Plant Physiol. (1997) 114: 1561–1566

Rapid Communication

Mechanosensitive Expression of a Lipoxygenase Gene in Wheat[1]

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Touch stimulation of wheat (*Triticum aestivum* L.) seedlings led to a strong and dose-dependent increase in the level of lipoxygenase mRNA transcripts. The touch-induced response occurred within 1 h and was transient. A similar response was observed after wind treatment and wounding. The mechanical strain-regulated lipoxygenase might translate mechanical strain into lipoxygenase pathway-dependent growth responses.

Mechanical signals have an important influence on the development and morphology of higher plants. In many cases, mechanical stress results in a reduction of growth rates that leads to a sturdier plant. These adaptive responses, termed thigmomorphogenesis, are displayed by a majority of plant species (Jaffe and Forbes, 1993; Mitchell and Myers, 1995). Recent evidence suggests that mechanical signals might also play a role in gravitropism and in the determination of plant form (Ding and Pickard, 1993; Trewavas and Knight, 1994). Whereas the phenotypical effects of mechanical strain on plant growth have been well documented, the molecular mechanisms underlying touch perception and mechanotransduction are not well understood. Braam and Davis (1990) described a number of genes in *Arabidopsis thaliana* that were up-regulated by touch and wind. Three of the five touch-induced genes encoded calmodulin and calmodulin-like proteins. Later, touch-induced calmodulin genes were also detected in potato (Takezawa et al., 1995) and mung bean (Botella et al., 1995). A second mechanical strain-induced gene was identified in mung bean as an ACC synthase gene (Botella et al., 1995).

Using the *WCI-2* cDNA, which represents a wheat (*Triticum aestivum* L.) transcript that accumulates upon treatment with the disease resistance-inducing chemicals DC-INA and BTH (Görlach et al., 1996), as a probe, we serendipitously detected a touch-regulated LOX gene in wheat. Similar to the known mechanical strain-induced genes, this wheat LOX gene was rapidly and transiently induced by touch, wind, and wounding. LOXs (EC 1.13.11.12) are a family of enzymes that catalyze the introduction of molecular oxygen into the *cis,cis*-1,4-pentadiene structure of polyunsaturated fatty acids (Vick and Zimmermann, 1987; Siedow, 1991). LOXs have been implicated in plant growth and development, mobilization of lipid reserves, senescence, ABA biosynthesis, wound responses, and resistance against pathogens and pests (Ocampo et al., 1986; Vick and Zimmerman, 1987; Creelman et al., 1992; Croft et al., 1993). The primary products of LOX-catalyzed reactions, fatty acid hydroperoxides, are often metabolized into molecules with known or suspected regulatory activity, such as jasmonic acid and traumatic acid (Vick and Zimmermann, 1987; Vick, 1991; Hamberg, 1993). Jasmonic acid and its methyl ester have been shown to affect many physiological processes (Koda, 1992; Staswick, 1992), including the induction of a large number of genes (Sembdner and Parthier, 1993). In particular, jasmonates have been implicated as signal transduction molecules in the response of plants to stress in the form of wounding and pathogen attack (Creelman et al., 1992; Farmer et al., 1992). It is interesting that individual LOX genes were shown to be induced by jasmonates, and it was suggested that the products of these genes are part of a jasmonate-based signal amplification mechanism (Bell and Mullet, 1991; Grimes et al., 1992; Melan et al., 1993). In this report we describe the regulation of the expression of a wheat LOX gene by different mechanical stimuli.

MATERIALS AND METHODS

Growth and Treatment of Wheat Seedlings

Wheat (*Triticum aestivum* L. cv 75141) plants obtained from the Swiss Federal Research Station for Agronomy (Zürich-Reckenholz, Switzerland) were grown at 21°C with a 12-h photoperiod (180 μE m^{-2}). Seven-day-old seedlings were inoculated in a moist chamber with conidiospores of *Erysiphe graminis* f. sp. *tritici* or *E. graminis*. f. sp. *hordei* by

[1] This work was supported by the Kommission zur Förderung der wissenschaftlichen Forschung and by Ciba-Geigy.

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Abbreviations: BTH, benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester; DC-INA, 2,6-dichloro isonicotinic acid; LOX, lipoxygenase.

brushing plants infected 7 d earlier over the test plants. Application of DC-INA was by soil drenching. The final concentration calculated for the entire soil volume was 0.5 mM DC-INA. Treatment with methyl jasmonate was performed in airtight plexiglass containers by adding the methyl jasmonate diluted in 50% ethanol to cotton balls. Mechanical stimulation was applied by horizontally moving a glass rod 10 times within 30 s through the seedlings. For stronger stimulation this treatment was repeated 10 times at 1-min intervals. Wounding was done by squeezing the seedlings with forceps 6 to 8 times over their length. For wind treatment seedlings were placed in front of a fan that generated a wind speed of 1 m s^{-1}. For all of the experiments the pots containing the seedlings were handled with the utmost care.

RNA Gel-Blot Analysis and Genomic DNA-Blot Analysis

Total RNA was isolated from the aboveground parts of 7-d-old seedlings, as previously described (Dudler et al., 1991). RNA samples (10 μg) were separated by electrophoresis through formaldehyde-agarose gels, as described in the protocol of the ZAP cDNA synthesis kit (Stratagene). The gels were blotted onto nylon membranes and hybridized to the random-primed ^{32}P-labeled cDNA probe, according to standard procedures. Ethidium bromide was included in the loading buffer to allow direct confirmation of equal sample loading and RNA transfer. An RNA ladder (Pharmacia) was used as a size standard. Total genomic DNA was isolated from 7-d-old wheat seedlings. Ten-microgram samples of DNA were digested with *Hin*dIII, *Eco*RI, or *Sal*I, separated by electrophoresis on a 0.8% agarose gel, transferred to nylon membranes by capillary transfer, and cross-linked to the membranes by UV irradiation. The filters were hybridized with either the coding region or the 3′ untranslated region of the LOX cDNA. The *Cla*I site overlapping with the stop codon was used to prepare a probe of the untranslated 3′ end of the cDNA clone.

LOX Enzyme Activity Measurement

The aboveground parts of wheat seedlings were pulverized in liquid nitrogen and homogenized with sand and 20 mg of polyvinylpolypyrrilidone in 2 mL of 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA. The homogenates were shaken for 15 min on a Eppendorf shaker and centrifuged at 12,000*g* for 20 min. LOX enzyme activity of the resulting supernatant was measured at 30°C with linoleic acid as a substrate (Ocampo et al., 1986). The molar extinction coefficient of the reaction product, 2.5 × 10^7 cm^{-1} mol^{-1}, was used for the calculation of the enzyme activity. The data represent the mean values and SDs of four independently processed samples per time point. A typical result is shown. The experiment was repeated once with similar results, although the absolute levels of LOX activity varied between the experiments.

RESULTS

Characterization of the *WCI-2* cDNA

The *WCI-2* cDNA (GenBank accession no. U32428) used as a probe was originally isolated by differential screening of a cDNA library constructed from wheat seedlings treated with the resistance-inducing compound BTH (Görlach et al., 1996). The longest open reading frame of the 1831-bp-long cDNA encodes a polypeptide of 517 amino acid residues. The absence of a start codon and the comparison to the length of 3 kb of the hybridizing mRNA suggested that the *WCI-2* cDNA is not full length. A comparison of the amino acid sequence of the longest open reading frame of the *WCI-2* cDNA with LOXs from rice and from soybean indicated that the *WCI-2* cDNA encodes 60% of the C-terminal end of a LOX (Fig. 1). The amino acid sequence of the *WCI-2*-encoded protein is 64% identical and 78% similar to LOX L-2 from rice (Ohta et al., 1992) and 60% identical and 74% similar to the LOX2 sequence of soybean (Shibata et al., 1987). The His residues conserved in mammalian and plant LOXs (Siedow, 1991) are also conserved in the *WCI-2* cDNA sequence. Together, these data suggest that the protein encoded by the *WCI-2* cDNA encodes a LOX. The results of genomic Southern experiments (data not shown) indicate that the allohexaploid wheat contains at least two copies of *WCI-2*-related genes per haploid genome. A presumably more specific probe derived from the untranslated 3′ end of the *WCI-2* cDNA still hybridized to four fragments larger than 4 kb, indicating that it is not a suitable gene-specific probe.

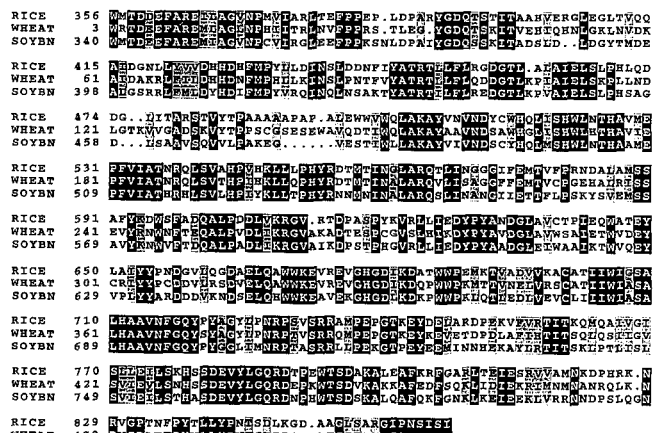

Figure 1. Amino acid sequence alignment of the *WCI-2*-encoded protein with two authentic LOXs. The deduced amino acid sequence of the *WCI-2*-encoded protein is aligned with the C-terminal protein sequences of LOX L-2 from rice (SwissProt accession no. P29250; Ohta et al., 1992) and of LOX1 from soybean (SwissProt accession no. P08170; Shibata et al., 1987). Periods indicate gaps inserted to optimize alignment. Identical amino acids are dark-shaded and similar amino acids are light-shaded. Sequence comparisons were made using the PileUp program of the GCG software package (Genetics Computer Group, Madison, WI).

Induction of LOX mRNA Accumulation by Inducers of Disease Resistance and Methyl Jasmonate

Figure 2 shows the induction of *WCI-2* homologous mRNA following treatment of wheat seedlings by a drench application of DC-INA. LOX mRNA started to accumulate 4 h after the application of DC-INA, and the level increased constantly throughout the next 20 h. Similar results were obtained by treatment with 0.5 mM BTH and with 0.5 mM salicylic acid, although the latter was a much weaker inducer (data not shown). Exposure of wheat seedlings to an atmosphere containing 1 μL L^{-1} methyl jasmonate also resulted in a strong accumulation of *WCI-2* homologous mRNA (Fig. 2). The induction by methyl jasmonate occurred within less than 2 h and mRNA levels started to decrease again after 24 h. The first two blots of Figure 2 were overexposed to demonstrate that only a small amount of *WCI-2* homologous mRNA was expressed in control plants. Under these conditions a higher-molecular-weight band hybridizing to the *WCI-2* probe became visible. Presently, the nature of this band is not clear. Since chemical inducers of resistance often activate the same set of genes that are activated by potential pathogens, we tested whether inoculation of wheat seedlings with a powdery mildew fungus had an effect on the expression of *WCI-2* homologous mRNA. Neither inoculation of wheat seedlings with *E. graminis* f. sp. *hordei* (incompatible interaction) nor inoculation with *E. graminis* f. sp. *tritici* (compatible interaction, data not shown) resulted in an accumulation of *WCI-2* homologous mRNA (Fig. 2). As a control for the inoculation process, the induction of a known pathogen-induced gene, *WIR1* (Bull et al., 1992), was demonstrated on the same blot. Positive control mRNA samples were included to verify the quality of the labeling reaction. We conclude from these experiments that *WCI-2* homologous transcripts rapidly accumulate after treatment with chemical inducers of disease resistance and with methyl jasmonate but not after inoculation with powdery mildew fungi.

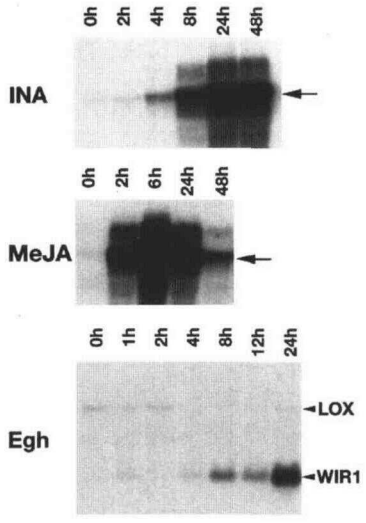

Figure 2. Induction of *WCI-2* homologous mRNAs by DC-INA, methyl jasmonate, or inoculation with *E. graminis* f. sp. *hordei*. Gel-blot analysis of *WCI-2* homologous mRNA expression after treatment with 0.5 mM DC-INA (INA) or 1 μL L^{-1} methyl jasmonate (MeJA) or inoculation with *E. graminis* f. sp. *hordei* (Egh). The time course in hours after treatment is indicated. The arrow indicates an RNA size of 3 kb. The blots were hybridized with either the ^{32}P-labeled *WCI-2* cDNA probe alone (INA, MeJA) or with a mixture of ^{32}P-labeled *WCI-2* cDNA and *WIR1* cDNA (Egh). Expression of the pathogen-induced *WIR1* gene served as a control for the inoculation process (Bull et al., 1992).

Induction of LOX mRNA Expression by Mechanical Stimulation

In the course of the above-described experiments, some problems were initially encountered with the reproducibility of the results. We first suspected that light might influence LOX expression. However, a careful examination revealed that the levels of *WCI-2* homologous mRNA were not regulated by light conditions but were strongly induced by mechanical stimuli. When the light was turned on in the growth chamber, the cooling system started to work, resulting in an increased air flow and an irregular pattern of local vibrations. To study the regulation of LOX expression the wheat seedlings had to be grown under carefully controlled conditions. Figure 3A shows the result of a controlled touch treatment on the levels of *WCI-2* homologous mRNA. Slowly moving a glass rod 10 times within 30s through the seedlings resulted in the rapid and transient accumulation of mRNA hybridizing to the *WCI-2* cDNA probe. Elevated mRNA levels were already detected 1 h after touch treatment and lasted for only a few hours. Accumulation of *WCI-2*-hybridizing transcripts was dose-dependent. A 10-fold repetition of the initial touch treatment resulted in a stronger and more prolonged accumulation of mRNA, indicating that the system that senses the touch stimulus is capable of detecting differences in the strength of stimulation. Clearly, wheat contains a LOX gene with an expression regulated by mechanical strain. This gene was named *TaLOX1*. The transient nature of mRNA accumulation indicates that the mRNA encoded by *TaLOX1* is rapidly turned over. Figure 3A demonstrates that wounding also resulted in a strong expression of *TaLOX1*. It is clear that mechanical wounding cannot be separated from touch stimulus. Squeezing of the seedlings with forceps did not result in a much higher expression above the levels detected in the seedlings treated with repeated touch stimulus.

The induction of *TaLOX1* expression was not dependent on direct physical touch, but the expression of *TaLOX1* could be induced by wind treatment. Constant wind conditions led to the prolonged accumulation of *TaLOX1*-encoded mRNA (Fig. 3B). Under alternating wind conditions transcript levels started to increase when the wind-generating fan was turned on and decreased again when the fan was turned off (Fig. 3C). Wind treatment also led to an increase in LOX enzyme activity, thus supporting the enzymatic identity of the mechanically induced gene product (Fig. 4). LOX activity started to increase relatively late following the beginning of wind treatment and reached about a 2-fold higher activity above the control values. This

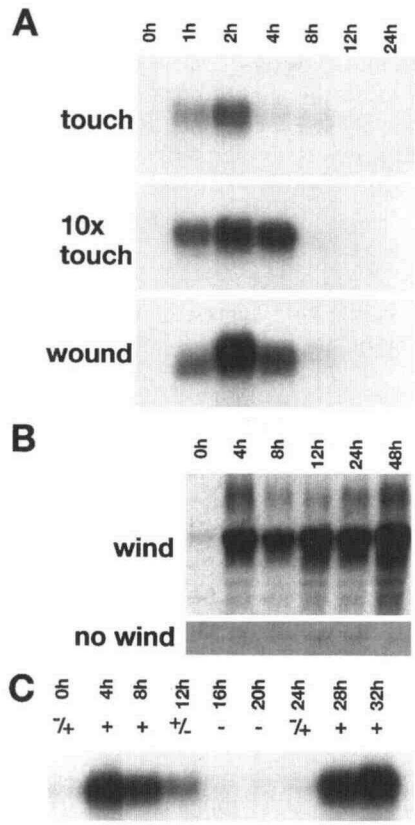

Figure 3. Effect of mechanical stimulation on the expression of *WCI-2* homologous mRNAs. RNA gel-blot analysis of *TaLOX1* expression following mechanical stimulation (A) by touch, prolonged touch treatment (10× touch), and wounding (wound). B and C, Induction of *TaLOX1* expression by wind. Wheat seedlings were exposed to constant wind (1 m s^{-1}) starting at 0 h or to no wind (B) or alternating wind (C). The time course in hours is indicated. Wind generated by a fan was switched on (−/+) and off (+/−) at the times indicated. The blot was probed with the ^{32}P-labeled *WCI-2* cDNA.

low factor of induction at the enzymatic level can be explained by the high constitutive LOX activity present in wheat seedlings. The constitutive LOX enzyme activity is unlikely to be the result of an earlier expression of *WCI-2* homologous genes. *WCI-2* homologous mRNA did not accumulate at any stage during the first 8 d of the development of control seedlings (data not shown). The constitutive LOX activity appears to be the result of the expression of additional LOXs that do not share sufficient sequence similarity to be detected with the *WCI-2* cDNA on RNA gel blots.

DISCUSSION

In this paper we describe the regulation of wheat LOX expression by various chemical compounds and by mechanical stress. Many different functions in plant metabolism have been proposed for LOXs (Siedow, 1991; Vick, 1991; Eiben and Slusarenko, 1994). The described mechanical strain-regulated LOX of wheat is hypothesized to be involved in linking mechanoperception to the growth responses observed in mechanically stressed plants.

In addition to mechanical stress situations, mRNAs homologous to the *WCI-2* cDNA accumulated rapidly in response to methyl jasmonate and in response to the three chemicals salicylic acid, DC-INA, and BTH, which are known to induce resistance against pathogens in many plant species (Kessmann et al., 1994). Surprisingly, no accumulation of *WCI-2*-related mRNAs could be observed within 24 h following inoculation of wheat with powdery mildew fungi. The induction of *WCI-2*-related proteins does not appear to play a role in the early events of an attempted infection. However, *WCI-2* homologous mRNAs are accumulating at much later stages of powdery mildew infection (Görlach et al., 1996).

The gene with the expression regulated by mechanical strain was called *TaLOX1* by definition. The *WCI-2* cDNA, and indeed also a fragment derived from the 3′ untranslated region, hybridized to multiple bands on genomic DNA gel blots, indicating that multiple copies of closely related genes exist in the wheat genome. Therefore, it is presently unclear whether the resistance-inducing chemicals and mechanical strain activate the expression of the same or different genes.

The increase in LOX enzyme activity observed following exposure of seedlings to wind confirms the presence of a mechanically regulated LOX in wheat. The low relative increase in enzyme activity contrasts the much higher relative increase reflected on the mRNA level. In addition, the high constitutive LOX enzyme activity contrasts with the low constitutive abundance of *WCI-2* homologous mRNA. The most likely explanation for this situation is that wheat contains additional LOXs that are not detected by the *WCI-2* cDNA probe on RNA blots. The high constitutive expression of this second group of LOXs is likely to mask the mechanical induction of *TaLOX1* at the enzymatic level, but it does not interfere with the estimation of *TaLOX1* expression at the transcript level. Two different classes of LOXs with little sequence similarity have been described in

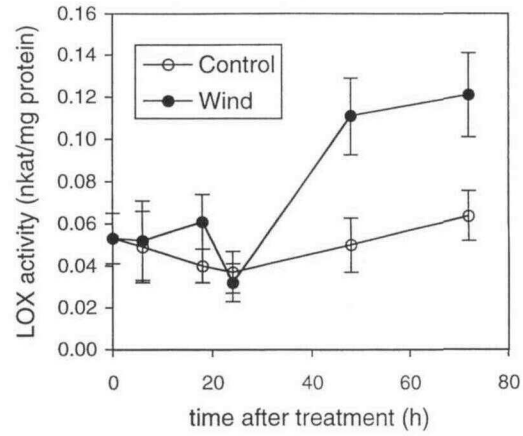

Figure 4. Wind-induced increase in LOX enzyme activity. Wheat seedlings were exposed to a constant wind of 1 m s^{-1} (●) or kept under windless conditions (○). Error bars denote the SE of the enzyme measurement of four independently processed samples.

rice (Peng et al., 1993). However, in wheat there is no positive proof yet for the existence of a second class of LOXs. No additional sequences for wheat LOXs are available.

Accumulation of *TaLOX1*-encoded mRNA is strongly induced by various mechanical stimuli, such as wind, touch, and wounding. The kinetics of induction of *TaLOX1* is typical for the few touch-induced genes previously described (Braam and Davis, 1990; Botella et al., 1995; Takezawa et al., 1995). The induction is very rapid, occurring within less than 1 h after stress application, and the accumulation is transient, indicating that the mRNA is rapidly turned over. Induction of LOXs by wounding has been reported in several plant species (Mason and Mullet, 1990; Bell and Mullet, 1993), and it has been postulated that the increased synthesis of LOX would lead to an increased production of jasmonic acid, which in turn would activate a range of jasmonate-inducible proteins (Creelman et al., 1992; Staswick, 1992; Farmer, 1994; Creelman and Mullet, 1995). A transient increase in jasmonic acid production could indeed be measured upon wounding (Creelman et al., 1992; Albrecht et al., 1993) and upon touch stimulation of plants (Weiler et al., 1993). In the winding plant *Bryonia dioica*, touch and metabolites of linolenic acid and jasmonic acid were shown to be highly effective inducers of tendril coiling (Falkenstein et al., 1991; Weiler et al., 1993). Endogenous levels of jasmonic acid raised dramatically at the time of coiling (Albrecht et al., 1993). It has thus been suggested that jasmonic acid, or one of its precursors, may couple the mechanostimulation to the coiling response. It is tempting to speculate that a touch-induced LOX similar to *TaLOX1* is responsible for the increased production of fatty acid-derived molecules in this system.

A number of LOX genes, including genes of wheat homologous to the *WCI-2* cDNA, are strongly induced by jasmonic acid or methyl jasmonate (Bell and Mullet, 1991, 1993; Grimes et al., 1992; Melan et al., 1993). This suggests that jasmonic acid might be a positive regulator of its own synthesis and that jasmonic acid-induced LOXs are part of a signal amplification mechanism. In this case, the transient nature of *WCI-2* homologous mRNA accumulation following touch treatment is somewhat surprising and suggests that either negative feedback regulation mechanisms exist or that the *TaLOX1*-encoded protein does not feed into the pathway leading to jasmonate production.

Little is known about the primary signal that leads from the mechanoperception event to the induction of *TaLOX1* expression. There is growing evidence that calcium signaling is an early response in the mechanotransduction process that leads to the expression of touch-regulated genes (Trewavas and Knight, 1994; Haley et al., 1995). Some touch-induced calmodulin-like genes of Arabidopsis were found to be regulated by calcium (Braam, 1992). Mechanosensitive calcium-selective cation channels have been described in a number of plant species (Cosgrove and Hedrich, 1991; Ding and Pickard, 1993). Together, these results suggest the possibility that mechanical strain-induced genes might be regulated by an increase in cytosolic calcium, which is triggered by the mechanical stimulation of stretch-gated calcium channels. However, it is not known whether changes in cytoplasmic calcium levels are also important for the mechanical induction of *TaLOX1*.

The proteins encoded by mechanical strain-induced genes often appear to have the potential to translate the mechanical stimulus into a multitude of secondary reactions by regulating the expression of a number of downstream genes. In this paper a mechanical strain-induced LOX is described, which could potentially influence plant growth via an increased production of fatty acid-derived compounds. In many plant species compounds such as jasmonic acid, traumatic acid, or ABA have been demonstrated to have dramatic effects on plant growth and development (Vick and Zimmerman, 1987; Falkenstein et al., 1991; Koda, 1992; Staswick, 1992). It will now be interesting to test whether mechanisms known to be involved in the regulation of plant growth are controlled by the touch- and LOX-dependent production of fatty acid-derived signaling molecules.

Received May 1, 1997; accepted May 16, 1997.

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