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The maize lipoxygenase, ZmLOX10, mediates green leaf volatile, jasmonate and herbivore#induced plant volatile production for defense against insect attack

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1 **Title:**

2 **The maize lipoxygenase, *ZmLOX10*, mediates green leaf volatile, jasmonate,**
3 **and herbivore-induced plant volatile production for defense against insect**
4 **attack**

5
6 **Authors:**

7 Christensen, Shawn A.^{1‡} Nemchenko, Andriy^{1†} Borrego, Eli¹ Murray, Ian¹ Sobhy
8 S., Islam^{2††} Bosak, Liz³ DeBlasio, Stacy⁴ Erb, Matthias^{2‡} Robert, Christelle
9 A.M.^{2‡} Vaughn, Kathy A.¹ Herrfurth, Cornelia⁵ Tumlinson, Jim³ Feussner, Ivo⁵
10 Jackson, David⁴ Turlings, Ted C.J.² Engelberth, Jurgen⁶ Nansen, Christian¹
11 Meeley, Robert⁷, and Kolomiets, Michael V.^{1*}

12
13 **Institution Addresses:**

14 ¹Department of Plant Pathology and Microbiology, Texas A&M University,
15 College Station, Texas 77843

16 ²Laboratory of Fundamental and Applied Research in Chemical Ecology, Institute
17 of Biology, University of Neuchatel, Rue Emile-Argand 11, CP158, CH-2009,
18 Neuchatel, Switzerland.

19 ³Center for Chemical Ecology, Penn State University, University Park, PA 16802

20 ⁴Cold Spring Harbor Laboratory, 1 Bungtown Rd., Cold Spring Harbor, NY
21 11724

[‡] Chemistry Unit, Center of Medical, Agricultural, and Veterinary Entomology, US Department of Agriculture, Gainesville, FL 32608

[†] University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd, Dallas, TX 75390

^{††} Department of Plant Protection, Suez Canal University, 41522 Ismailia, Egypt

[‡] Max Planck Institute for Chemical Ecology Hans-Knoell Str. 8 07745 Jena, Germany

2

1 ⁵Dept. of Plant Biochemistry, Justus-von-Liebig-Weg, 11 37077, Göttingen,
2 Germany

3 ⁶Department of Biology, University of Texas at San Antonio, San Antonio, Texas
4 78249

5 ⁷Pioneer – a DuPont Company, Johnston, Iowa 50131

6 *Corresponding author: Michael Kolomiets; Department of Plant Pathology and
7 Microbiology, Texas A&M University, College Station, Texas 77843; Telephone:
8 +1.979.458.4624; Fax: +1.979.845.6483; E-mail: kolomiets@tamu.edu

9

10 **Email Addresses of Authors:**

11 Christensen, Shawn A. Shawn.Christensen@ars.usda.gov

12 Nemchenko, Andriy nemchenko2000@gmail.com

13 Borrego, Eli eli.borrego@neo.tamu.edu

14 Murray, Ian IVMurray@medicine.tamhsc.edu

15 Soby, Islam islam.sobhy@unine.ch

16 Bosak, Liz ejb246@psu.edu

17 DeBlasio, Stacy_deblasio@csihl.edu

18 Erb, Matthias merb@ice.mpg.de

19 Robert, Christelle christelle.robert@unine.ch

20 Tumlinson, Jim jht2@psu.edu

21 Vaughn, Kathy KMVaughn@ag.tamu.edu

22 Herrfurth, Connie cgoebel@gwdg.de

23 Jackson, David_jacksond@csihl.edu

3

- 1 Feussner, Ivo ifeussn@gwdg.de
- 2 Turlings, Ted ted.turlings@unine.ch
- 3 Nansen, Christian cnansen@ag.tamu.edu
- 4 Engelberth, Juergen Jurgen.Engelberth@utsa.edu
- 5 Meeley, Robert bob.meeley@pioneer.com
- 6 Kolomiets, Michael kolomiets@tamu.edu

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ZmLOX10 mediates GLVs, JA, and HIPVs

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1 compromised resistance to insect feeding, both under laboratory and field
2 conditions, which is strong evidence that LOX10-dependent metabolites confer
3 immunity against insect attack. Hence, this comprehensive gene-to-
4 agroecosystem study reveals the broad implications of a single LOX isoform in
5 herbivore defense.

7 INTRODUCTION

8
9 In response to insect attack, plant tissues undergo significant reprogramming of
10 genetic and metabolic processes, resulting in direct and indirect defense
11 responses (Maffei et al., 2006). As direct countermeasures, toxins and defensive
12 proteins are produced to repel pests and impede digestibility. As a mode of
13 indirect defenses, herbivore induced plant volatile (HIPV) emissions attract insect
14 predators and parasitoids (D'Alessandro and Turlings 2006; Heil 2008; Dicke,
15 2009). While our knowledge of the detailed molecular and biochemical
16 regulation of these responses is still incomplete, it is widely known that much of
17 the essential defense signals are produced in the fatty acid oxidation pathways
18 including metabolites derived from lipoxygenases (LOXs; Matsui, 2006; Howe
19 and Jander, 2008; Mosblech et al., 2009).

20 Initiation of the LOX pathway begins when polyunsaturated fatty acids
21 [linoleic (18:2) and α -linolenic (18:3) acid] are cleaved from cell membranes by
22 diverse lipases and dioxygenated by either 9- or 13-LOXs to form 9- and 13-
23 hydroperoxides, respectively. These hydroperoxides act as a substrate for seven

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3 1 downstream branches of the LOX pathway, namely peroxygenases, divinyl ether
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5 2 synthases, reductases, epoxy alcohol synthases, hydroperoxide lyases (HPL),
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7 3 allene oxide synthases (AOS), and additional LOX reactions, which collectively
8
9 4 produce numerous oxylipins (Feussner and Wasternack, 2002; Mosblech et al.,
10
11 5 2009). While there is still much to unveil in terms of the physiological roles of
12
13 6 specific oxylipins in response to stresses, the HPL and AOS pathways
14
15 7 responsible for green leaf volatile (GLV) and jasmonic acid (JA) production,
16
17 8 respectively, are the predominant and better understood pathways activated in
18
19 9 the wound and herbivore response.

10 The synthesis of GLVs begins with the cleavage of 13-hydroperoxy
11 octadecatrienoic acid (13-HPOTE) by HPL to form (3Z)-hexenal, which is
12 enzymatically converted to other C6-compounds including (3Z)-hexenol and (3Z)-
13 hexenyl acetate (Blée, 2002; D'Auria et al., 2002; Matsui, 2006). GLVs act as
14 signals that induce expression of defensive genes (Bate and Rothstein, 1998),
15 regulate plant-plant communication after insect elicitation (Arimura et al., 2000;
16 Farag and Pare, 2002; Engelberth et al., 2004), and attract parasitoid wasps
17 (Whitman and Eller, 1990). The exposure of plants to exogenous GLVs induces
18 JA and HIPV production, and more importantly, enhances the JA response to
19 herbivore attack (Farag and Pare, 2002; Engelberth et al., 2004). GLVs also
20 possess antimicrobial properties, although in some plant pathogen systems they
21 may serve as signals to facilitate pathogenesis processes (Prost et al., 2005;
22 Christensen and Kolomiets, 2011). These herbivore and microbial related
23 examples show the large number of organisms that respond to GLVs and

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1 demonstrate the varied effects GLVs can have, based on the co-evolution of the
2 plant and species involved.

3 Similar to GLVs, the formation of JA begins with LOX-derived 13-HPOTE,
4 which is catalyzed into allene oxide by AOS and subsequently transformed into a
5 cyclopentenone by allene oxide cyclase. The resulting (+)-12-oxo phytodienoic
6 acid (OPDA) is further reduced by 12-oxo-phytodienoate reductase isoform 3
7 (OPR3), and then truncated by three beta-oxidation steps to form (+)-7-*iso*-JA
8 (Wasternack, 2007). JA and/or its derivatives play important roles in growth and
9 development (Creelman and Mullet, 1997; Acosta et al., 2009; Yan et al., 2012)
10 and in microbial- and herbivore-induced defense responses (Browse 2009; Koo
11 and Howe 2009). The rapid accumulation of JA in response to wounding or
12 insect attack is essential for direct defenses at the site of attack and systemic
13 defense signaling (Howe and Jander, 2008). A major ecological significance of
14 JA is found in its regulation of HIPVs (Dicke et al., 1999; Koch et al., 1999;
15 Bruinsma et al., 2009), which play an important role in indirect defense
16 responses. The majority of HIPVs are synthesized via the isoprenoid pathway
17 where terpene synthases enzymatically convert their pyrophosphate-substrates
18 into diverse homo-, mono-, sesqui-, and diterpenes, which are sensed as
19 foraging cues for natural enemies i.e. predators (Kessler and Baldwin, 2001;
20 Turlings et al., 2004). An interesting feature of HIPVs is their diurnal emissions
21 during herbivory. While this emissions pattern has long been established
22 (Louhgrin et al., 1994; Turlings et al., 1995; Martin et al., 2003), the mechanism
23 behind it is not fully understood.

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3 1 The significance of lipid derivatives in diverse biological and ecological
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5 2 functions has been demonstrated by studies of LOX mutants in several plant
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8 3 species. For example, analysis of transgenic lines in tomato *TomLOXC*, potato
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10 4 *LOX-H1*, tobacco *NaLOX2* and rice *OsLOX1* show that these genes specialize in
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12 5 GLV biosynthesis (Chen et al., 2004; Leon et al., 2002; Allmann et al., 2010 and
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15 6 Wang et al., 2008). Furthermore, measurement of wound- and/or herbivore-
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17 7 induced JA accumulation has shown that *Arabidopsis AtLOX2*, rice *OsHI-LOX*
18
19 8 and *OsLOX1*, and tobacco *NaLOX3* all contribute to JA biosynthesis (Bell et al.,
20
21 9 1995; Zhou et al., 2009; Wang et al., 2008; Halitschke and Baldwin, 2003).
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24 10 Beyond functions in GLVs and JA biosynthesis, LOXs also regulate downstream
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26 11 defense responses and biological resistance. Antisense plants downregulated
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28 12 for *Arabidopsis LOX2* are more susceptible to *Pieris rapae* (Zheng et al., 2011)
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30 13 than their corresponding wild type. Silenced *Nicotiana attenuata* plants at the
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32 14 *NaLOX3* locus are impaired in nicotine, trypsin protease inhibitors (TPI), as well
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34 15 as terpene volatile emissions (Halitschke and Baldwin, 2003). *OsHI-LOX* and
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36 16 *OsLOX1* were also shown to be involved in resistance through assays with stem
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38 17 borers or brown plant hoppers (Zhou et al., 2009; Wang et al., 2008). While the
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40 18 collective characterization of these and other JA and GLV producing LOXs has
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42 19 contributed to our understanding of plant-insect defense in various plant species,
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44 20 there is a dearth of genetic evidence for the functions of defense genes in the
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46 21 economically important crop maize. Moreover, despite the diverse functions of
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48 22 LOXs in regulating different aspects of the GLV, JA, or HIPV pathways, evidence
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50 23 for a single LOX isoform that modulates all three remains unseen.
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4 1 Although GLVs and JA are commonly synthesized by the LOX pathway,
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6 2 the mode by which the final products are produced is diverse and appears to be
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8 3 species-dependent. For instance, the chloroplast-localized rice LOX, OsLOX1,
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10 4 has been shown to supply both the AOS and HPL branches of the LOX pathway
11
12 5 (Wang et al., 2008). In other reported cases, separate LOX isoforms provide
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14 6 pathway specific 13-hydroperoxide substrates for either the JA or the GLV
15
16 7 cascade (Halitschke and Baldwin, 2003; Halitschke et al., 2004; Chehab et al.,
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18 8 2008; Allman et al., 2010). Despite the variation that exists in the number of
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20 9 LOXs required to feed substrate to the GLV and JA pathway, the HPL and AOS
21
22 10 branches are commonly co-localized to the same organelle, the chloroplast
23
24 11 (Froehlich et al., 2001; Farmaki et al., 2007). To the best of our knowledge, the
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26 12 only reported crosstalk between these co-localized pathways has been at the
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28 13 linolenic fatty acid or 13-hydroperoxide substrate levels (Halitschke et al., 2004;
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30 14 Chehab et al., 2008; Wang et al., 2008). Surprisingly, the intermediates and final
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32 15 products of these branches are known for their function as potent signaling
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34 16 molecules, yet there is no evidence suggesting signaling interaction between
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36 17 these pathways. For example, GLVs have been shown to be strong inducers of
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38 18 JA when pharmacologically applied (Engelberth et al., 2004, 2007), but the
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40 19 endogenous presence or absence of these metabolites due to anti-sense or
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42 20 overexpression (Halitschke et al., 2004; Chehab et al., 2008; Allman et al., 2010)
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44 21 does not seem to affect the production of JA, even though both pathways reside
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46 22 in the same organelle.
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1 Here we provide evidence that, in maize, GLV and JA biosynthesis occurs
2 in separate and distinct organelles and show that wound-induced JA is
3 dependent on signaling from GLVs and/or oxylipins derived from the GLV-
4 producing LOX. Specifically, we show that LOX10 provides substrate to the GLV
5 biosynthesis pathway and is localized to organelles distinct from chloroplasts.
6 Furthermore, we demonstrate that the chloroplast localized LOX8 (also known as
7 tasselseed1, LOX-ts1; Acosta et al., 2009) is responsible for wound-induced JA,
8 yet LOX8-mediated JA production is dependent on signaling from LOX10-derived
9 oxylipins. The elimination of LOX10 by *Mu*-transposon insertional mutagenesis
10 compromises resistance to insects under both laboratory and field conditions,
11 and leads to the impairment of ecologically important HIPV emissions, making
12 plants less appealing to the parasitoid wasp *C. marginiventris*. Collectively, our
13 results show that *ZmLOX10* is a key modulator of insect defense in maize by
14 regulating JA, GLV, and HIPV production in response to herbivory.

16 RESULTS

18 Generation of transposon-insertional *lox10* mutants

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20 Nemchenko et al. (2006) reported on the two segmentally duplicated LOX
21 paralogs, *ZmLOX10* and *ZmLOX11*, which share 90% identity at the amino acid
22 level. To identify putative mutant alleles, we screened the *Mutator*-transposon
23 insertional genetics resource at Pioneer Hi-Bred for insertions in these two genes.

11

1 Unfortunately, there were no *Mu*-insertions identified in the *ZmLOX11* gene.
2 However, three independent alleles were identified at the *ZmLOX10* locus (*lox10-*
3 *1*, *lox10-2*, and *lox10-3*). Sequencing of the regions flanking insertion sites
4 showed that the *lox10-1* allele harbored a *Mu*-element in the first intron while
5 *lox10-2* and *lox10-3* had *Mu*-insertions in exon III (Figure 1a). The original
6 mutants were backcrossed into the B73 (*lox10-2* and *lox10-3*) and W438 (*lox10-*
7 *2*) genetic backgrounds and genetically advanced to create near-isogenic mutant
8 and wild-type lines (BC₃F₄ – BC₅F₄; as designated below) suitable for functional
9 analysis. To test if *lox10-2* and *lox10-3* are null-alleles, transcript accumulation
10 was measured 8 h post mechanical wounding, the time point at which LOX10 is
11 expressed at the greatest levels (Nemchenko et al., 2006). WT plants showed a
12 clear induction of *ZmLOX10* transcripts, whereas no detectable hybridization
13 signal was observed for both *lox10-2* and *lox10-3* alleles (Figure 1b). These data
14 demonstrate that *lox10-2* and *lox10-3* mutants are null alleles.

16 **The characterization and subcellular localization of LOX10**

18 The clustering of LOX10 with other GLV-producing isoforms (Figure S1;
19 Nemchenko et al., 2006) prompted the hypothesis that LOX10 feeds substrate to
20 the HPL pathway. Since GLVs are emitted rapidly upon mechanical damage, we
21 cut leaf tissue and collected head-space volatiles from WT, *lox10-2* and *lox10-3*
22 mutant seedlings for 10 min. Gas chromatography mass spectrometry (GC/MS)
23 analyses showed no detectable amounts of (3Z)-hexenal, (3Z)-hexenol, and

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1 (3Z)-hexenyl acetate in wounded *lox10-2* and *lox10-3* mutant leaves, whereas
2 WT plants emitted normal levels of all three volatiles (Figure 2a). GLV emission
3 was further measured in response to *Spodoptera exigua* feeding on seedlings 2-
4 4 h post infestation. Normal levels of total GLVs (combined values for (3Z)-
5 hexenal, (3Z)-hexenol, (3Z)-hexenyl acetate and (2E)-hexenal were observed in
6 infested WT plants (156 ng/h – B73 background; 230ng/h – W438 background),
7 whereas, *lox10-2* and *lox10-3* mutant emissions were comparable to background
8 levels (Figure 2b). These results show that LOX10 is the sole 13-LOX isoform
9 that provides substrate for the HPL pathway for GLV production in maize leaves
10 in response to wounding or herbivory by *S. exigua*.

11 Because LOX10 was not observed in chloroplasts (Mohanty et al., 2009),
12 the expected site for GLV and JA biosynthesis, we further characterized
13 subcellular localization using a *ZmLOX10*:YFP fluorescent protein tagged gene,
14 driven by the endogenous *ZmLOX10* promoter. Our results showed that
15 fluorescence from the YFP-tagged protein is, indeed, emitted from organelles
16 lacking chlorophyll autofluorescence (Figure 2c, panel E and F), indicating that
17 LOX10 is localized to non-chloroplast organelles.

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19 ***ZmLOX8* is responsible for wound-induced JA in maize**

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21 With the functional role of LOX10 in GLV production confirmed, our next step
22 was to determine which LOX provides 13-hydroperoxy substrate to the JA
23 biosynthesis pathway. To test this, we wounded WT seedlings and measured

13

1 transcript accumulation in candidate maize 13-LOXs (*ZmLOX7*, *ZmLOX8*, and
2 *ZmLOX9*). Of the maize genes that clustered with JA producing LOXs from other
3 plant species (Figure S1), *ZmLOX8* was strongly induced by wounding at early
4 time points (Figure 4c). This data, coupled with the knowledge that *ZmLOX8* is
5 required for JA-mediated tassel development (LOX8 was designated as *ts1* in
6 Acosta et al., 2009), led us to hypothesize that LOX8 provides substrate for
7 wound-induced JA biosynthesis. Using the publicly available *tasselseed1-ref*
8 (*ts1-ref*) allele, a knockout mutant of the *LOX8* locus (Acosta et al., 2009), we
9 measured wound-induced JA levels in homozygous WT and mutant seedlings
10 from the F2 segregating family. Figure 3 shows that *lox8/ts1-ref* mutants (here
11 after denoted as *lox8*) have 66% less JA than WT in response to wounding
12 ($P < 0.05$). JA levels between *lox8* wounded and unwounded controls were not
13 significantly different, indicating that LOX8 provides substrate to the wound-
14 induced JA biosynthesis pathway, but is not responsible for basal JA in
15 unwounded leaves (Figure 3). Unfortunately, *Mu*-insertion mutants in *ZmLOX9*
16 are not available, but analysis of mutants in the *ZmLOX7* gene showed that
17 LOX7 is not responsible for either basal or wound-induced JA in leaves.

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19 **LOX10-derived signaling is required for wound-induced JA via regulation of**

20 **the JA biosynthesis genes *ZmLOX8* and *ZmOPR7/8***

21

22 Because GLVs have been shown to play a strong signaling role for the induction
23 of JA in maize (Engelberth et al., 2004, 2007), we hypothesized that *lox10*

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1 mutants would have decreased wound-induced JA levels. To test this hypothesis,
2 OPDA and JA levels were measured in mechanically wounded leaves of WT and
3 *lox10-2* and *lox10-3* mutants. Accumulation of OPDA, the natural JA precursor,
4 was not different between mutants and WT at the 0 h time point ($P \geq 0.20$), but
5 was significantly higher in WT compared to the mutants 2 h after wounding ($P \leq$
6 0.01; Figure 4a). Twelve hours post wounding, OPDA levels in WT plants were
7 still significantly higher than *lox10-2* and *lox10-3* mutants ($P \leq 0.01$). Similar to
8 OPDA, there was no significant difference in the levels of JA for non-wounded (0
9 h time point) WT and *lox10-2* and *lox10-3* mutants ($P \geq 0.25$; Figure 4b).
10 However, 2 h post wounding, WT levels of JA were approximately 79% higher
11 than *lox10-2* and *lox10-3* mutants ($P \leq 0.001$). By 12 h post-treatment, WT JA
12 levels were still 50% higher than levels in *lox10-2* and *lox10-3* mutants ($P \leq 0.05$).
13 This data strongly suggests that *ZmLOX10* is involved in the wound-induced
14 regulation of OPDA and JA biosynthesis, but not in the production of basal levels
15 of these octadecanoids.

16 To elucidate the potential mechanism of LOX10 involvement in the
17 regulation of wound-induced JA biosynthesis, transcript accumulation of known
18 JA-producing genes was measured in wounded leaves over a time course
19 spanning 8 h. In addition to *ZmLOX8*, there are two other previously
20 characterized JA producing genes in the maize genome, *ZmOPR7* and *ZmOPR8*
21 (Zhang et al., 2005; Yan et al., 2012). These two genes are highly homologous
22 (>95% nucleotide sequence homology) and are the only maize OPRs that
23 phylogenetically cluster with the Arabidopsis JA producing *AtOPR3* (Zhang et al.,

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1 2005; Yan et al., 2012; Schaller et al., 2000; hereafter denoted as *ZmOPR7/8*).
2 Northern blot analysis showed that WT seedling transcript levels of *ZmLOX8*
3 increased strongly as early as 0.5 h after wounding and then returned to basal
4 levels by 4 h post treatment (Figure 4c). Although similar in kinetics, there was a
5 notable decrease in the expression levels of *ZmLOX8* in *lox10-2* and *lox10-3*
6 mutant plants, as compared to WT. Similar differences between WT and *lox10-2*
7 and *lox10-3* were seen in *ZmOPR7/8* gene expression at 2 h post wounding, the
8 time of maximal transcript accumulation for these genes in WT plants. Combined,
9 these results suggest that LOX10-mediated signaling is required for normal
10 wound-induced expression of the JA-producing genes *ZmLOX8* and *ZmOPR7/8*
11 and normal levels of JA biosynthesis in response to mechanical damage.

12 To determine if LOX10-derived GLVs regulate wound-induced JA
13 biosynthesis, we attempted to complement JA levels in *lox10* mutants with GLVs.
14 Because of the rapid volatilization of GLVs and to better mimic the direct release
15 of these compounds from damaged tissue, a substantial amount of GLVs was
16 applied directly to the wound site immediately after wounding. Two hours post-
17 treatment, we observed the partial restoration (~50% recovery) of JA in *lox10-2*
18 mutant plants (Figure 4d). This data suggests that GLVs may play a role in
19 wound-induced JA biosynthesis, but signaling from additional LOX10-derivatives
20 may be required.

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22 **LOX10 mediates HIPV emissions**

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1 To test if disruption of LOX10 alters HIPV emissions, WT and *lox10-2* and *lox10-*
2 *3* mutant seedlings in the B73 background were infested with 3rd instar *S. exigua*
3 larvae and GLVs, monoterpenes, homoterpenes, and sesquiterpenes were
4 collected over a 2 h period. As expected, total GLVs (3Z)-hexenal, (2E)-hexenal,
5 (3Z)-hexen-1-ol, and (3Z)-hexenyl acetate) were essentially absent in *lox10-2*
6 and *lox10-3* mutants as compared to the WT plants ($P < 0.001$; Figure 2b).
7 Emissions of monoterpenes (α -phellandrene, 3-carene, α -terpinene, limonene,
8 ocimene, geranyl acetate, β -pinene, and β -myrcene) and sesquiterpenes
9 (copaene, cedrene, caryophyllene, bergamotene, *E*- β -farnesene, and
10 bicyclosesquiphellandrene) in *lox10-2* and *lox10-3* mutants were about 60%
11 lower than emissions from WT plants ($P \leq 0.03$; $P = 0.066$, respectively; Figure
12 5a). Moreover, levels of homoterpenes (4,8-dimethyl-1,3,7-nonatriene and
13 4,8,12-trimethyl-1,3,7,11-tridecatetraene) were approximately 73% lower in *lox10*
14 mutants ($P \leq 0.02$). In addition to B73, we ran a 7.5 h time course on WT and
15 mutant plants from the W438 genetic background, which produced greater levels
16 of GLVs during *S. exigua* feeding (Figure 2b). Here, even greater differences in
17 HIPVs were observed between WT and *lox10-2* mutant plants (Figure 5b). As
18 expected, GLVs were essentially absent in *lox10-2* mutants at all time points.
19 Monoterpenes and homoterpenes both showed strong induction 2-4 h post
20 infestation demonstrating significant differences between WT and mutant ($P \leq$
21 0.0076 ; $P \leq 0.001$, respectively). Moreover, WT levels for all three classes of
22 volatile terpenes were significantly higher than the mutant at 4-6 h
23 (monoterpenes, $P \leq 0.001$; homoterpenes, $P \leq 0.001$; sesquiterpenes $P \leq 0.001$;

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1 Figure 5b) and 6-7.5 h (monoterpenes, $P \leq 0.001$; homoterpenes, $P \leq 0.001$;
2 sesquiterpenes $P \leq 0.001$) post-infestation. The results from these two
3 experiments in two genetic backgrounds strongly advocate that *ZmLOX10* has
4 an important function in the mediation of HIPV production in response to insect
5 herbivory.

7 **The circadian rhythm regulated *ZmLOX10* and HIPV diurnal emissions**

8
9 While running the 7.5 h time course in the W438 genetic background, we
10 observed an increase in HIPV emissions during mid-day and then a decrease in
11 emissions late in the afternoon and early evening, which was reminiscent of daily
12 volatile patterns observed in diurnal emission studies (Louhgrin et al., 1994;
13 Turlings et al., 1995; Martin et al., 2003). In our previous work, we showed that
14 expression of *ZmLOX10* is regulated by circadian rhythm, with transcript levels
15 peaking in the afternoon at 2pm and decreasing to their lowest levels in the early
16 morning at 2am (Nemchenko et al., 2006; Figure 6). With maize plants
17 dependent on LOX10 for normal HIPV production, we questioned if the
18 significant reduction of HIPV emissions in *lox10* mutants would be sustainable
19 over an extended period of time. To test if mutant plants could recover and
20 produce diurnal emissions despite the loss of LOX10, we infested WT and *lox10*-
21 2 mutants with *Spodoptera littoralis* and monitored HIPV emissions over a 2.5 d
22 time course. Other than modest increases in WT GLVs, HIPV emissions
23 remained low during the first night. During the second day, levels of HIPV in WT

18

1 infested plants were increased to high levels by the afternoon (12:30 – 15:30;
2 GLVs, >15ng/h; Monoterpenes, >80ng/h; homoterpenes >80ng/h;
3 Sesquiterpenes >170ng/h) and then tapered off to low levels again during the
4 evening (Figure 6). On the third day, levels of GLVs, monoterpenes, and
5 homoterpenes, again, increased to high levels in WT plants during the day,
6 however, only a minor increase was observed in sesquiterpenes. Levels of all
7 four classes of volatiles were reduced to low levels by the evening on the third
8 day. For *lox10-2* mutant infested plants, levels of homoterpenes remained low
9 throughout the time course. Increases in monoterpenes and sesquiterpenes
10 were observed during the second day (<40ng/h and <150ng/h, respectively), but
11 remained low throughout the remainder of the time course. There were no GLVs
12 observed throughout the 2.5 d period for *lox10-2* infested plants, and both WT
13 and *lox10-2* controls remained relatively low for all classes of volatiles measured
14 throughout the time course. Collectively, these results suggest that mutation in
15 *ZmLOX10* is sufficient to maintain the disruption of herbivore-induced diurnal
16 emissions over several days.

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18 ***ZmLOX10* mediates parasitoid attraction to HIPVs**

19

20 To determine the behavioral significance of LOX10 in parasitoid attraction, we
21 subjected naïve *Cotesia marginiventris* females to volatiles emitted by W438 WT
22 and *lox10-2* mutants infested with *Spodoptera littoralis* in a six-arm olfactometer
23 system. Odors emitted from WT plants were significantly more attractive to *C.*

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1 *marginiventris* compared to those produced by mutants (Figure 7a). Non-
2 infested WT and mutant genotypes were comparable to mutant infested plants in
3 appeal, but more attractive than empty controls. While plant-emitted volatiles are
4 known to be attractive to parasitoids, the specific maize-derived signal(s) that
5 wasps use to cue in on their prey remains unknown (D'Alessandro et al., 2009).
6 To genetically test for the impact that maize GLVs have on wasp attraction, we
7 used only mutant plants in the six-arm olfactometer, allowing wasps to choose
8 between odors emitted from infested and non-infested *lox10* mutants. In the
9 absence of WT plants, the infested mutants were significantly more attractive
10 than non-infested mutants and empty controls (Figure 7b). These results
11 suggest that GLVs alone are not responsible for parasitoid attraction. Volatile
12 collections during the olfactometer assay revealed several other HIPVs that may
13 be significant attractants for wasps (Figure 7c). HIPVs that had significantly
14 higher emissions in the infested mutants compared to non-infested mutants
15 include linalool ($P = 0.036$), 2-phenethyl acetate ($P = 0.036$), indole ($P = 0.036$),
16 and β -farnesene ($P = 0.016$). Collectively, the data indicate that the volatile suite
17 responsible for *C. marginiventris* attraction is made up of a complex blend, in
18 which GLVs play an important role. The results also implicate that infested *lox10*
19 mutants are not repellent by nature, but are less attractive because of the
20 absence of GLVs and reduced levels of other volatile attractants.

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22 ***lox10* mutants are more susceptible to chewing insects under laboratory**
23 **and field conditions**

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5 2 The orchestrating effect of LOX10 on herbivore-induced defense-related
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8 3 compounds prompted us to measure its biological relevance to insect resistance.
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10 4 We infested near-isogenic WT in the B73 background and *lox10-2* and *lox10-3*
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12 5 mutant plants with 2nd instar *S. exigua* and monitored caterpillar performance by
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14 6 measuring weight gain 6 d after feeding. *S. exigua* that fed on WT plants gained
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16 7 significantly less weight than both *lox10-2* ($P = 0.0265$) and *lox10-3* ($P = 0.0407$)
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18 8 mutants (Figure 8a). To measure herbivore damage on the infested plants, we
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20 9 compared fresh weight of the control non-infested WT and *lox10-2* and *lox10-3*
21
22 10 mutant plants to their counterpart infested plants 6 d after feeding. While the
23
24 11 weight of the WT infested plants was not significantly different from that of the
25
26 12 control non-infested plants after *S. exigua* feeding (Figure 8b; $P = 0.5456$), both
27
28 13 infested *lox10-2* and *lox10-3* mutant plants weighed significantly less than their
29
30 14 non-infested equivalents ($P = 0.03$ and $P = 0.05$, respectively), indicating that the
31
32 15 *S. exigua* consumed more plant tissue when feeding on mutant plants than on
33
34 16 WT. The feeding preference of *S. exigua* on WT and *lox10* mutants was
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36 17 measured by placing larvae in pots with WT and *lox10-2*, or WT and *lox10-3*
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38 18 plants. Six days post-infestation, weights of WT and mutant infested plants were
39
40 19 compared to non-infested plants to estimate weight loss incurred by larval
41
42 20 feeding. Figure 8c shows that both *lox10-2* and *lox10-3* mutant plants decreased
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44 21 in weight over the 6 d feeding period, whereas WT plants showed increased
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46 22 weight gain. To see how *lox10* mutants would perform under field conditions, we
47
48 23 planted WT and *lox10-2* and *lox10-3* mutant plants in the B73 background in the
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1 College Station, TX field in 2010 and WT and *lox10-2* mutants in the W438
2 background in 2011. In 2010, larvae consumed 36% and 39% more leaf tissue
3 on *lox10-2* and *lox10-3* mutant plants, respectively, than WT (Figure 8d). In 2011,
4 insects consumed 76% more leaf tissue from *lox10-2* mutants than WT plants in
5 the W438 background. These field results implicate a role for LOX10 in both
6 direct and possibly indirect defense responses. Collectively, our laboratory and
7 field results show that *ZmLOX10* plays a significant biological role in resistance
8 to herbivory.

10 DISCUSSION

11
12 Plants have evolved the ability to resist insect attack by inducible direct and
13 indirect defense responses. The characterization of these responses in maize is
14 fragmentary, yet emerging evidence suggests that the major strategy of maize
15 involves diverse lipid oxidation products that serve as defense signals, including
16 those derived from the HPL and AOS branches of the LOX pathway (Farag and
17 Pare, 2002; Engelberth, et al., 2004; Ton et al., 2007; Engelberth, 2011). Several
18 studies have described inducible genes associated with these defense signals in
19 an effort to exploit the information to enhance agronomic prosperity. While these
20 advances in scientific research have shed a much needed light on the potential
21 biological and ecological roles of these genes, genetic studies elucidating the
22 specific genes that generate and regulate these defense signals in the
23 agronomically important crop maize remain unknown. In this study, we provide

22

1 genetic and biochemical evidence for the individual LOX isoforms that provide
2 substrate for the GLV and JA biosynthesis pathways and elucidate the significant
3 modulating role that *ZmLOX10* plays in direct and indirect defense responses,
4 both under laboratory and field conditions.

5 One of the principle findings of this study is that LOX10 is the sole isoform,
6 among the 13 individual LOX genes identified in the maize genome, responsible
7 for generating 13S-HPOTE for the GLV biosynthesis pathway. Evidence for this
8 was generated in both wounding and herbivore related treatments where *lox10-2*
9 and *lox10-3* mutants were GLV-deficient. Surprisingly, this occurs in the
10 presence of five other functional 13-LOXs including *ZmLOX11*, which is a
11 segmentally duplicated gene that shares 90% identity at the deduced amino acid
12 level with LOX10 (Nemchenko et al., 2006). Nemchenko et al. (2006) showed
13 that *ZmLOX10* and *ZmLOX11* are differentially expressed under diverse
14 treatments and in an organ-specific manner, which suggests that the biochemical
15 and perhaps physiological functions of these paralogs diverged since their
16 duplication event, possibly as a result of minimal selection pressure on
17 *ZmLOX11* in the presence of a functional *ZmLOX10*. The fact that *ZmLOX10* is
18 uniquely responsible for GLV biosynthesis in leaves in the presence of *ZmLOX11*
19 and the four other 13-LOXs underscores the functional significance of the
20 differential transcriptional regulation, subcellular localization, and tissue-specific
21 spatial organization that exists among different LOX isoforms in planta
22 (Bannenberget al., 2009).

1 Differential LOX function due to distinct localizations is further exemplified
2 by the diverse roles of LOX8 and LOX10. In dicots, the JA and HPL pathways
3 have been shown to compete for the same substrate, 13-hydroperoxide of
4 linolenic acid. Evidence of this can be seen when one pathway is shut down and
5 the other produces more product due to diminished substrate competition
6 (Halitschke and Baldwin, 2003; Halitschke et al., 2004; Chehab et al., 2008). This
7 negative correlation is most likely due to co-localization between the JA- and
8 HPL-associated enzymes in dicots, both being in the chloroplast (Froehlich et al.,
9 2001; Farmaki et al., 2007). The GLV and JA pathways in maize, however, are
10 physically separated from each other, as evidenced by the localization of the JA
11 producing LOX8 to chloroplasts (Acosta et al., 2009) and the GLV producing
12 LOX10 to organelles distinct from chloroplasts (Figure 2c). The physical
13 separation of these two LOXs in distinct organelles prevents competition for the
14 JA and GLV pathway substrate, suggesting that any interaction between these
15 two LOX pathway branches would be due to signaling cross-talk. Decreased
16 wound-induced transcript levels of the JA producing genes *ZmLOX8* and
17 *ZmOPR7/8* (Figure 4c) in *lox10* mutants, plus the partial restoration of wound-
18 induced JA in *lox10* mutants with GLVs (Figure 4d), indicates that wound-
19 induced JA production is mediated by oxylipin signals derived from LOX10. To
20 date, the intermediates and final products of the JA and GLV LOX branch
21 pathways have never been reported to engage in signaling crosstalk. This is
22 rather surprising given the well-known potency of these signals, their induction by
23 the same stresses, and their proximity *in cell*. This study presents evidence that

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3 1 in maize, the products of LOX10 have a dramatic impact on wound-induced JA
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5 2 biosynthesis.
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8 3 Another important discovery of this study was the finding that *lox10*
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10 4 mutants are reduced in HIPV production. Mono-, homo-, and sesquiterpene
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12 5 levels were reduced in *lox10* mutants during herbivore feeding assays run in both
13
14 6 the B73 and W438 genetic backgrounds. During an extended time course we
15
16 7 also tested for a possible function of LOX10 in diurnal emissions. Our monitoring
17
18 8 of HIPV emissions infested WT and *lox10* mutant plants over a 2.5 d period
19
20 9 showed that clock-regulated expression of *ZmLOX10* (Nemchenko et al., 2006)
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22 10 may be a mechanistic link for circadian GLV emissions and explains, at least in
23
24 11 part, the well characterized HIPV diurnal emissions. Our study genetically shows
25
26 12 that GLVs may contribute to the induction of HIPV, supporting previous results
27
28 13 obtained using exogenous GLVs (Farag and Pare, 2002; Engelberth et al., 2004,
29
30 14 2007). Other reports have shown the significance of JA in mediating HIPV
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32 15 emissions (Dicke et al., 1999; Koch et al., 1999; Bruinsma et al., 2009) and a
33
34 16 recent study showed that JA production follows a diurnal pattern during herbivory
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36 17 (Godspeed et al., 2012). Whether or not GLVs have a direct impact on HIPV
37
38 18 production in maize or if HIPVs are solely JA-regulated needs further
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40 19 investigation, using appropriate mutants and/or double mutants in the respective
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42 20 pathways.
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50 21 Since LOX10 is required for GLV, JA, and HIPV production, it is not
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52 22 surprising that *lox10* mutants were more susceptible to insect feeding. Under
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54 23 laboratory conditions, the increase in mutant plant consumption paralleled the
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3 1 increase in larval weight gain and the mutants were significantly preferred over
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5 2 WT by *S. exigua*, indicating a role for LOX10 in direct defense. This preference
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7 3 for, and lack of resistance in, *lox10* mutants was also observed under field
8
9 4 conditions, which may have been a result of both direct and indirect defense
10
11 5 responses. In parasitoid attraction assays, WT plants had a significantly greater
12
13 6 appeal to *C. marginiventris* than *lox10-2* mutants, genetically confirming the
14
15 7 importance of HIPVs for wasp attraction in WT maize plants. Further tests using
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17 8 GLV-deficient *lox10* mutants (Fig. 9) showed that HIPVs other than GLVs are
18
19 9 important for parasitoid attraction in maize. Indeed, attraction appears to be due
20
21 10 to a complex volatile blend, of which, GLVs play an important role. Collectively,
22
23 11 our parasitoid data suggests that LOX10 has a strong ecological function in
24
25 12 parasitic wasp behavior.
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32 13 Based on our finding that LOX10 regulates, at least in part, JA- and HIPV-
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34 14 mediated defense responses for resistance to insect attack, we propose a model
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36 15 that describes this interaction (Figure 9). It is proposed that α -linolenic acid is
37
38 16 catalyzed by LOX10 to produce 13S-HPOTE, which is utilized by the HPL branch
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40 17 of the LOX pathway for GLV production. Upon wounding/herbivory, LOX10
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42 18 derivatives are produced in damaged tissue, which induce transcription of
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44 19 *ZmLOX8* and *ZmOPR7/8* for increased levels of JA production. Because both
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46 20 exogenous GLVs and JA have been proposed to induce HIPV emissions (Farg
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48 21 and Pare, 2002), it is further suggested that GLVs may, along with JA, contribute
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50 22 to the release of HIPVs for indirect defense. The collective induction of these
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3 1 important defensive compounds results in heightened wasp attraction and
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6 2 increased resistance to herbivory.

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8 3 In summary, although maize is an important agro-economical crop with
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10 4 broad applications in the food, chemical, livestock, and biofuel industries, little is
11
12 5 understood in terms of defensive gene function because of the difficulty in
13
14 6 generating knockout mutants and the resulting lack of genetic studies. In this
15
16 7 report we show that LOX10 provides substrate to the GLV biosynthesis pathway
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18 8 and is localized to organelles distinct from chloroplasts. Furthermore, we
19
20 9 demonstrate that the chloroplast localized LOX8 is responsible for wound-
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22 10 induced JA, yet LOX8-mediated JA production is dependent on signaling from
23
24 11 LOX10-derived oxylipins. The functional role that LOX10 plays in direct defense
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26 12 responses is evident by the susceptibility levels of *lox10* mutants to insect
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28 13 feeding during the described biological assays in this study. Additionally, LOX10
29
30 14 plays a key role in indirect defense, as evidenced by the reduced levels of HIPV
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32 15 in *lox10* mutants and the resulting diminished attraction of *C. marginiventris*.
33
34 16 These findings denote *ZmLOX10* as an important herbivore defense-related
35
36 17 gene that plays a central role in herbivore-induced defense mechanisms in maize.
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38 18 Knowledge gained from the characterization of *ZmLOX10* in plant defense
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40 19 against herbivory will contribute to improve our knowledge about plant-insect
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42 20 interaction evolution, and may help to develop marker-assisted selection
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44 21 strategies in breeding for insect resistance.
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55 23 **MATERIALS AND METHODS**
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2 Plant materials and subcellular localization

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4 The reverse genetics resource [Trait Utility System for Corn (TUSC)] at Pioneer
5 Hi-bred Inc. was used to generate mutant alleles as described by Gao et al.
6 (2007), using a *Mu* (*Mutator*) specific primer and *ZmLOX10* gene-specific primers
7 (see Methods S1). Three *Mu*-insertional alleles were detected for the *ZmLOX10*
8 locus (*lox10-1*, *lox10-2* and *lox10-3*) and the flanking regions of each insertion
9 site was cloned with a pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA, U.S.A.)
10 and sequenced to determine the precise location of the insertion sites. The *Mu*-
11 insertion site for *lox10-1* positioned 342 bp inside the first intron and *lox10-2* and
12 *lox10-3* insertion sites positioned 253 and 278 bp inside the third exon,
13 respectively. Original mutants were backcrossed into the B73 and W438 genetic
14 backgrounds and genetically advanced to eliminate unwanted *Mu* insertions
15 throughout the genome and to create near-isogenic mutant and wild-type lines
16 (BC₃F₄ – BC₅F₄; as designated below) suitable for functional analysis. The *lox8*
17 mutant was acquired from the Maize Genetics Cooperation Stock Center at The
18 University of Illinois at Urbana-Champaign (Maize COOP,
19 <http://maizecoop.cropsci.uiuc.edu>) as a segregating 1:1 heterozygous: mutant
20 population. Heterozygous individuals were selfed and a subsequent 1:2:1
21 segregating population was genotyped with gene specific primers (see Methods
22 S1). For the *ZmLOX10*pro:*ZmLOX10*-YFP transgenic line, the construct was
23 generated using the MultiSite Gateway® Three-Fragment Vector Construction Kit

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1 (Invitrogen). The complete coding sequence of *ZmLox10* plus 2.609 kilobases of
2 upstream and 1.589 kilobases of downstream genomic sequence were
3 recombined into the maize pTF101.1 binary vector, tagging *ZmLox10* with YFP
4 citrine at the C-terminus. *Agrobacterium*-mediated transformation of maize Hill
5 was performed by the Iowa State University Plant Transformation Facility. T0s
6 were crossed to the inbred line B73 and T1s imaged live using a Zeiss 510
7 confocal laser-scanning microscope. Construct sequences and image metadata
8 are available at <http://maize.jcvi.org/cellgenomics/index.shtml>.

10 **Phylogenetic analysis**

11
12 A maximum likelihood tree was built by aligning protein sequence from maize 13-
13 LOXs (ZmLOX7, ZmLOX8, ZmLOX9, ZmLOX10, ZmLOX11, ZmLOX13) and
14 other GLV- or JA-relevant 13-LOXs in tomato (TomLOXC and TomLOXD), potato
15 (StLOXH-1 and StLOXH-3), and tobacco (NaLOX3) using Clustal W software
16 (Guindon and Gascuel 2003). Phylogeny was reconstructed using the LG
17 substitution model implemented in PHYLM 3.0 software.

19 **Quantification of GLVs and HIPV**

20
21 Two grams of leaf-cuttings from V2 stage WT and *lox10-2* and *lox10-3* maize
22 plants were placed into glass cylinders connected to a Super Q filter trap (Alltech
23 Associates) and methods were followed as described by Engerlberth et al.,

1 (2004). For HIPV emission collections, *S. exigua* were reared on pinto-based
2 artificial diet (Southland Products Inc., Lake Village, AR) at 25° C to the 3rd instar
3 developmental stage. The night before volatile collection, *S. exigua* were fed in
4 individual diet cups on corn leaf cuttings. Experimental treatments (consisting of
5 infested and non-infested WT and *lox10* mutant plants) and sample extractions
6 were carried out in the J. Tumlinson lab at Penn State University, as previously
7 described by Cardoza et al., (2006).

9 **Oxylipin profiling of wounded and GLV treated plants**

10
11 WT (BC₅F₂) and mutant allele *lox10-2* and *lox10-3* (BC₄F₂ and BC₅F₂,
12 respectively) maize seedlings were grown between 25-28°C in commercial soil
13 (Metro-Mix 366; Scotts-Sierra Horticultural Products, Marysville, OH, U.S.A.)
14 under a 12 hour photoperiod (120 μmol m⁻² s⁻¹; Quantum Meter; Apogee
15 Instruments, Logan, UT, U.S.A.). V2 seedlings were placed in the dark for a
16 period consisting of two consecutive nights to circumvent the circadian rhythm
17 regulated gene expression of *ZmLOX10* (Nemchenko et al. 2006). Under green
18 light, seedlings were wounded seven times in the mid portion of the second leaf
19 (three wound sites on one side of the midvein and four on the other side), using a
20 hemostat (wound sites approx. 1-2cm apart). The second leaf from WT and
21 *lox10-2* and *lox10-3* seedlings were harvested in liquid N₂ at the 0hr, 2hr, and
22 12hr time points and subsequently analyzed for free oxylipins (JA and OPDA) as
23 outlined in the methods from the University of Göttingen oxylipin database

30

1 website (<http://www.oxylipins.uni-goettingen.de/>), with minor modifications. For
2 *ZmLOX8*, JA was measured by LC/MS in negative phase mode using methods
3 outlined by Pan et al., (2008) with minor modifications (Methods S2).

5 Northern blot analysis

6
7 For northern blots, total RNA was extracted using the standard TRI reagent
8 protocol (Molecular Research Center Inc., Cincinnati, OH). Following extraction,
9 RNA (12ug) was separated by a 1.5% formaldehyde/1X MOPS gel and
10 transferred to a MagnaGraph nylon membrane (Micron Separations Inc.,
11 Westboro, MA) in 10XSSC (1500 mM sodium chloride, 150 mM sodium citrate)
12 overnight. Blots were prehybridized with UltraHyb hybridization solution (Ambion,
13 Austin, TX) and probed overnight with a ³²P-labeled *ZmLOX10*, *ZmLOX8*, or
14 *ZmOPR7/8* gene-specific probe as indicated below. Blots were washed 2 X 5min
15 (or 2 X 15min for *ZmLOX8*) with a 2×SSC and 0.1% SDS (sodium dodecyl
16 sulfate) solution (an additional 2 X 5min or 3 X 15 min wash with 0.1×SSC and
17 0.1% SDS was performed for the blots hybridized to *ZmLOX8* gene specific and
18 *ZmOPR7/8* probes, respectively) and exposed to BioMax X-ray film (Kodak,
19 Rochester, NY) at –80 °C for 1 to 6 days prior to developing the films. rRNA
20 loading controls were visualized with ethidium bromide staining and UV
21 transillumination. For gene expression of *ZmLOX8* and *ZmOPR7/8* in both WT
22 (BC₄F₂) and *lox10-2* and *lox10-3* (BC₄F₂ and BC₃F₇, respectively) mutant
23 backgrounds, seedlings were grown at 25-29°C in commercial soil (SB300

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1 Universal Mix; Sungro Sunshine, Belle Vue, WA, U.S.A.) under a 12 hour
2 photoperiod. V-2 stage plants were wounded using the treatment described
3 above and immediately harvested in liquid N₂ at 0 h, 30 min, 1 h, 2 h, 4 h and 8 h
4 for northern blot analysis.

5 **Biological assays and Field trials**

6
7
8 Preference and performance feeding assays were carried out with WT and *lox10-*
9 *2* and *lox10-3* seedlings in the B73 genetic background. Five replicates, each
10 with seedlings planted in 5-inch pots, were randomly arranged to have treated
11 and untreated lines consisting of individual WT, *lox10-2* or *lox10-3* seedlings
12 (performance test) or all three genotypes together (preference test) in each
13 replicate. When seedlings reached the V-3 stage, the three plants were loosely
14 put together to form a trio. A cage sleeve was then carefully placed over the
15 pot/plants and an individual 2nd instar *S. exigua* larva was weighed and placed in
16 the middle of the trio before the cage was fastened at the top. Six days post
17 infestation, *S. exigua* were removed and weighed to determine total weight gain
18 and seedlings were cut at the soil level and fresh weight was taken. For field
19 studies, two to four WT and mutant rows (7.62 m /row) were planted (25 seeds
20 per row) in test plots and exposed to natural infestation. Leaf chewing herbivores
21 identified in the College Station field include, fall armyworm, sugarcane corn
22 borer, European corn borer, and southwestern corn borer. % damage was

1 estimated by a scoring system ranging from 0-6, where 0 = no damage; 1 = ~7%;
2 2 = ~14%; 3 = ~21%; 4 = ~28%; 5 = ~35%; and 6 = ~42% damaged area.

3

4 **Wasp attraction and circadian rhythm assays**

5
6 Rearing of the caterpillar *S. littoralis* and the endoparasitoid *C. marginiventris*
7 were carried out as described by Turlings et al., (2004). Plants for wasp
8 attraction and circadian rhythm assays were grown according to methods
9 described by Erb et al., (2010) and olfactometer bioassays and odor collections
10 were done as previously described by Turlings et al., (2004). For circadian
11 rhythm, volatiles were collected for three hour periods over a 2.5 d time course at
12 the 1930, 0130, 0730, and 1330 times of day under continuous feeding with 10 *S.*
13 *littoralis* 2nd instar caterpillars or no-treatment (control) conditions (n=6).

14

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16

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4
5
6 2 Dr. Jurgen Engelberth.
7

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10 4 **SUPPORTING INFORMATION LEGEND**
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13 5 Figure S1. Phylogeny of GLV- and JA-associated LOXs from different plant
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CONFIDENTIAL

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3 **1 FIGURE LEGENDS**
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8 Figure 1. *Mu*-insertions in the *lox10-2* and *lox10-3* mutant alleles resulted in
9
10 suppression of *ZmLOX10* gene expression suggesting that they are null alleles.

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12 (a) Schematic diagram of *Mu*-insertion sites in the *ZmLOX10* gene. *lox10-2* and
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15 *lox10-3* are exonic insertions.
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18 (b) Northern blot analysis of *ZmLOX10* transcript levels in WT and *lox10-2* and
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21 *lox10-3* mutants 8 h post-wounding.
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24 **9**

25 Figure 2. LOX10 is responsible for GLVs in leaves of wounded and *S. exigua*
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27 infested maize plants and is localized to organelles lacking chlorophyll
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30 autofluorescence.
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32 (a) A representative chromatogram of GC/MS analysis showing volatile
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34 emissions from mechanically-wounded leaves of WT and *lox10-2* and *lox10-3*
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36 mutant plants. Volatiles are labeled numerically as follows: (1) (3Z)-hexenal, (2)
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38 (3Z)-hexenol, and (3) (3Z)-hexenyl acetate. U = Unknown compound.
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41 (b) Quantification of total GLV emissions in *lox10-2* and *lox10-3* mutant and WT
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43 seedlings 2-4 h post-infestation with *Spodoptera exigua* in WT and mutant alleles
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45 in the B73 and W438 genetic backgrounds. Measurement of selected volatile
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47 emissions from maize seedlings presented as total GLVs (B73: (3Z)-hexenal,
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49 (2E)-hexenal, (3Z)-hexen-1-ol, and (3Z)-hexenyl acetate; W438: (3Z)-hexenal,
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51 (2E)-hexenal, (3Z)-hexen-1-ol, (3Z)-hexenyl isobutyrate, and (3Z)-hexenyl
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53 acetate). Data represented as means \pm standard deviation (SD).
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3 1 (c) Localization of LOX10-YFP to organelles lacking standard chlorophyll
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5 2 autofluorescence. Confocal micrographs of representative images comparing WT
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7 and LOX10-YFP, showing chlorophyll autofluorescence (left panel, A and D),
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9 3 YFP fluorescence (middle panel, B and E), and merged chlorophyll
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11 4 autofluorescence and YFP fluorescence (right panel, C and F). Scale bars =
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13 5
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15 6 5 μ m.
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19
20 8 Figure 3. *ZmLOX8* (*tassel seed 1*) mutants produce lower levels of JA in
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22 9 response to mechanical wounding of leaves. JA was measured in wounded
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24 10 leaves of WT and *lox8* mutant seedlings 1 h post treatment (mean \pm SD;
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26 11 ANOVA; * $P \leq 0.05$).
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31
32 13 Figure 4. LOX10-derived signaling regulates wound-induced JA and expression
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34 14 of the JA biosynthesis genes *ZmLOX8* and *ZmOPR7/8*.

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36 15 (a) and (b) Mutant *lox10-2* and *lox10-3* alleles in the B73 background produce
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38 16 reduced levels of OPDA and JA in response to mechanical wounding.

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40 17 Quantification of OPDA (a) and JA (b) levels (mean \pm SD) in maize seedlings 0, 2,
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42 18 and 12 h post wounding by hemostat (n = 4). Asterisks specify significant
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44 19 differences between WT and mutants under comparable treatments (ANOVA,
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46 20 Tukey pairwise comparison; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).
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50 21 (c) *ZmLOX8* and *ZmOPR7/8* transcript accumulation in response to wounding
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52 22 of *lox10-2* and *lox10-3* mutant and WT seedlings. The second leaf from maize
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54 23 seedlings were mechanically wounded with a hemostat and collected at 0, 0.5, 1,
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1 2, 4, and 8 h post treatment. Total RNA was transferred to a nylon membrane
2 and hybridized with either a *ZmLOX8* or *ZmOPR7/8* probe. Equal loading of
3 RNA was visualized by ethidium bromide staining and UV transillumination.

4 (d) Exogenously applied GLVs partially restore WT levels of JA in *lox10* mutants.
5 WT and *lox10* mutant plants were exposed to either ethanol solvent (control) or
6 Z-3-hexenyl acetate dissolved in ethanol (GLVs). Plants were mechanically
7 wounded (MW) and GLVs or the control was immediately applied to each wound
8 site. Treated leaf tissue was collected 2 h post treatment. Letters represent
9 significant differences between bars (mean \pm SE; ANOVA; * $P \leq 0.05$).

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11 Figure 5. Herbivore-induced plant volatile (HIPV) emissions from beat armyworm
12 infested maize plants.

13 (a) HIPV emissions (mean \pm SD; ng per hour) in beat armyworm (*Spodoptera*
14 *exigua*) infested wild type and *lox10-2* and *lox10-3* mutant plants in the B73
15 genetic background. Bars represent mono-, homo-, and sesquiterpene emissions
16 during a 2 h collection period.

17 (b) Emanation of HIPVs (mean \pm SD; ng per hour) from WT and *lox10-2* mutant
18 *S. exigua* infested maize plants in the W438 genetic background over a 7.5 h
19 time course. Significant differences in GLVs, monoterpenes, homoterpenes, and
20 sesquiterpenes are indicated by asterisks (ANOVA, Tukey pairwise comparison;
21 * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

22

50

1 Figure 6. Circadian rhythm regulation of *ZmLOX10* and diurnal emissions of
2 herbivore-induced plant volatiles (HIPVs) in WT and *lox10-2* mutants.
3 The circadian rhythm regulated gene expression of *ZmLOX10* (center) (modified
4 from Nemchenko et al., 2006, by quantifying expression levels). HIPV emissions
5 were collected from infested and non-infested WT and *lox10-2* mutants over a
6 2.5 day time course. Diurnal emission patterns are shown for GLVs (top left),
7 monoterpenes (top right), homoterpenes (bottom left), and sesquiterpenes
8 (bottom right). Shaded grey areas represent night/dark, non-shaded area
9 represent day/light. X-axis = time of day; y-axis = specified volatiles emitted in
10 ng per hour.

11
12 Figure 7. Olfactometer responses of naive *C. marginiventris* females to WT and
13 *lox10-2* mutant plants in the W438 background. Values shown are number of
14 attracted parasitoids to each treatment. (a) Wasps were allowed to choose
15 between odors from WT Infested, *lox10-2* infested, WT non-infested, *lox10-2*
16 non-infested, and empty controls. Sixteen groups of 6 female parasitoids were
17 tested each experimental day at two fixed times. A total of 288 wasps were
18 released over the course of three consecutive experimental days. Pie-chart
19 indicates the percent response of wasps that made a choice. Different letters on
20 the same colored bars indicate significant differences ($P < 0.05$). (b) Wasps were
21 allowed to choose between odors from *lox10-2* mutant infested and *lox10-2*
22 mutant non-infested plants and empty controls. Six groups of 6 female
23 parasitoids were tested each experimental day at a fixed time (1-3 p.m.). (c)

51

1 Mean amount (\pm SE) (ng) of HIPVs trapped by super-Q filters from *Spodoptera*-
2 induced infested and non-infested *lox10* mutant plants during a 3 hr collection
3 period. Asterisks above bars indicate significant differences (Kruskal-Wallis One
4 Way Analysis of Variance on Ranks * $P < 0.05$).

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6 Figure 8. Herbivore performance and preference bioassays in the laboratory and
7 the field.

8 (a) and (b) Performance bioassays. Separate pots of wild type (WT), *lox10-2*, or
9 *lox10-3* maize plants were infested with a single 2nd instar *Spodoptera exigua*
10 larva. Six days post-infestation, plants and larvae were collected and weighed.
11 (a) Infested plants (grey) compared to non-infested (black) controls. (b) Larval
12 weight gain 6 d post infestation.

13 (c) Preference bioassays. Pots containing WT and *lox10-2*, or WT and *lox10-3*
14 were infested with a single *S. exigua* larva. Plant weight was measured 6 d post
15 infestation. Graph represents plant weight loss as determined by comparison of
16 infested plants with their respective non-infested controls (mean \pm SD; Kruskal-
17 Wallis Test; * $P \leq 0.05$).

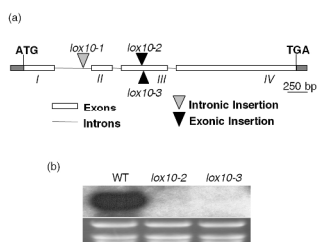
18 (d) Field experiment consisting of WT and *lox10-2* and *lox10-3* mutant plants in
19 the B73 background in the College Station 2010 field and WT and *lox10-2*
20 mutants in the W438 background in the College Station 2011 field. WT and
21 mutant plants were scored for % leaf damage.

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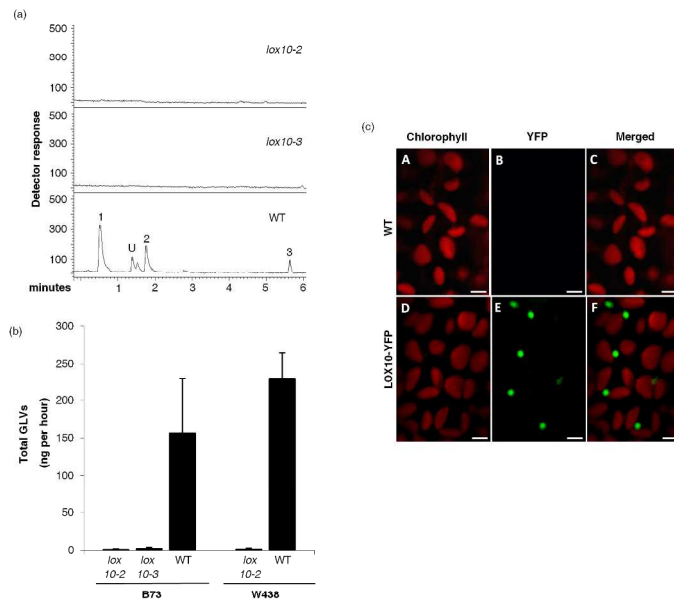
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3 1 Figure 9. A working model for the role of *ZmLOX10* in GLV biosynthesis and the
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5 2 regulation of JA and HIPVs. AOS, allene oxide synthase; AOC, allene oxide
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7 3 cyclase; OPR, oxophytodienoic acid reductase; HPL, hydroperoxide lyase. Solid
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9 4 black arrows indicate known mechanisms; dashed black arrows designate
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11 5 hypothetical roles of LOX10 in JA biosynthesis and HIPV production.
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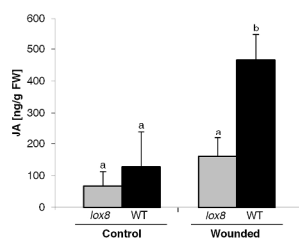
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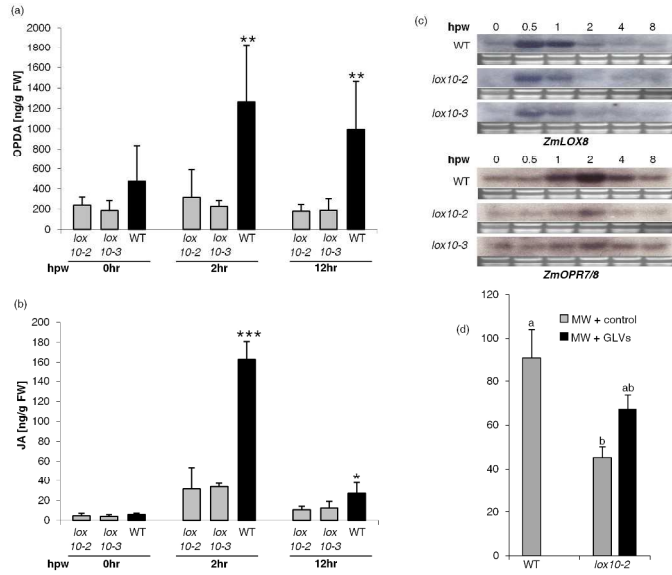


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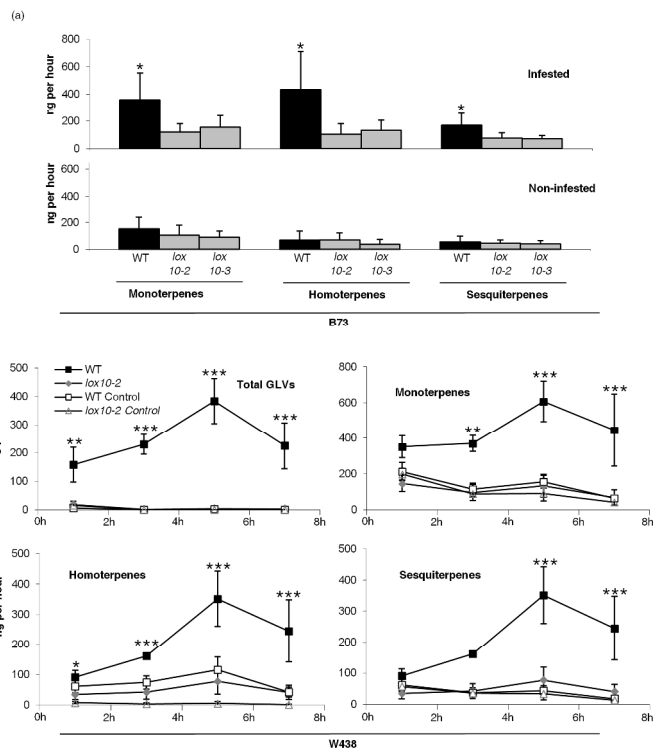


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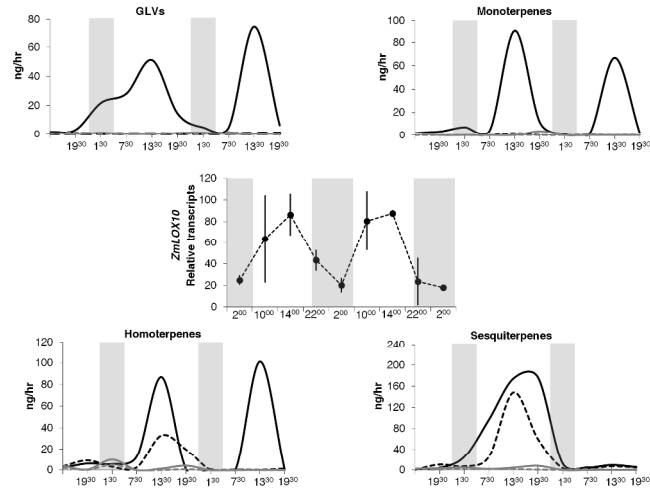


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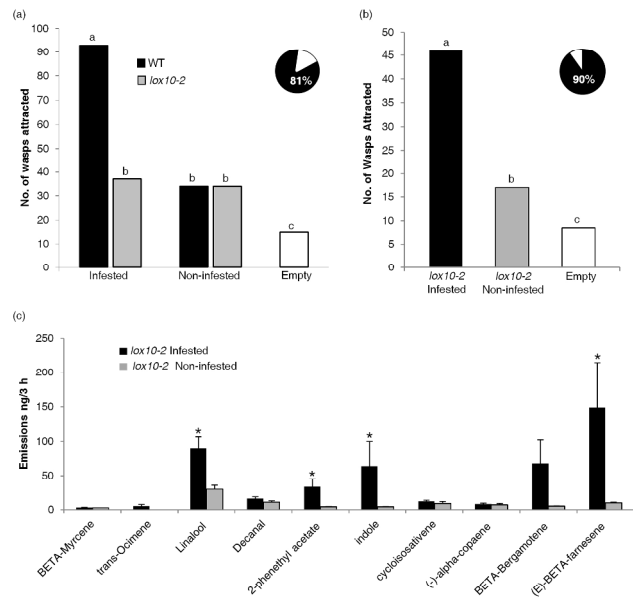
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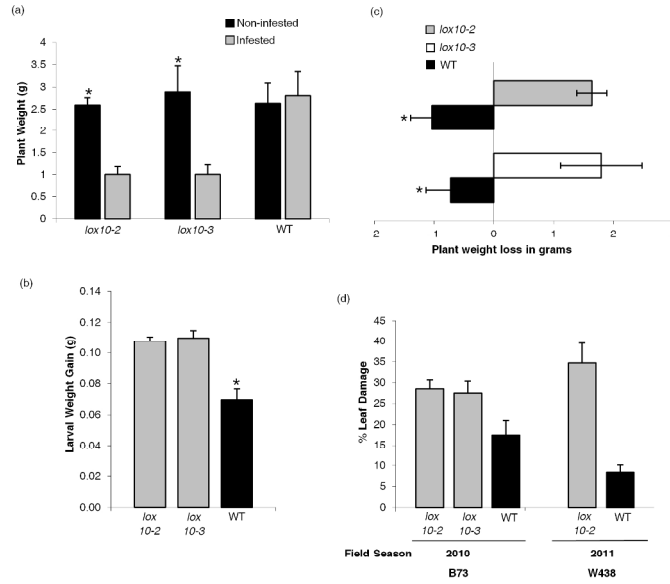
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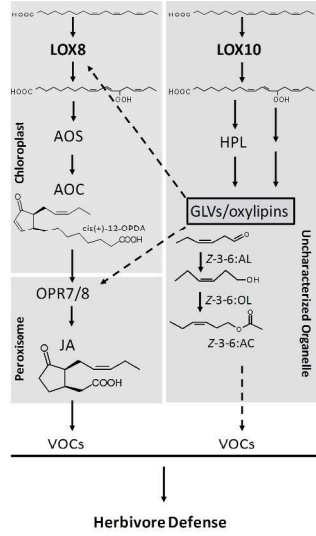


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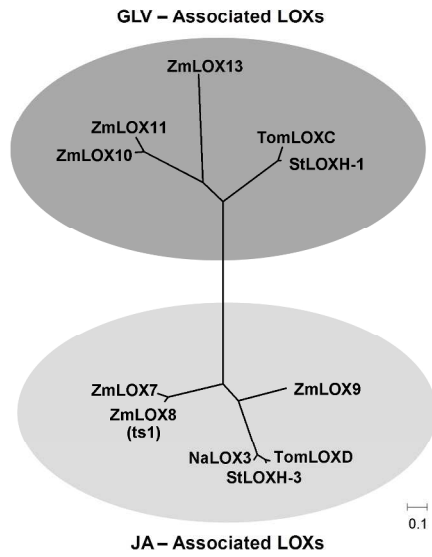
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Supplemental Methods

S1. Gene-specific primers

Sequence Description	Primer Name	Sequence
Mutator Transposon	9242	AGA-GAA-GCCAAC-GCC-AWC-GCC-TCY-A
ZmLOX10	61560 F	CGC-TAG-CTT-AGC-CAC-CAG-TAG-TCC
	61561 R	GCG-CTG-GAA-GTA-CAT-CTG-CCC-GA
	61563 F	GCC-ATC-GGG-CAG-ATG-TAC-TTC-CAG
	61562 R	GGA-AGC-GCA-GAT-CCT-TCT-TGT-TGA-TGA-G
	61564 R	TAG-TCG-TAG-ACG-CGC-TCG-AAC-ACC-TT
	61565 R	AAC-TCC-TCG-TCC-TTG-AAC-CAC-GAG-AAC
	61566 F	TTC-TCG-TGG-TTC-AAG-GAC-GAG-GAG-TTC
	61567 R	CCT-CTG-CAT-GCA-ATA-ATA-GTA-CAC-CCC-C
	61568 F	CGG-CTG-TTC-ATC-CTG-GAC-TAC-CAC
	61569 R	CAG-GTT-GTT-GTT-GCA-CTC-GTC-GAT-GAA-C
ZmLOX8	P1767	CTAGTCCACCGGCGATTTGTTG
	P1768	ACGATGATTCGGTAAGATCGATG

S2. Measurement of jasmonic acid by LC/MS

~100mg of leaf tissue was placed in a 2-ml screw-cap Fast-Prep tube (Qbiogene, Carlsbad, CA, U.S.A.) with 10-50ng of the standard dihydro JA and 0.5-1g of Zirmil beads (1.1 mm; SEPR Ceramic Beads and Powders, Mountainside, NJ, U.S.A.). Samples were homogenized by a Precellys® 24 tissue homogenizer (Bertin Technologies, Saint-Quentin-en-Yvelines-Cedex, France) and shaken for 30min at 4°C. Dichloromethane was then added (1ml) and then samples were reshaken for 30min at 4°C, followed by centrifugation for 5min at 13,000g. The bottom organic phase was then transferred into 1.8ml LOV autosampler glass vials (WVR International, West Chester, PA), evaporated by continuous air flow, and then dissolved in 300ul of methanol. JA was measured on a QTrap 2000 (Applied Biosystems, Foster, CA) LC/MS in multiple reaction mode, using a Discovery C18 HPLC column (5 cm×2.1, 5µm particle size; Supelco, Bellefonte, PA) at a 100ul/min flow rate. For GLV rescue treatments, we attempted to mimic basal levels of GLVs by placing WT and *lox10* mutant plants in containers completely open to the air and exposing them to exogenous GLVs (50ul of 100ug/ul on a cotton swab) once a day for four consecutive days prior to wounding treatment. However, this attempt to more closely replicate WT conditions may be superfluous as experiments without pretreatment of

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GLVs also partially restored JA levels in *lox10* mutants. To restore wound-induced JA to *lox10* mutants, we wounded the third leaf of V-3 plants as described above and applied 110 nL of Z-3-hexenyl acetate to each wound site. 2hr post-treatment, tissue was collected and JA was measured by LC/MS.

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