

## A role for 9-lipoxygenases in maize defense against insect herbivory

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### ABSTRACT

Feeding by *Spodoptera exigua* (beet armyworm) larvae on *Zea mays* (maize) induces expression of 9-lipoxygenases to a greater extent than 13-lipoxygenases. Whereas 13-lipoxygenases have an established role in the synthesis of jasmonates that serve as defense signaling molecules in many plant species, relatively little is known about the role of 9-lipoxygenases in herbivore defense. Phylogenetic analysis of lipoxygenases from maize inbred lines B73 and W22 shows that, although most *Lox* genes are present in both lines, *Lox12*, a 9-lipoxygenase that has been implicated in fungal defense, is truncated and unlikely to encode a functional protein in W22. Two independent *Mutator* transposon insertions in another 9-lipoxygenase, *Lox4*, caused improved *S. exigua* growth on the mutant lines relative to wildtype W22. This observation suggests a function in herbivore defense for metabolic products downstream of maize *Lox4*, either through direct toxicity or a perhaps an as yet unknown signaling function.

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### Introduction

Plants respond to insect herbivory with a large variety of metabolic changes. Some induced metabolites have toxic or deterrent effects against the herbivores, whereas others act as signaling molecules to induce defense responses in parts of the plant that are not yet under attack. Two well-studied plant metabolic pathways, those of indole glucosinolates in *Arabidopsis thaliana*<sup>1</sup> and benzoxazinoids in *Zea mays* (maize),<sup>2,3</sup> produce metabolites with both direct defensive properties and defense signaling molecules.

Jasmonic acid and its derivatives methyl jasmonate and jasmonate-isoleucine are well-studied components of the defense signaling pathway in many plant species.<sup>4</sup> The first reaction in the synthesis of jasmonic acid from  $\alpha$ -linolenic acid is catalyzed by 13-lipoxygenases (Fig. 1).<sup>5</sup> In the sequenced B73 maize genome,<sup>6</sup> six genes are predicted to encode 13-lipoxygenases (*Lox7*, *Lox8*, *Lox9*, *Lox10*, *Lox11*, and *Lox13*; Fig. 2). *Lox7* and *Lox8*, are involved in jasmonate biosynthesis, thereby contributing to inflorescence development and plant defense, respectively.<sup>7,8</sup> *Lox10* activity leads to the release of herbivore-induced plant volatiles.<sup>8</sup>


In addition to 13-lipoxygenases, the B73 genome contains seven genes encoding predicted 9-lipoxygenases (*Lox1*, *Lox2*, *Lox3*, *Lox4*, *Lox5*, *Lox6*, and *Lox12*; Fig. 2), which oxidize 18:3  $\alpha$ -linolenic acid and 18:2  $\alpha$ -linoleic acid to produce 10-oxo-11-phytodienoic acid (10-OPDA; Fig. 1) and 10-oxo-11-phytoenoic acid (10-OPEA), respectively. Fungal infection, mechanical wounding, and feeding by *Helicoverpa zea* (corn earworm)

induce 10-OPEA accumulation in maize.<sup>9,10</sup> Furthermore, *in vitro* experiments show that 10-OPEA inhibits the growth of fungal pathogens (*Aspergillus flavus* and *Fusarium verticillioides*) and *H. zea* at physiologically relevant concentrations. Similarly, assays with maize 9-lipoxygenase mutants have demonstrated functions in plant defense. For instance, a *lox12* mutation reduced *F. verticillioides* resistance<sup>11</sup> and *Meloidogyne incognita* (root-knot nematode) grew better on a *lox3* mutant line.<sup>12</sup>

In recent research, we investigated the transcriptional and metabolic responses of inbred line B73 to feeding by *Spodoptera exigua* (beet armyworm) caterpillars.<sup>13</sup> As expected, insect herbivory induced the expression of 13-lipoxygenases (Fig. 3) and increased the accumulation of jasmonic acid and its derivatives. However, *S. exigua* growth was not affected by a *lox8* mutation, suggesting possible redundancy in maize 13-lipoxygenase activity. Compared to 13-lipoxygenases, 9-lipoxygenases were induced to an even greater extent by *S. exigua* feeding (Fig. 3). Expression of *Lox1/2*, *Lox3*, *Lox4*, and *Lox5* was increased up to 100-fold within an hour of caterpillar feeding and remained at a high level for the duration of the 24-hour experiment. In other research, *Lox1* and *Lox5* expression was induced by *Ostrinia furnicalis* (Asian corn borer) feeding.<sup>14</sup> Previous experiments using RNA blots to measure induction of gene expression in response to *Spodoptera frugiperda* (fall armyworm) feeding showed increased expression of *Lox5*, but not *Lox4* after 1, 4, and 8 days of caterpillar feeding.<sup>15</sup> Differences in the observed induction of *Lox4* expression (Fig. 3 vs.<sup>15</sup>) could result from the use of different maize lines, different herbivore species, or perhaps a transient expression of *Lox4* early

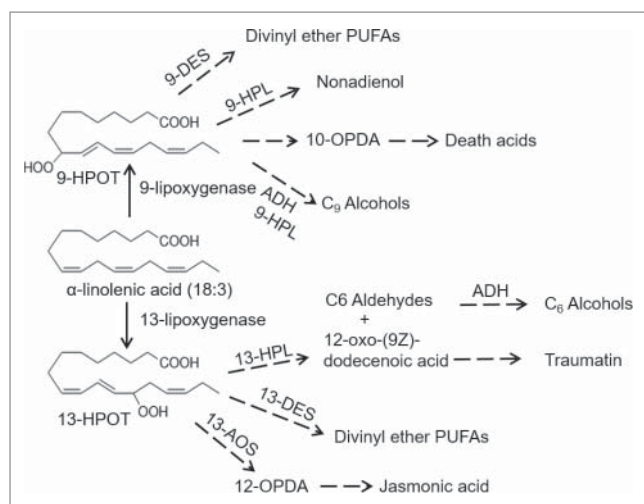
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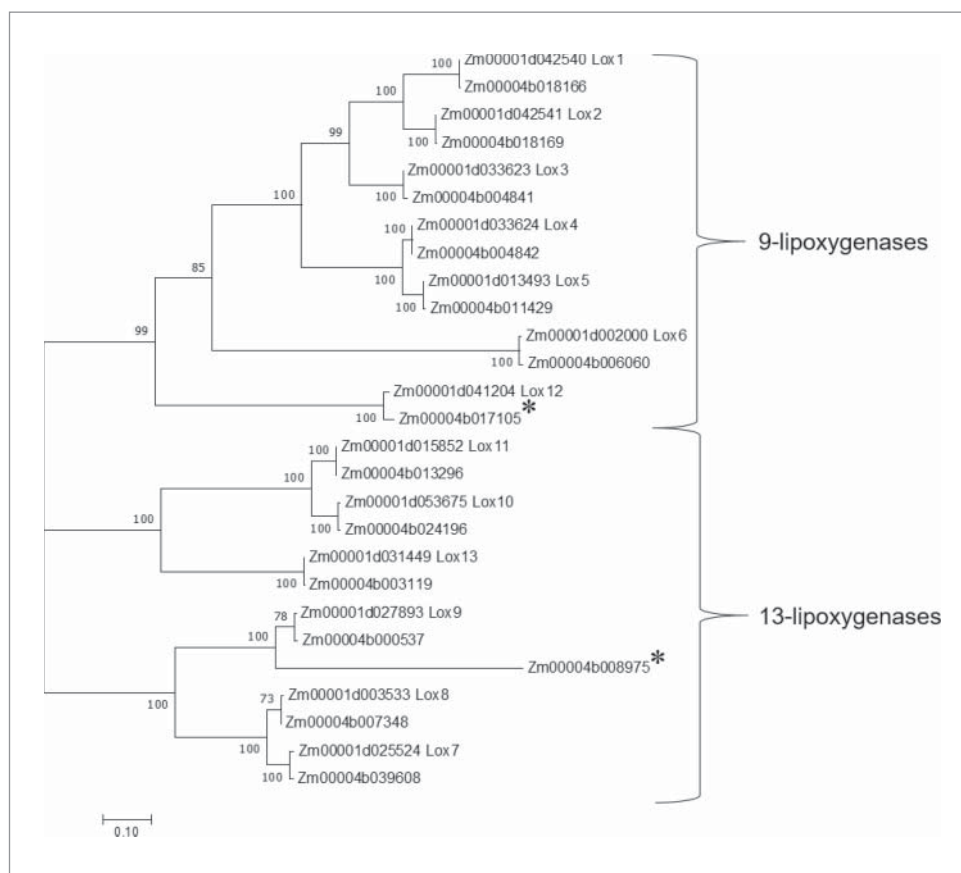
**Figure 1.** 9-Lipoxygenase and 13-lipoxygenase oxygenate  $\alpha$ -linolenic acid at different positions to produce (10E,12Z)-9-Hydroperoxy-10,12,15-octadecatrienoic acid (9-HPOT) and (10E,12Z)-13-hydroperoxy-10,12,15-octadecatrienoic acid (13-HPOT), respectively. 9-HPOT and 13-HPOT serve as precursors for differing sets of bioactive plant metabolites. 10-OPDA = 10-oxophytodienoic acid; 12-OPDA = 12-oxophytodienoic acid; DES = divinyl ether synthase; AOS = allene oxide synthase; HPL = hydroperoxide lyase. Modified from Schiller et al. (2015).

during herbivory. Together, these gene expression results suggested that the activity of 9-lipoxygenases might also make significant contributions to maize defense against caterpillar herbivory.

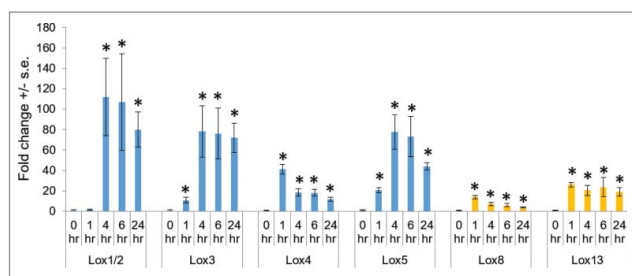
## Results and discussion

Whereas our gene expression analysis<sup>13</sup> was conducted with maize inbred line B73, better genetic resources, in particular, collections of *Mutator* (*Mu*), *Activator* (*Ac*) and *Dissociation* (*Ds*) transposon insertions are available for the W22 inbred line.<sup>16-20</sup> To facilitate the identification of a 9-lipoxygenase knockout mutation in W22, we first compared the complement of Lox proteins encoded in the B73 genome<sup>6</sup> and the recently assembled W22 genome.<sup>21</sup>

The protein sequences of 14 predicted W22 lipoxygenases and 13 predicted B73 lipoxygenases were aligned (Fig. S1). This showed that about the W22 homolog of B73 Lox12 (Zm00004b017105) is truncated by about 50% and is likely to be non-functional. As a *Lox12* mutation in the B73 genetic background increases susceptibility to *Fusarium verticilloides*,<sup>11</sup> W22 might also show susceptibility to this fungal pathogen. Zm00004b008975, a Lox9-like protein that is present in W22



**Figure 2.** Dendrogram of predicted protein sequences of 27 lipoxygenases from the maize inbred line B73 v4 assembly (Zm00001d numbers) and the inbred line W22 v2 assembly (Zm00004b numbers). The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. Evolutionary analyses were conducted in MEGA7. The tree with the highest log likelihood is shown. Bootstrap values are based on 1000 replications. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 80% site coverage were eliminated. There were a total of 835 positions in the final dataset. Proteins that are truncated and likely non-functional in W22 are marked with asterisks.



**Figure 3.** Caterpillar-induced expression of 9-lipoxygenases (blue bars) and 13-lipoxygenases (orange bars) in maize inbred line B73. Mean  $\pm$  s.e. of 4 independent samples. \* $P$  value < 0.05, Dunnett's test relative to 0-hour controls. Data are adapted from Tzin et al. 2017.

but not in B73 is similarly truncated and likely to be non-functional. A phylogenetic analysis of the B73 and W22 lipoxygenase proteins (Fig. 2) showed that, with the exception of *Lox12*, there is a full-length W22 homolog for each of the 13 known B73 lipoxygenases (Fig. 2). The two pairs of tandem-duplicated lipoxygenases in B73, *Lox1/2* and *Lox3/4* are similarly localized as gene pairs in the W22 genome.

Analysis of publicly available *Mu* transposon insertions identified lines UFMu-01831 and UFMu-03303 as having an insertion in the W22 *Lox4* gene (Fig. 4A). Homozygous *Mu* insertions in *Lox4* were verified by PCR amplification and DNA sequencing. Both transposon insertions are in coding regions and lead to predicted C-terminal truncations of the conserved lipoxygenase domain of the protein. Analysis of

*Lox4* gene expression by quantitative PCR using primers that bind upstream of the transposon insertion sites showed reduced transcript levels in UFMu-01831 but not UFMu-03303, with and without caterpillar feeding. It is possible that the two *Mu*-induced truncations of the gene have differing effects on *Lox4* transcript stability. Relative to W22, *S. exigua* caterpillars gained more mass over a five-day period on both UFMu-01831 and UFMu-03303, indicating that maize defense responses are compromised by the *Lox4::Mu* mutations that truncate the C-terminus of this protein.

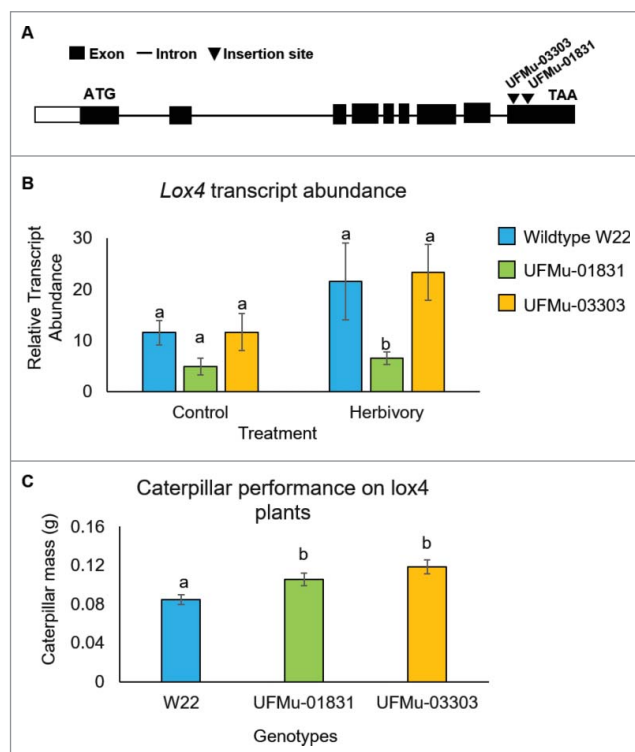
Compared to the extensive research on the role of 13-lipoxygenases for jasmonate-mediated plant defense signaling, there is relatively little published information on the role that 9-lipoxygenases play in defense against insect herbivory. Altered caterpillar performance due to the *lox4* mutation could be due to either changes in the production of toxic or deterrent metabolites, e.g. 10-OPEA,<sup>9</sup> or perhaps changes in plant defense signaling. For instance, just as 12-OPDA is a precursor for the production of jasmonic acid, 10-OPDA or 10-OPEA could lead to the formation of mobile signaling molecules that lead to the induction of defense responses in maize.

Given the large caterpillar-induced changes in gene expression (Fig. 3) and the improved growth of *S. exigua* on *lox4* mutants (Fig. 4), further research on the role of 9-lipoxygenase in maize defense against insects is warranted. In addition to *Lox4::Mu* insertion lines that we have investigated, *Mu* insertions in the W22 genetic background are available for two other 9-lipoxygenases, *Lox2* and *Lox5*, in public collections. Investigation of herbivore resistance, gene expression changes, and metabolite accumulation in these transposon insertion lines likely will identify new pathways of maize defense against insect herbivory.

## Methods

**Molecular Phylogeny.** Protein sequences of the 13 known lipoxygenase proteins (*Lox1* – *Lox13*) in maize inbred line B73 Ref-Gen v4 were downloaded from the Maize Genetics and Genomics Database ([www.maizegdb.org](http://www.maizegdb.org)). Sequence comparisons of B73 genes from the v2 assembly of the W22 genome<sup>21</sup> identified 14 predicted lipoxygenase genes. Protein sequences of these 27 identified lipoxygenases were aligned with Muscle<sup>22</sup> using the following program parameters: gap penalties – gap open -2.9, gap extend 0, hydrophobicity multiplier 1.2; max iterations 8; clustering method UPGMB; and min diag length 24. A dendrogram of protein sequences was prepared using MEGA7.<sup>23</sup> The evolutionary history was inferred using the Maximum Likelihood method based on the JTT matrix-based model.<sup>24</sup> Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. All amino acid positions with less than 80% site coverage among the 27 analyzed proteins were eliminated. There were a total of 835 positions were included in the final dataset.

*Mu* transposon insertions in *Lox4*. Line UFMu-01831 and UFMu-03303 were obtained from Maize Genetics COOP (<http://maizecoop.cropsci.uiuc.edu/>). *Mu* transposon insertions in the *Lox4* gene were confirmed by PCR



**Figure 4.** Analysis of a W22 *Lox4* *Mu* insertion lines (A) Location of the UFMu-01831 and UFMu-03303 insertions in the *Lox4* gene and positions of primers that were used for quantitative RT-PCR gene expression analysis. (B) Expression level of the *Lox4* gene, with and without six hours of *S. exigua* feeding. Mean  $\pm$  s.e. of 5 independent samples. (C) Mass of *S. exigua* caterpillars after five days of feeding on W22, UFMu-01831, and UFMu-03303 seedlings. Mean  $\pm$  s.e. of 57 samples. \* $P$  value < 0.05, ANOVA followed by Tukey's HSD test.

amplification using a Biorad C1000 thermal cycler the gene-specific primer (GGCGACGATAATCCTGGACCATA-CAAGG) and the transposon-specific primer TIR6 primer (AGAGAAGCCAACGCCAWCGCCTCYATTTCGTC), followed by DNA sequencing. *Lox4* expression levels were measured by quantitative RT-PCR using an Applied Biosystems 7900HT instrument and the primers GCTGAACCTGAA-CATCTACG and GTAGAGCCCAAGGATGTCTT.

Insect bioassays. Eggs of *S. exigua* were obtained from Benzon Research ([www.benzonresearch.com](http://www.benzonresearch.com)). Eggs were hatched at 28°C and moved to *S. exigua* artificial diet (Southland Products, <http://www.tecinfo.com/~southland/>). After five days, larvae were moved onto 17-day-old maize plants that were grown in a Conviron walk-in growth room at 23°C with 16:8 h light:dark cycle and 180 mmol photons m<sup>-2</sup>s<sup>-1</sup> light intensity. After 5 days on the plants, caterpillars were removed and their mass was measured using a Sartorius precision balance.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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