 4A).

Accumulation of *AtLox2* mRNA was also induced by wounding (Fig. 4B). This accumulation occurred within 6 h, both in the leaves that had been wounded and in adjacent leaves on the same plant. No analogous induction of *AtLox1* mRNA was detected.

DISCUSSION

We have determined that *AtLox2* encodes a LOX based on the similarity of its deduced amino acid sequence to those of other LOXs. This similarity occurs throughout the sequence, and AtLOX2 contains the features that have so far been identified as being important for LOX enzyme function. Although this homology is significant, AtLOX2 shows less

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1  aagccttataagaagaaaaaatatgtattgtagagagtccttctgcagtcctcagacttgaacgtagcaaaagagctcagctcttggctccctaaacaatcagctctcatcaacc
   M Y C R E S L S S L Q T L N V A K S L S S L F P K Q S A L I N P
121 gatctccgctggagctcgaaaacactgcctcgtccaaacctcagaagacgatgtaaggtaccgcttccagggctaatattgaacaagaaggttaacacagtgaaagaaccattcagaa
   I S A G R R N N L P R P N L R R R C K V T A S R A N I E Q E G N T V K E P I Q N
241 tattaaagtaaaggatacataacggcccaagaaggttctctggagggcataacttggctcgagaggtctcgcgatgacattgctgatatccgaggagatcattgctcgtcagcttattag
   I K V K G Y I T A Q E E F L E G I T W S R G L D D I A D I R G R S L L V E L I S
361 cgccaagactgaccacggattacggtagaagactacgcacaacgtgtatggccgaagccctgatgagaagtacgagtgcgagtttgagatgcctgaagactttggaccagtgaggc
   A K T D Q R I T V E D Y A Q R V W A E A P D E K Y E C E F E M P E D F G P V G A
481 catcaagattcagaaccaataccatcgacagttgttctcctcaaggggtagagcttaaattaccggcgggtcgataacgctttacatgtgagctcatgggtggccccaagctccgttgacc
   I K I Q N Q Y H R Q L P L K G V E L K L P G G S I T F T C E S W V A P K S V D P
601 aaccaagcgatattctctccagaactcctactgcctcccaaacaccagagcctctctaaagatcgaaaagaggagctcgagaccttacaagcaagaaccggaggaagttgg
   T K R I F F S D K S Y L P S Q T P E P L K K Y R K E E L E T L Q G K N R E E V G
721 tgaatttccaagtttgagcgcatttacgactatgatgtgtacaacgatgttggtagccctgacaatgatcctgactgctcgtccggtaattggaggcctcacacatccatccaag
   E F T K F E R I Y D Y D V Y N D V G D P D N D P B L A R P V I G G L T H P Y P R
841 gcgttgaagaccggctgaaacctgtgagactgacctcttcagagcaacgctacgggggagagttctatgtaccaagagacgaggagttctctacagccaagggcacttcattc
   R C K T G R K P C E T D P S S E Q R Y Y G G E F Y V P R D E E F S T A K G T S P T
961 agccaagcgcgtactggcagctcttccctccatcttccctcagatcgagctgttctattgagctcccaagaacctttccacacttcaagccatacaaaatctcttgaagaaggcat
   G K A V L A A L P S I F P Q I E S V L L S P Q E P F P H F K A I Q N L P E E G I
1081 tcagcttcccaagatgctggccttctacctcgtctcccaagaatcatcaagctcttggcgaagctcaagatgatattctccagtttgatgccccagttctcattaacagggatagatt
   Q L P K D A G L L P L L P R I I K A L G E A Q D D I L Q F D A P V L I N R D R F
1201 ttcatggttacgagacgagatttgcctccagacacttgcggccttaactctatagcattcagctagttgaagagtgccgtaataagaactagaccggcgtttatggtga
   S W L R D D E F A R Q T L A G L N P Y S I Q L V E E W P L I S K L D P A V Y G D
1321 tcccacctcactcattctgggaaattgtggaagagaagttaaaggaacatgacagttgatgaggtcctaaagaataagagattgtctggttgattatcatgattgcttacc
   P T S L I T W E I V E R E V K G N M T V D E A L K N K R L F V L D Y H D L L L P
1441 atatgtgaacaaagtgagagattaaacaataccacattatagcttctagaacaattcttctcagcagatagacacattgagcctgttgccattgagttgactgtccacaaa
   Y V N K V R E L N N T T L Y A S R T L F F L S D D S T L R P V A I E L T C P P N
1561 tatcaacaagcccaatggaacaagtcttcacgcccagctatgatgctacgtcagctggttgaatcttgtaagactcatgctatctctcatgacgcccggttatcatcagcttat
   I N K P Q W K Q V F T P G Y D A T S C W L W N L A K T H A I S H D A G Y H Q L I
1681 ttcccattggtgaggactcagctgtacggagccataataagcggcaaatagacaactaagtcacatgccatttataggtctttgcatcctcattccgctacaccatgga
   S H W L R T H A C T E P Y I I A A N R Q L S A M H P I Y R L L H P H F R Y T M E
1801 aatcaagctcgtgcacgccaagtctgtcaacgggtggaatcattgagactgttctctgcccgggaagtatgcattagagctaagtcagcgcgtctatggtaaactatggaggtt
   I N A R A R Q S L V N G G G I I E T C F W P G K Y A L E L S S A V Y G K L W R F
1921 tgaccaggaaggtctaccgcggtatcctcaaaaggggttgctgaggaagataagaccgcagaacatggagtagctctgacgataccagactatccattgcaaatgatggtcta
   D Q E G L P A D L I K R G L A E B D K T A E H G V R L T I P D Y P F A N D G L I
2041 attgtggatgcaattaaagaatgggtgacagactatgtaaacattactatcctgatgaagaactgatcacatcgagcaggaactccaaggatgggtgagtgaaagtgcggaacatagg
   L W D A I K E W V T D Y V K H Y Y P D E E L I T S D E E L Q G W W S E V R N I G
2161 ccacggagacaagaagacgaaccttgggtgcctcctcaaacacaagatgactgtggtgtggttaactacgattgcatgggtcaccctcaggtcaccatgacagctgtaaaccttgg
   H G D K K D E P W W P V L K T Q D D L I G V V T T I A W V T S G H H A A V N F G
2281 acagtcggataggagatacttcccaaccgaccaacgacaacaaggataagaatgccaacggaagatccgacagatgaagccttaaaagagttctatgagtcgccgaaaagagttt
   Q Y G Y G G Y F P N R P T T T R I R M P T E D P T D E A L K E F Y E S P E K V L
2401 gcttaagacataccatcgagaaacagggcaccctagtgatggtcagctggtatctttatcaacacattcactgacgaagagatcattggagaacaacaagaagcattctgggccaa
   L K T Y P S Q K Q A T L V M V T L D L L S T H S P D E E Y I G E Q Q E A S W A N
2521 tgagcctgtatcaatgctgcaattgaaagattcaaaagcaagctccaatataagaggagtgatagatgagagaacgtgaacattacttaagaatagagctggagctggtggtgt
   E P V I N A A F E R F K G K L Q Y L E G V I D E R N V N I T L K N R A G A G V V
2641 taagatgagctttgaaagcctcctcgtgagcaggtgttaccggaatgggtgttcttatagattttctattgatctatgcttttcttctcaaaatgcttcttaattttctgt
   K Y E L L K P T S E H G V T G M G V P Y S I S I
2761 atctctgctcttgataaaatgaataaaaacaaatgctccttctctcc 2809

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Figure 1. Nucleotide and deduced amino acid sequences of the *AtLox2* cDNA. The open reading frame encodes a 896-amino acid polypeptide.

identity with other available plant LOX sequences than they do with each other. For example, the derived amino acid sequence of the other *Arabidopsis* LOX, *AtLOX1*, is 56 to 62% identical with LOX sequences from legumes and rice but only 45% identical with *AtLOX2*. Thus, *AtLOX2* may represent a category of plant LOX types that have not previously been characterized.

Another interesting feature of the *AtLOX2* sequence is that it is more than 30 amino acids longer at the amino terminus than the other plant LOX sequences. This extension may function in localization of the protein. The amino terminus has some of the general features described for chloroplast transit peptides (von Heijne and Nishikawa, 1991), including a high proportion of Ser and Thr residues and a near lack of

soy LOX3	DSCYHQLVSH	WLNTHAVVEP	FIIATNRHLS	VVHPIYKLLH	PHYRDTMNIN	GLARLSLVND	568
At LOX1	DSGNHQLISH	WMQTHASIEP	FVIATNRQLS	VLHPVFKLLE	PHFRDTMNIN	ALARQILING	569
rice LOX	DYCWHQLISH	WLNTHAVMEP	FVIATNRQLS	VAHPVHKLLL	PHYRDTMTIN	GLARQTLING	571
At LOX2	DAGYHQLISH	WLRTHACTEP	YIIAANRQLS	AMHPIYRLLH	PHFRYTMFIN	ARARQSLVNG	604
human 5-LOX	DFHVHQITTH	LLRTHLVSEV	FGIAMRYQLP	AVHPIFKLLV	AHVRPTIAIN	TKAREQLICE	417
Consensus	D---HQ--- <u>H</u>	---T <u>H</u> ---E-	--IA--R-L-	--HP---LL-	-H-R-T--IN	--AR--L---	
soy LOX3	. . .	CAIIIWTASA	LHAAVNFQGY	PYGGILNRP	727		
At LOX1	. . .	CTIIIWVASA	LHAAVNFQGY	PVAGYLPNRP	729		
rice LOX	. . .	CATIIWIGSA	LHAAVNFQGY	PYAGYLPNRP	729		
At LOX2	. . .	VTIIAWVTSG	HAAVNFQGY	GYGGYFPNRP	764		
human 5-LOX	. . .	LTVVIFTASA	QHAAVNFQGY	DWCWIPNAP	568		
Consensus		-----S-	- <u>H</u> AAVNFQGY	-----N-P			

Figure 2. Amino acid sequence comparison of specific regions of several LOXs. Numbers indicate the position of each block in the corresponding sequence; the two blocks shown are not contiguous. His residues underlined in the consensus line are those shown by mutagenesis to be required for enzyme activity (Steczko et al., 1992; Zhang et al., 1992). Alignment is with soybean (soy) LOX3 (Yenofsky et al., 1988), *Arabidopsis* (At) LOX1 (Melan et al., 1993), rice LOX (Ohta et al., 1992), and human 5-LOX (Dixon et al., 1988).

acidic residues, suggesting the possibility that AtLOX2 is chloroplast localized. LOX protein (Grimes et al., 1992) and activity (Vick and Zimmerman, 1987) have been reported in a number of subcellular compartments including chloroplasts, and it may be that the location of individual isozymes is important for specific physiological functions.

AtLox2 mRNA is not detectable in seeds, but levels slowly increase during the first few days of seedling development. The level of *AtLox2* mRNA in seedlings is less than that seen in leaves or inflorescences of mature plants. This mRNA was faintly detectable in roots only after a long exposure of the blot to film. The expression pattern seen for *AtLox2* is another distinguishing feature between it and *AtLox1*, which is most

strongly expressed in roots and in young seedlings (Melan et al., 1993).

The lack of *AtLox2* mRNA in roots is not overcome by treatment with meJA, which induces *AtLox2* mRNA accumulation in leaves to near-maximal levels within 2 h. The tissue-specific effect of meJA on LOX induction is also seen

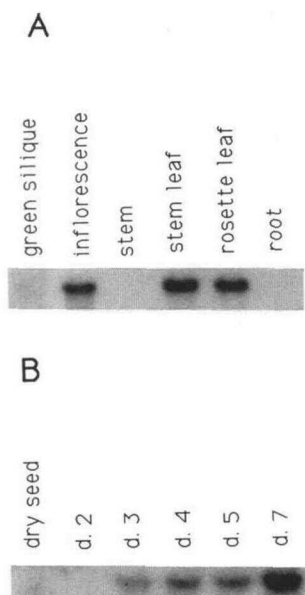


Figure 3. *AtLox2* mRNA accumulation in *Arabidopsis* tissues. Blots were hybridized with an *AtLox2* probe as described in "Materials and Methods." A, Tissue specificity of *AtLox2* expression. Nucleic acid was loaded at 5 μ g/lane. B, Developmental induction of *AtLox2* mRNA. Times shown are days after sowing seeds. Nucleic acid was loaded at 7.8 μ g/lane.

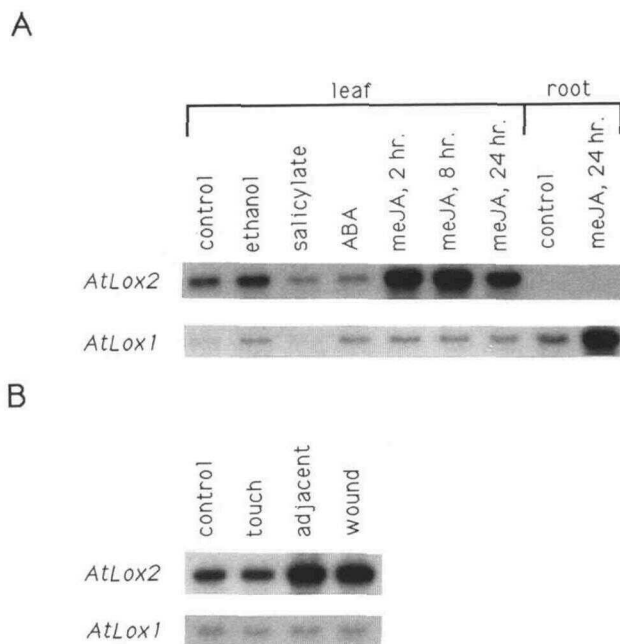


Figure 4. *AtLox2* and *AtLox1* mRNA accumulation in treated plants. Blots were hybridized with an *AtLox2* probe or an *AtLox1* probe as indicated. A, Effect of specific compounds on *AtLox2* and *AtLox1* mRNA levels. Cultured plants were untreated (control) or treated with meJA (20 μ M), ABA (20 μ M), sodium salicylate (30 μ M), or ethanol as described in "Materials and Methods." Incubation was for 8 h or for the time indicated, following which leaves and roots were harvested. Nucleic acid was loaded at 5 μ g/lane. B, Accumulation of *AtLox2* and *AtLox1* mRNA in wounded leaves. Wound and control plants were treated as described in "Materials and Methods" and then incubated for 6 h, after which leaves were harvested. The "adjacent" sample is from leaves that were on the same plant as the wounded leaves. Nucleic acid was loaded at 5 μ g/lane.

for *AtLox1*, which is induced in roots but not in leaves. The meJA-responsive *AtLox2* mRNA accumulation is more rapid than that previously observed in soybean cell cultures, where LOX mRNA reached maximal levels more than 6 h following exposure to meJA (Bell and Mullet, 1991). There have now been several reports of LOX induction by meJA (Bell and Mullet, 1991; Grimes et al., 1992; Melan et al., 1993). LOX is involved in jasmonate biosynthesis (Vick and Zimmerman, 1983), and the induction of LOX by jasmonate may be important for increasing jasmonate levels under certain growth conditions. In soybean a LOX has also been classified as one of several vegetative storage proteins (Tranbarger et al., 1991) that are coordinately induced under distinct growth conditions, one of which is exposure to meJA (Grimes et al., 1992).

Two other compounds, salicylate and ABA, which have been characterized as modulators of stress responses in plants, appeared to slightly reduce *AtLox2* expression. At this point the possible significance of this is not clear. We saw no effect of these compounds on *AtLox1* expression in leaves, but Melan et al. (1993) reported substantial *AtLox1* induction by ABA in roots. Given the differences in experimental design, it will require further work to determine whether ABA, like meJA, has a tissue-specific effect on *AtLox1* expression.

AtLox2 mRNA accumulation is induced by wounding both in leaves that are directly wounded and in unwounded leaves on the same plant. As observed in soybean (Creelman et al., 1992b), wounding of *Arabidopsis* leads to an increase in JA/meJA levels in the plant within 3 h (R. Creelman, personal communication). Thus, it is possible that the wound induction of *AtLox2* is a consequence of increasing jasmonate concentrations in the plant following wounding. Alternatively, another wound-induced compound that is capable of movement through the plant could be affecting LOX expression.

As described here and in the report of Melan et al. (1993), two *Arabidopsis* LOX genes have been identified. These genes encode proteins with surprisingly divergent sequences and show different patterns of expression within the plant. Although both are responsive to meJA, the pattern of meJA induction is distinct for each gene. These differences support the hypothesis that individual LOX isozymes have unique physiological roles. Available evidence indicates that the *Arabidopsis* LOX gene family is small, making this an ideal system in which to investigate the role of specific LOX isozymes in growth and stress responses.

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