Facilitation of *Fusarium graminearum* Infection by 9-Lipoxygenases in *Arabidopsis* and Wheat

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Fusarium graminearum causes *Fusarium* head blight, an important disease of wheat. *F. graminearum* can also cause disease in *Arabidopsis thaliana*. Here, we show that the *Arabidopsis* *LOX1* and *LOX5* genes, which encode 9-lipoxygenases (9-LOXs), are targeted during this interaction to facilitate infection. *LOX1* and *LOX5* expression were upregulated in *F. graminearum*-inoculated plants and loss of *LOX1* or *LOX5* function resulted in enhanced disease resistance in the corresponding mutant plants. The enhanced resistance to *F. graminearum* infection in the *lox1* and *lox5* mutants was accompanied by more robust induction of salicylic acid (SA) accumulation and signaling and attenuation of jasmonic acid (JA) signaling in response to infection. The *lox1*- and *lox5*-conferred resistance was diminished in plants expressing the SA-degrading salicylate hydroxylase or by the application of methyl-JA. Results presented here suggest that plant 9-LOXs are engaged during infection to control the balance between SA and JA signaling to facilitate infection. Furthermore, since silencing of *TaLpx-1* encoding a 9-LOX with homology to *LOX1* and *LOX5*, resulted in enhanced resistance against *F. graminearum* in wheat, we suggest that 9-LOXs have a conserved role as susceptibility factors in disease caused by this important fungus in *Arabidopsis* and wheat.

Fungi in genus *Fusarium* include several important phytopathogens. In wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), *Fusarium* head blight (FHB), which is an important disease of floral tissues, is caused by a complex of closely related *Fusarium* species. The principal causal agent for the disease in North America is *Fusarium graminearum* (Bai and Shaner 2004; McMullen et al. 1997a; Xu and Nicholson 2009). FHB epidemics in the past have resulted in losses ranging from \$0.3 to \$3 billion (Bai and Shaner 2004; Johnson et al. 2003). Infection initiates in the florets at anthesis from which the fungus spreads to additional spikelets causing them to bleach

and undergo necrosis (McMullen et al. 1997a). The infected tissues and the resulting grain contain mycotoxins that further limit acceptability of grain by the flour and malting industry and as a component in animal feed (Bai and Shaner 2004; McMullen et al. 1997b). Compounding the problem further is the nonavailability of wheat cultivars that are highly resistant to FHB. In addition, the use of fungicides for controlling FHB is constrained by cost, limited understanding of factors that affect disease, and practical difficulties with the efficient application of fungicide to floral tissues (McMullen et al. 1997b; Pirgozliev et al. 2003).

Limited information is available on the mechanisms employed by the host plant to control disease and how the fungus targets host physiology to promote infection. Although long considered a necrotrophic pathogen, on wheat, during early stages of infection, the fungus is restricted to extracellular spaces and fungal penetration of the host cells is not observed, suggesting the existence of a transient biotrophic phase in the infection cycle (Brown et al. 2010; Goswami and Kistler 2004). The subsequent switch to necrotrophy is associated with increased vigor of colonization and disease spread. Fungal infection is accompanied by changes in salicylic acid (SA) and jasmonic acid (JA) content and the activation of SA and JA signaling (Ding et al. 2011; Li and Yen 2008; Makandar et al. 2006, 2012; Pritsch et al. 2000; Yu and Muehlbauer 2001). Experiments with wheat plants expressing the bacterial *nahG* gene, which encodes a salicylate hydroxylase that degrades SA, confirmed that SA has an important role in controlling FHB severity (Makandar et al. 2012). Transgenic NahG plants exhibited heightened severity of FHB compared with the control nontransgenic wheat. The importance of SA signaling in limiting *F. graminearum* infection is further evident from the reduced severity of infection observed in wheat plants constitutively expressing the *Arabidopsis* *NPR1* and *PAD4* genes, which are important regulators of SA signaling and accumulation, respectively (Gao et al. 2013; Makandar et al. 2006, 2012, 2015). More recently, allelic variants of *NPR1*-like genes were shown to be associated with FHB resistance in winter wheat (Diethelm et al. 2014). In contrast to SA, JA was suggested to have dichotomous involvement in wheat-*F. graminearum* interaction, contributing to susceptibility during the early stages of infection via its antagonism of SA signaling and subsequently contributing to resistance during the later stages of infection (Makandar et al. 2012). Exposure of wheat plants to methyl-jasmonate (MeJA) vapors prior to fungal inoculation attenuated basal resistance (Makandar et al. 2012). However, when applied later during infection or when applied as repeated applications, MeJA limited FHB severity (Li and Yen 2008; Makandar et al. 2012). Several

Nucleotide sequence data is available under the following accession numbers: *LOX1* (At1g55020), *LOX5* (At3g22400), *PR1* (At2g14610), *ICS1* (At1g74710), *PDF1.2* (At5g44420), *EF1a* (At5g60390), *TaLpx-1* (GQ166692), *F. graminearum nahG* (XM_011322400.1).

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studies have shown that *F. graminearum* is capable of colonizing *Arabidopsis* leaves and floral tissues (Chen et al. 2006, 2009; Cuzick et al. 2008; Makandar et al. 2006, 2010, 2015; Savitch et al. 2007; Skadsen and Hohn 2004; Urban et al. 2002; Van Hemelrijck et al. 2006). Similar conclusions on the involvement of SA and JA signaling in plant interaction with *F. graminearum* have been derived from studies involving *Arabidopsis* in which SA was shown to promote resistance (Makandar et al. 2006, 2010; Savitch et al. 2007) and JA signaling was shown to attenuate the activation of SA signaling during the early phases of infection and, thus, facilitate infection (Makandar et al. 2010). However, later during infection, JA in conjunction with SA promoted resistance (Makandar et al. 2010). These studies have demonstrated that the *Arabidopsis*–*F. graminearum* interaction is a good model pathosystem for characterizing the molecular and physiological basis of plant response to *F. graminearum*.

Oxylipins include an array of oxidized fatty acid–derived metabolites that have important roles in plant growth, development, and stress response. The first step in the synthesis of oxylipins involves oxygenation of polyunsaturated fatty acids to yield fatty acid hydroperoxides. This oxygenation of fatty acids can result from autooxidation or catalysis by enzymes like lipoxygenases (LOXs) and α -dioxygenases (Feussner and Wasternack 2002). Based on whether they incorporate oxygen at the C-9 or C-13 position of the fatty acid to yield 9- or 13-fatty acid hydroperoxides, LOXs can be broadly categorized as 9-LOXs and 13-LOXs, respectively. The fatty acid hydroperoxides can be further modified, either spontaneously or enzymatically, to yield an array of oxylipins (Feussner and Wasternack 2002; Mosblech et al. 2009). JA, which is one of the best-studied oxylipin with roles in plant development and stress response, is derived from the 13-LOX pathway (Browse 2009; Howe and Jander 2008; Wu and Baldwin 2010). 9-LOXs have also been linked to plant development, e.g., the development of lateral roots (Gao et al. 2008; Vellosillo et al. 2007). In addition, stress response in plants also engages the 9-LOX pathway. 9-LOX participation occurs in defense against bacterial and fungal pathogens as well as nematodes (Gao et al. 2008, 2009; Göbel et al. 2001; Hwang and Hwang 2010; López et al. 2011; Marmey et al. 2007; Montillet et al. 2013; Rustérucci et al. 1999; Vellosillo et al. 2007). 9-LOX-derived oxylipins have also been implicated in suppressing basal and inducible defense mechanisms in maize (*Zea mays*) (Constantino et al. 2013; Gao et al. 2007; Isakeit et al. 2007) and as signal molecules in cross-kingdom communication (Christensen and Kolomiets 2011).

Among the cereal crops, three LOX-encoding genes have been reported from barley (*LoxA*, *LoxB*, and *LoxC*) (Doderer et al. 1992; van Mechelen et al. 1999) and three LOX-encoding loci (*Lpx-1*, *Lpx-2*, and *Lpx-3*) have been reported from common wheat (Carrera et al. 2007; Hart and Langston 1977; Hessler et al. 2002). Based on protein sequence similarity, wheat *Lpx-3* corresponds to barley *LoxB*, wheat *Lpx-1* (hereafter referred as *TaLpx-1*) is related to barley *LoxA*, which encodes a 9-LOX, and wheat *Lpx-2* corresponds to barley *LoxC*, which encodes a 13-LOX (Carrera et al. 2007; Doderer et al. 1992; Hessler et al. 2002; van Mechelen et al. 1999). Previously, LOXs have been linked with wheat defense against pests. For example, LOX activity is stimulated in wheat leaves treated with MeJA and fungal elicitors (Bohland et al. 1997; Mauch et al. 1997). The application of BTH (benzo [1,2,3]thiadiazole-7-carbothioic acid *S*-methyl ester), a synthetic analog of SA that promotes resistance against several fungal pathogens (Görlach et al. 1996; Makandar et al. 2012), induced expression of *WCI-2* (wheat chemically-induced 2), which encodes a putative LOX (Görlach et al. 1996). *WCI-2*

expression was also strongly upregulated during feeding by Hessian fly (*Mayetiola destructor*) larvae (Sardesai et al. 2005). In contrast, expression of a putative wheat LOX was repressed in response to septoria leaf blotch caused by *Mycosphaerella graminicola* (Ray et al. 2003). Microarray studies also have indicated differential expression of wheat genes involved in oxylipin metabolism, including LOXs in wheat spikes challenged with *F. graminearum* (Kruger et al. 2002; Li and Yen 2008). However, the biological significance of altered expression or activity of these LOXs to the outcome of wheat interaction with pests is unclear.

Here, we show that, in both *Arabidopsis* and wheat, 9-LOXs have an important role in plant response to *F. graminearum* infection. *F. graminearum* disease severity was reduced in *Arabidopsis* and wheat plants in which expression of 9-LOXs was knocked down, thus confirming that these 9-LOXs facilitate *F. graminearum* infection in wild-type (WT) plants. Furthermore, the knockdown of *LOX1* and *LOX5* expression in *Arabidopsis* resulted in the attenuation of JA signaling and, simultaneously, more robust accumulation of SA and activation of SA signaling, which was required for the full extent of resistance in *lox1* and *lox5* mutant plants. Taken together with the observation that *LOX1* and *LOX5* expression was upregulated in *F. graminearum*–inoculated tissues, results presented here suggest that 9-LOX-derived oxylipins are engaged by the fungus to control balance between SA and JA signaling to facilitate infection.

RESULTS

LOX1 and *LOX5* expression is induced in response to *F. graminearum* infection.

Expression of the 9-LOX-encoding *Arabidopsis LOX1* (At1g55020) and *LOX5* (At3g22400) genes (Bannenberg et al. 2009) was upregulated in response to *F. graminearum* infection (Fig. 1A). However, the temporal pattern of *LOX1* and *LOX5* induction in response to *F. graminearum* infection differed. While *LOX5* expression was transiently induced, *LOX1* expression showed a sustained induction response. The expression of *LOX1* and *LOX5* in response to *F. graminearum* infection was also examined in transgenic plants expressing a chimeric construct in which the expression of the β -glucuronidase (GUS) reporter was driven from either the *LOX1* or *LOX5* promoters (Vellosillo et al. 2007). In contrast to the temporally distinct pattern of *LOX1* and *LOX5* induction in response to *F. graminearum* infection, histochemical analysis for GUS activity indicated an overlap in the spatial pattern of *LOX1* and *LOX5* promoter activity in *F. graminearum*–inoculated tissues (Supplementary Fig. S1). Strongest expression of the *LOX1_p::uidA* and *LOX5_p::uidA* reporters was observed in the minor veins of the *F. graminearum*–inoculated leaves.

The upregulation of *LOX1* and *LOX5* expression in *F. graminearum*–inoculated WT plants was accompanied by an increase in the content of the 9-LOX-derived oxylipin 9-HOD (9-hydroxyoctadecadienoic acid) (Fig. 1B). In contrast, this increase in 9-HOD was not observed in *F. graminearum*–inoculated leaves of the *lox1-1 lox5-1* double mutant, thus confirming that these 9-LOXs are required for *F. graminearum* infection–associated increase in 9-HOD content.

Arabidopsis 9-LOXs promote severity of *F. graminearum* infection.

The increase in *Arabidopsis LOX1* and *LOX5* expression and accumulation of 9-LOX-derived oxylipins in WT *Arabidopsis* could contribute to plant defense against *F. graminearum*. Alternatively, the expression of these genes might be targeted by the pathogen to facilitate infection. To discriminate between

these possibilities, *F. graminearum* disease severity was compared between the WT *Arabidopsis* accession Columbia (Col-0) and the *lox1-1 lox5-1* double mutant. As shown in Figure 2A, *F. graminearum* disease severity in the *lox1-1 lox5-1* double mutant was significantly lower than in the WT plant. This reduction in disease severity correlated with lowered fungal growth in the *lox1-1 lox5-1* mutant than the WT, which was monitored as the relative amount of *F. graminearum* DNA that accumulated in leaf tissues at 5 days postinoculation (dpi) (Fig. 2B). Pretreatment with 9-HOD or 9-HOT (9-hydroxyoctadecatrienoic acid) restored disease severity and fungal growth in the *lox1-1 lox5-1* plants (Fig. 2A and B), thus confirming that one or more 9-LOX-derived oxylipins or the products thereof promotes *F. graminearum* infection in *Arabidopsis*.

Fig. 1. *LOX1* and *LOX5* expression in response *Fusarium graminearum* infection. **A**, *LOX1* and *LOX5* expression in response *F. graminearum* inoculation. *LOX1* (upper panel) and *LOX5* (lower panel) expression in leaves of *F. graminearum*-inoculated (+Fg) and mock-inoculated (-Fg) wild-type (WT) plants. Quantitative real-time reverse-transcription-polymerase chain reaction was performed on RNA from leaves harvested at 0, 6, 12, 24, and 48 h postinoculation (hpi). The expression levels of *LOX1* and *LOX5* relative to expression levels of *EF1a* $\pm$ standard error at each time point is presented ($n = 3$). Asterisks above bars indicate values that are significantly different from the mock-inoculated for that time point ($P < 0.05$). **B**, 9-Hydroxyoctadecadienoic acid (9-HOD) and 13-HOD content in *F. graminearum*-inoculated *Arabidopsis*. Levels of 9- and 13-HOD were monitored in leaves of the WT and the *lox1-1 lox5-1* double mutant at 12 and 24 hpi with *F. graminearum* (+Fg) and, as a control, in mock-inoculated (-Fg) plants. Error bars represent standard error. Asterisks above bars indicate values that are significantly different from the mock-inoculated for that time point ($P < 0.05$).

To determine the individual contribution of *LOX1* and *LOX5* in promoting *F. graminearum* infection, disease severity and fungal growth was monitored on the *lox1-1* and *lox5-1* single mutants. Disease severity and fungal growth were lower on the *lox1-1* and *lox5-1* mutants compared with the WT (Fig. 2A and B; Supplementary Fig. 2A and B). Similarly, disease severity

Fig. 2. *Arabidopsis lox1* and *lox5* mutants exhibit reduced severity of *Fusarium graminearum* infection. **A**, Upper panel, *F. graminearum* disease severity in leaves of the *Arabidopsis lox1-1 lox5-1* double mutant plants infiltrated with distilled water (DW), and 9-hydroxyoctadecadienoic acid (9-HOD) and 9-hydroxyoctadecatrienoic acid (9-HOT) solutions (1 μ M) prepared in DW. Wild-type (WT) Col-0 plants infiltrated with DW provided the control. Lower panel, *F. graminearum* disease severity in leaves of *Arabidopsis* WT Col-0, *lox1-1* and *lox5-1* mutant, and *lox1-1 lox5-1* double mutant plants. The percentage of inoculated leaves exhibiting necrosis covering <25%, 25 to 50%, 50 to 75%, and >75% of the leaf area was determined for each genotype at 5 days postinoculation (dpi). Different letters next to the bars indicate values that are significantly different ($P < 0.05$) from each other. **B**, Quantitative polymerase chain reaction analysis for content of the *F. graminearum nahG* gene relative to the *Arabidopsis ACT8* gene $\pm$ standard error ($n = 3$) in leaves of WT Col-0 and the *lox1-1 lox5-1* double mutant at 5 dpi with *F. graminearum* (Fg).

was lower on additional T-DNA knockdown alleles (*lox1-3* and *lox5-3*) of *LOX1* and *LOX5*. *LOX1* and *LOX5* expressed from the heterologous *Cauliflower mosaic virus 35S* gene promoter restored *F. graminearum* disease severity in the *lox1-1* and *lox5-1* mutants, respectively, thus confirming the influence of these two 9-LOXs in facilitating *F. graminearum* infection in *Arabidopsis*. Since *F. graminearum* infection is a major disease of the spikes (floral tissues) of wheat and barley and *F. graminearum* can also infect *Arabidopsis* flowers, the effect of the *lox1-1*, *lox1-3*, *lox5-1*, and *lox5-3* mutant alleles on *F. graminearum* disease severity was also evaluated in floral tissues, as previously described (Urban et al. 2002). Compared with the WT plants, the *Fusarium-Arabidopsis* disease (FAD) score was lower for the *lox1-1*, *lox1-3*, *lox5-1*, and *lox5-3* mutants (Table 1), thus indicating a higher level of resistance to *F. graminearum* in the floral tissues of *Arabidopsis* mutants lacking these 9-LOXs.

Attenuation of JA signaling is associated with the enhanced disease resistance observed in the 9-LOX mutants.

In addition to 9-LOX-derived 9-HOD, fungal infection of WT plants was also accompanied by an increase in the level of 13-HOD, a 13-LOX-derived oxylipin (Fig. 1B), thus suggesting increased 13-LOX activity. However, in comparison with the WT plant, in which 13-HOD levels were elevated at 12 and 24 h postinoculation (hpi), the accumulation of 13-HOD was transient in the fungus-inoculated *lox1-1* *lox5-1* mutant (Fig. 1B), thus suggesting that 9-LOXs, although not essential to the increase of 13-LOX-derived products, promoted the sustained activation of the 13-LOX pathway in *F. graminearum*-inoculated tissues. Since signaling mediated by JA, an important product of the 13-LOX pathway, has previously been shown to influence *Arabidopsis* interaction with *F. graminearum*, expression of the JA-responsive *PDF1.2* gene was compared between the WT and the *lox1-1* and *lox5-1* mutants. As previously shown (Makandar et al. 2010), *PDF1.2* expression in the WT plants showed a continual increase during the course of infection (Fig. 3A). In contrast, the induction of *PDF1.2* was slower and significantly lower in the fungus-inoculated leaves of the *lox1-1* and *lox5-1* plants. These results suggest that, in WT *Arabidopsis*, *LOX1* and *LOX5* positively influence the robustness of JA signaling in response to *F. graminearum* infection.

The weaker induction of JA signaling in the 9-LOX mutants could result from the lowered severity of infection in these mutants. Alternatively, the heightened resistance against *F. graminearum* in the *lox1-1* and *lox5-1* mutants may result from the attenuation of JA signaling in these mutants. To further test these possibilities, WT and the *lox1-1* and *lox5-1* mutant plants were exposed to MeJA vapors, for a 24-h period immediately prior to inoculation with *F. graminearum*. As previously shown (Makandar et al. 2010), pretreatment with MeJA compared with ethanol, which was used as a solvent for MeJA, resulted in a significant increase in *F. graminearum* disease severity in WT plants (Fig. 3B). Similarly, MeJA treatment, as compared with ethanol treatment, attenuated the *lox1-1*- and

lox5-1-conferred enhanced resistance against *F. graminearum* (Fig. 3B). Disease severity was comparable between the MeJA-treated WT and the MeJA-treated *lox1-1* and *lox5-1* mutants, thus indicating that activation of JA signaling prior to fungal inoculation breaks down the *lox1*- and *lox5*-determined resistance against *F. graminearum*. Taken together, these results suggest that the attenuation of JA signaling in response to fungal infection is associated with enhanced resistance against *F. graminearum* in the *lox1* and *lox5* mutants compared with the WT.

lox1- and *lox5*-conferred enhanced resistance is coupled with robust activation of SA signaling.

SA accumulation has an important function in limiting *F. graminearum* infection in *Arabidopsis* and wheat (Makandar et al. 2010, 2012, 2015). Previous studies have shown that enhancing sensitivity to SA by increasing expression of *NPR1* resulted in enhanced resistance against *F. graminearum* (Makandar et al. 2006, 2012). Here, we show that the temporal pattern of upregulation of *PR1* in response to the exogenous application of SA was comparable between the *lox1-1* and *lox5-1* mutants and the WT plants (Supplementary Fig. S3). Furthermore, in comparison with the WT, pretreatment with SA

Fig. 3. Attenuation of jasmonic acid (JA) signaling is associated with the *lox1*- and *lox5*-conferred enhanced resistance against *Fusarium graminearum*. **A**, Expression of the jasmonate-responsive *PDF1.2* gene in leaves of *F. graminearum*-inoculated (+Fg) *Arabidopsis* wild-type (WT) accession Col-0, *lox1-1*, and *lox5-1* plants. Quantitative real-time reverse-transcription-polymerase chain reaction was performed on RNA extracted from leaves of *F. graminearum*-inoculated plants harvested at 6, 24, and 48 h postinoculation (hpi). As a control, RNA was also extracted at the same time points from leaves of mock-inoculated plants (–Fg). The expression level of *PDF1.2* relative to expression level of *EF1α* ± standard error at each time point is presented ($n = 3$). Asterisks indicate values that are significantly different ($P < 0.05$) from the WT for that time point and treatment. **B**, *F. graminearum* disease severity in leaves of WT accession Col-0, *lox1-1*, and *lox5-1* plants that were exposed to methyl-jasmonate (MeJA) vapors for a 24-h period immediately prior to inoculation with *F. graminearum*. Plants exposed to ethanol (EtO), which was used as a solvent for MeJA, provided the controls. Disease severity was recorded at 5 days postinoculation. Different letters next to the bars indicate values that are significantly different ($P < 0.05$) from each other.

Table 1. *Fusarium graminearum* disease on *Arabidopsis* inflorescence

Genotype	FAD ^a
WT (Col-0)	4.1 ± 0.6
<i>lox1-1</i>	2.7 ± 0.3
<i>lox1-3</i>	2.9 ± 0.4
<i>lox5-1</i>	1.5 ± 0.6
<i>lox5-3</i>	1.2 ± 0.3

^a *Fusarium-Arabidopsis* disease (FAD) value was evaluated 7 days postinoculation. This experiment was repeated twice with similar results.

did not result in any significant increase in disease resistance in the *lox1-1* and *lox5-1* mutants (Fig. 4A), thus suggesting that SA-mediated defenses are likely fully engaged in the 9-LOX mutants. Although basal content of SA was comparable between the WT and the *lox1-1* and *lox5-1* mutants in response to *F. graminearum*-inoculation, SA accumulation was higher in the *lox1-1* and *lox5-1* mutants than in the WT (Fig. 4B). Expression of the SA biosynthesis gene *ICS1* and the SA-inducible *PR1* gene were also higher in the *F. graminearum*-inoculated *lox1-1* and *lox5-1* mutants compared with the WT plants (Fig. 4C; Supplementary Fig. S4). These results suggest that although the absence of 9-LOX function does not significantly affect basal SA content and signaling, in response to *F. graminearum* infection, the SA pathway is hyperactivated in the *lox1-1* and *lox5-1* mutants and is associated with

significantly greater accumulation of SA and stronger activation of SA signaling compared with the WT.

We postulated that if SA is critical for the *lox1-1*- and *lox5-1*-conferred enhanced resistance to *F. graminearum*, then expression of the *Pseudomonas putida nahG* gene-encoded SA hydroxylase, which degrades SA, should diminish the *lox1-1*- and *lox5-1*-conferred resistance. Indeed, as shown in Figure 4D, the presence of *nahG* tempered the *lox1-1*- and *lox5-1*-conferred enhanced resistance, thus confirming the importance of SA to the *lox1-1*- and *lox5-1*-conferred enhanced resistance against *F. graminearum* infection.

Silencing of *TaLpx-1* expression in wheat is accompanied by reduced severity of FHB disease.

Wheat *TaLpx-1* encodes a predicted 622-amino acid protein (molecular mass = 73.5 kDa) that exhibits 57 and 58% homology to *Arabidopsis* LOX1 and LOX5, respectively, in the region containing the lipoxygenase domain (PFAM00305) (Supplementary Fig. S5). The predicted coding sequence of *TaLpx-1* also exhibits 95% homology to the barley 9-LOX LoxA (data not shown). 9-LOXs catalyze the oxidation of polyunsaturated fatty acids to yield 9-hydroperoxy fatty acids, which can be further converted to the relatively more stable 9-hydroxy fatty acids. To determine if *TaLpx-1* is a bona fide 9-LOX, the *TaLpx-1* coding sequence was cloned from the wheat cv. Bobwhite, the corresponding protein was expressed in *Escherichia coli*, and its ability to utilize linoleic acid as a substrate to yield 9-HOD was determined. The *Arabidopsis* LOX1 provided the positive control for this assay. LOX1 and *TaLpx-1* activity on linoleic acid resulted in the accumulation of 9-HOD (Fig. 5), thus confirming that *TaLpx-1*, like LOX1, contains 9-LOX activity. *TaLpx-1* action also resulted in the accumulation of 13-HOD. However, the 13-HOD produced was only 5% of the amount of 9-HOD produced by *TaLpx-1*, suggesting that *TaLpx-1* is a 9-LOX with minor 13-LOX activity. Since *TaLpx-1* is expressed in wheat spikes (Supplementary Fig. S6), we predicted that, like *LOX1* and *LOX5* in *Arabidopsis*, *TaLpx-1* is involved in wheat-*F. graminearum* interaction. To test this hypothesis, wheat plants (cv. Bobwhite) were generated in which RNA interference (RNAi) technology was utilized to silence expression of *TaLpx-1* (discussed below). Compared with the nontransgenic parental cv. Bobwhite, expression of *TaLpx-1* was knocked down in transgenic lines

Fig. 4. *lox1* and *lox5* mutants exhibit stronger accumulation of salicylic acid (SA) in response to *Fusarium graminearum* infection. **A**, *F. graminearum* disease severity in *Arabidopsis* wild-type (WT) accession Col-0 and the *lox1-1* and *lox5-1* mutant plants that were treated with a 200-μM solution of SA or, as control, with water (W) 24 h prior to inoculation with *F. graminearum*. Disease severity was recorded at 5 days postinoculation (dpi). Different letters next to the bars indicate values that are significantly different ($P < 0.05$) from each other. **B**, SA content $\pm$ standard error in leaves of *Arabidopsis* WT Col-0, *lox1-1* and *lox5-1* plants that were inoculated with *F. graminearum* (+Fg), or as control in mock-inoculated (-Fg) plants, 24 h postinoculation (hpi). Different letters above the bars indicate values that are significantly different ($P < 0.05$) from each other ($n = 3$). FW, fresh weight of leaf tissue. **C**, Quantitative real-time polymerase chain reaction analysis of *PR1* expression in leaves of *F. graminearum*-inoculated and mock-inoculated *Arabidopsis* wild-type (WT) accession Col-0, *lox1-1* and *lox5-1* plants at 12 and 24 hpi. Level of *PR1* transcript relative to that of *EF1 α* $\pm$ standard error at each time point is presented ($n = 3$). Asterisks represent values that are significantly different ($P < 0.05$) from the corresponding WT for that treatment and time point. **D**, *F. graminearum* disease severity in *Arabidopsis* WT accession Col-0, the *lox1-1*, *lox5-1*, *lox1-1 nahG*, and *lox5-1 nahG* plants. Disease severity was recorded at 5 dpi. Disease severity in the *lox1-1 nahG* and *lox5-1 nahG* plants is significantly higher ($P < 0.05$) than that in the *lox1-1* and *lox5-1* mutants, respectively.

1097B, 1073A, and 1218A (Fig. 6A). Spikes of these *TaLpx-1*-silenced lines were challenged with *F. graminearum* to determine if reduction of *TaLpx-1* expression had any impact on *F. graminearum* disease severity. *F. graminearum* disease severity was monitored over a 21-day period, as previously described (Makandar et al. 2006, 2012). Compared with the nontransgenic plants, disease severity, represented as percentage of infected spikelets, was significantly lower in all three *TaLpx-1*-silenced lines (Fig. 6B), thus confirming that reduction in *TaLpx-1* expression is accompanied by enhanced resistance to *F. graminearum* in wheat. These results strongly suggest conservation in the involvement of 9-LOXs in *Arabidopsis* and wheat susceptibility to *F. graminearum*.

DISCUSSION

9-LOXs catalyze the synthesis of 9-hydroperoxy fatty acids, which serve as precursors for a variety of oxylipins and products thereof. Our results indicate that 9-LOXs in *Arabidopsis* and wheat facilitate infection by *F. graminearum*, an important pathogen of small grain cereals. We have shown that T-DNA insertion-mediated knockdown of *LOX1* and *LOX5*, which encode the two 9-LOXs in *Arabidopsis* (Bannenberg et al. 2009), resulted in reduced severity of disease caused by *F. graminearum* (Fig. 2A and B). Similarly, RNAi-mediated knockdown of *TaLpx-1*, which encodes a protein with homology to the *Arabidopsis* LOX1 and LOX5 proteins and has 9-LOX activity (Fig. 5B), was accompanied by reduced severity of FHB in wheat (Fig. 6B). These

results indicate that one or more 9-LOX products or factors dependent on them is a susceptibility factor that promotes *F. graminearum* infection. Indeed, pretreatment with 9-HOD or 9-HOT attenuated the enhanced resistance phenotype conferred by 9-LOX deficiency in the *lox1-1 lox5-1* mutant plant (Fig. 2A and B). Expression of both *LOX1* and *LOX5* in *Arabidopsis* is upregulated in response to *F. graminearum* infection (Fig. 1A), thus suggesting that *F. graminearum* might target expression of these 9-LOX genes to facilitate infection. Similarly, upregulation of *LOX5* expression and accumulation of 9-LOX products in roots promotes colonization of *Arabidopsis* by the green peach aphid (Nalam et al. 2012, 2013), and in maize, expression of the 9-LOX-encoding *ZmLOX3* gene that facilitates infection by *F. verticillioides* is upregulated in the fungus-inoculated kernel (Gao et al. 2007; Wilson et al. 2001). The contribution of lipids to wheat susceptibility to *F. graminearum* is further supported by the fact that one of the pathogenicity factors secreted by *F. graminearum* is the FGL1 lipase (Blümke et al. 2014; Voigt et al. 2005). The *fgl1* mutant fungus, although capable of infecting wheat, was unable to spread from the inoculated site, suggesting the involvement of *FGL1* in promoting fungal spread through the host. It is plausible that a FGL1-released fatty acid functions as the substrate for TaLpx1 in wheat.

The comparable level of resistance against *F. graminearum* observed in the *Arabidopsis lox1-1 lox5-1* double mutant and the single mutant plants (Fig. 2B) leads us to suggest that these genes have a common role in *Arabidopsis* interaction with *F. graminearum* and together contribute to a minimum threshold level of 9-LOX activity that is involved in facilitating

Fig. 5. Positional specificity of lipoxygenase activity of TaLpx-1. Gas chromatography-mass spectrometry analysis of trimethylsilyl derivatives of 9-HOD (9-hydroxyoctadecadienoic acid) and 13-HOD standard and products of TaLpx-1 and *Arabidopsis* LOX1 action on linoleic acid. The diagnostic ions for 9-HOD and 13-HOD are *m/z* 317 and 369, respectively. **A**, Fragmentation pattern for 9-HOD and 13-HOD standards. Insets indicate the key diagnostic ions for each species. **B**, Fragmentation pattern of 9-HOD and 13-HOD produced by recombinant TaLpx-1. **C**, Fragmentation pattern of 9-HOD produced by recombinant *Arabidopsis* LOX1.

F. graminearum infection. When either of these gene functions is knocked down, there is insufficient accumulation of one or more 9-LOX-dependent factors that promote *F. graminearum* infection. The strong expression of both these genes in the vasculature indicates an overlap in their major expression domain in response to *F. graminearum* infection. *LOX5*, which reaches peak expression levels by 12 hpi, might contribute early during the infection process compared with *LOX1*, which is induced later, but shows a more sustained induction during the course of the experiment (Fig. 1A). Furthermore, since the *LOX1* protein localizes to chloroplasts, as compared with the extraplastidic location of *LOX5* (Nalam et al. 2012), we suggest that the intracellular origin of these 9-LOX products is not critical for facilitating *F. graminearum* infection. This is in striking contrast to the role of 9-LOX function in *Arabidopsis* interaction with the green peach aphid, in which only *LOX5* is involved in facilitating insect infestation (Nalam et al. 2012).

Previously, it was shown that *Arabidopsis* and wheat accumulate elevated levels of SA in response to *F. graminearum* infection and that SA signaling is critical for limiting *F. graminearum* infection in these plants (Gao et al. 2013; Makandar et al. 2006, 2010, 2012, 2015; Savitch et al. 2007). In contrast, JA was shown to have a dichotomous role in *Arabidopsis* and wheat interaction with *F. graminearum* (Makandar et al. 2010, 2012). By constraining the activation of SA signaling during the early stages of infection, JA functioned as a susceptibility factor. However, later during infection, JA and SA had an additive effect on defense against *F. graminearum* (Makandar et al. 2010, 2012). Similar antagonistic and synergistic interactions between SA and JA have been reported by others with other pathosystems (Kunkel and Brooks 2002; Mur et al. 2006). The basal content of SA and level of *PR1* transcript were comparable in WT and *lox1* and *lox5* mutant plants (Fig. 4B and C), thus indicating that *LOX1* and *LOX5* do not

influence basal SA accumulation and signaling. However, the *F. graminearum*-inoculated *lox1* and *lox5* plants contained significantly higher levels of SA than *F. graminearum*-inoculated WT plants (Fig. 4B). The higher content of SA correlated with the stronger expression of *PR1* in *F. graminearum*-inoculated *lox1* and *lox5* mutants compared with the WT plant (Fig. 4C). Above all, expression of the *nahG* gene attenuated *lox1-1*- and *lox5-1*-conferred resistance (Fig. 4D), thus indicating that, in the WT plants, these 9-LOXs limit the accumulation of SA and the strength of downstream defense signaling in response to fungal infection. The stronger expression of *ICS1* in the fungus-inoculated *lox1* and *lox5* mutants compared with the WT plant suggests that this robust increase in SA in the *lox1* and *lox5* mutant plants is likely due to increased synthesis of SA in response to fungal infection. However, it is equally possible that, in the *F. graminearum*-inoculated *lox1* and *lox5* mutants, as compared with the WT plant, SA turnover is lower. 9-LOX-synthesized lipids can influence fungal physiology, including toxin production and development (Brodhagen and Keller 2006). Similarly, it is plausible that plant 9-LOX-synthesized oxylipins influence production of factors by *F. graminearum* that either limit SA production, promote SA turnover in the infected plant tissue, or both. Indeed, pathogenic fungi are known to secrete enzymes, like chorismate mutase, that limit SA production in the host and, thus, facilitate infection (Djamei et al. 2011). Furthermore, the *F. graminearum* genome contains a homolog of the bacterial *nahG* gene, which is predicted to encode a secreted salicylate hydroxylase that could potentially promote SA turnover in infected tissue (Brown et al. 2012).

In contrast to the robust induction of SA signaling in response to *F. graminearum* infection, the induction of *PDF1.2*, a marker gene for JA signaling activation, was attenuated in the fungus-infected *lox1* and *lox5* mutants (Fig. 3A), thus suggesting that, in the WT plants, 9-LOX products are required for the full extent of JA signaling. A sustained accumulation of 13-HOD that is observed in *F. graminearum*-inoculated WT plants was not observed in the *lox1-1* and *lox5-1* mutants (Fig. 1C), thus, further suggesting that in *F. graminearum*-infected plants, 9-LOX activity promotes activity of the 13-LOX pathway. A weak JA response could account for the stronger accumulation of SA in the 9-LOX mutants. JA signaling is known to antagonize the activation of SA signaling during plant defense (Pieterse et al. 2009), and previous studies have shown that JA signaling impairs the induction of SA accumulation and signaling in *F. graminearum*-infected *Arabidopsis* and wheat (Makandar et al. 2010, 2012). Alternatively, in the *lox1* and *lox5* mutants, the stronger activation of SA signaling could adversely impact activation of JA signaling. It is also possible that the weaker induction of the JA pathway in *F. graminearum*-inoculated *lox1-1* and *lox5-1* is due to lowered fungal growth and spread on this mutant and not due to a more direct impact of 9-LOX products on JA signaling.

The influence of plant 9-LOXs on control of SA and JA signaling has also been reported in other pathosystems. For example, in maize, a *ZmLOX3*-dependent mechanism that promotes resistance against the root knot nematode *Meloidogyne incognita* limits biosynthesis of both SA and JA (Gao et al. 2008). In case of *Arabidopsis* interaction with the bacterial pathogen *Pseudomonas syringae*, 9-LOX-synthesized oxylipins are required for controlling infection by suppressing JA signaling and simultaneously promoting SA signaling (Vicente et al. 2012). These physiological changes are in contrast to the results presented here showing the promotion of JA signaling and the attenuation of SA signaling by *LOX1*- and *LOX5*-dependent mechanisms in *F. graminearum*-infected *Arabidopsis*. Thus, depending on the host and pathogen, 9-LOXs have different effects on modulating SA and JA signaling,

Fig. 6. *Fusarium* head blight (FHB) resistance is enhanced in wheat plants silenced for expression of *TaLpx-1*. **A**, Reverse-transcription-polymerase chain reaction (RT-PCR) analysis showing knockdown of *TaLpx-1* expression in the wheat RNA-interference (RNAi) lines 1097B, 1073A, and 1218A. Nontransgenic cv. Bobwhite (Bw) plants provided the control for *TaLpx-1* expression. Expression of wheat tubulin (*Tub*) gene provided the control for RT-PCR. **B**, FHB severity in *TaLpx-1* RNAi lines. FHB disease rating was monitored in Bw and the three independently derived *TaLpx-1* RNAi lines 1097B, 1073A, and 1218A, in which *TaLpx-1* expression was attenuated. Disease incidence was recorded as the percentage of infected spikelets in each spike at 5, 9, 15, and 21 days postinoculation (dpi) with *F. graminearum* spores. Bars represent means $\pm$ standard error. Asterisks identify values that are significantly different ($P < 0.05$) from the non-transgenic Bw for that time point. Bw ($n = 26$); 1097B ($n = 18$); 1073A ($n = 5$); 1218A ($n = 8$).

suggesting that other factors associated with microbial infection influence the impact of 9-LOXs on host hormonal physiology.

In summary, this study has revealed that the *Arabidopsis*–*F. graminearum* pathosystem, which previously has been used to study plant defense against *F. graminearum*, also provides a good model system for the identification of factors that contribute to host susceptibility to *F. graminearum*, an agronomically important pathogen. Genetic studies indicate that *Arabidopsis* 9-LOXs facilitate *F. graminearum* infection. These 9-LOXs control the balance between SA and JA signaling in *F. graminearum*-infected plants. The reduced FHB severity observed in *TaLpx-1*-silenced wheat lines reveals conservation in the engagement of 9-LOX-dependent mechanisms for facilitating *F. graminearum* infection in *Arabidopsis* and wheat.

MATERIALS AND METHODS

Plant material and growth conditions.

Arabidopsis was cultivated in a growth room set at 22°C under a 14-h light (100 $\mu\text{E m}^{-2}\text{s}^{-1}$) and 10-h dark regime. Approximately 4-week-old plants were used for all experiments. The *lox1-1* (SALK_059431), *lox1-3* (SALK_012188), *lox5-1* (SALK_044826), and *lox5-3* (SALK_050933) T-DNA insertion alleles, which are in *Arabidopsis* accession Col-0, have been previously described (Nalam et al. 2012). The *LOX1_p::GUS* and *LOX5_p::GUS* lines in which the GUS reporter is expressed from the *LOX1* and the *LOX5* gene promoters were previously described (Vellosillo et al. 2007). The *lox1-1 nahG* and *lox5-1 nahG* lines were generated by transforming the *lox1-1* and *lox5-1* mutant plants with a T-DNA construct containing the *Pseudomonas putida nahG* gene (Shah et al. 1999). Wheat was grown in a greenhouse with a 21°C day and 18°C night temperature regime. Three plants were grown per gallon-capacity pot. The compost and peat-based Fafard #2 soil mix (Sunagro) was used for cultivating *Arabidopsis* and wheat. Prior to use, it was sterilized by autoclaving.

Fungal cultivation and plant infections.

F. graminearum isolate Z-3639 was cultured on half-strength potato dextrose agar (Difco Laboratories) at 22°C. Fungal inoculations of *Arabidopsis* leaves were carried out as previously described (Makandar et al. 2010). The fungal suspension was infiltrated into the abaxial surface of four to five leaves per *Arabidopsis* plant with a needleless syringe. The inoculated plants were left covered with a clear plastic dome in the growth room for 2 days, after which the dome was removed and the infection was allowed to proceed for 3 additional days at ambient humidity. The percentage of inoculated leaves exhibiting chlorosis covering <25% (category I), 25 to 50% (category II), 50 to 75% (category III), and >75% (category IV) of the leaf area were determined for each genotype, as previously described (Makandar et al. 2010). A minimum of 60 leaves of each genotype were analyzed for each experiment. Disease severity among different genotypes and treatments was compared by single factor analysis of variance (ANOVA) ($P < 0.05$) (SAS v5.1). Inoculations of *Arabidopsis* inflorescence with *F. graminearum* spores and disease rating expressed as the FAD score were conducted as previously described (Makandar et al. 2010; Urban et al. 2002).

Inoculation of wheat spikes with *F. graminearum* spore suspension have been previously described (Makandar et al. 2006, 2012). Disease was evaluated as percentage of diseased spikelets over a 21-day period. The significance of the data was tested by *t* test and ANOVA, using the GLM procedure available in Minitab version 15 (Minitab Inc.).

GUS staining.

Staining for GUS activity in leaves of the *LOX1_p::Uida* and *LOX5_p::Uida* lines was performed as previously described (Malamy and Benfey 1997).

RNA and DNA isolation and analysis.

DNA and RNA extraction from plant tissues (*Arabidopsis* leaves and wheat spikelets) and cDNA synthesis were performed as previously described (Makandar et al. 2010, 2012; Nalam et al. 2012). All RNA samples were treated with RNase-free DNase to destroy contaminating DNA. For each time point and treatment, RNA was collected from three biological replicates, each consisting of leaves/spikes pooled from four plants. The AtRTPrimer database (Han and Kim 2006) was used to design gene-specific primers (Supplementary Table S1) for reverse transcription polymerase chain reaction (RT-PCR) and quantitative real time PCR. PCR conditions were as follows: 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 58°C for 45 s, and 72°C for 45 s, followed by a final extension step of 72°C for 5 min. Quantitative (q)PCR was performed with Sybr green PCR master mix (Applied Biosystems) on an Eco Illumina system, using the following amplification protocol: 10 min at 95°C for polymerase activation and denaturation of nucleic acids, followed by 40 cycles of 95°C for 10 s, 58°C for 30 s, and 72°C for 30 s. This was followed by a product melt to confirm a single PCR product. The level of gene expression was normalized to that of *EF1 α* by subtracting the cycle threshold (C_T) value of *EF1 α* from the C_T value of the gene of interest. The $\Delta\Delta C_T$ method (Livak and Schmittgen 2001) was used to calculate relative fold changes.

qPCR was used to detect the presence of fungus in plant tissue. DNA from fungus-infected and mock-inoculated leaves was used for qPCR analysis. Primers designed to the *F. graminearum NahG* gene were used to monitor amount of fungus relative to *Arabidopsis EF1 α* .

Chemical and hormone treatment.

A 200- μM working solution of MeJA (Sigma Aldrich) was prepared in 0.1% ethanol. Cotton plugs soaked with 1 ml of the MeJA solution or, as control, with 0.1% ethanol were placed in a petri plate at the center of an airtight plexiglass chamber (48-liter volume) in which the plants to be treated were placed. The plants were left in the chamber exposed to MeJA vapors or, as control, to 0.1% ethanol for 24 h, after which they were removed and inoculated with the fungus.

SA treatment of *Arabidopsis* was initiated by irrigating and spraying plants to run-off with a 200- μM solution of sodium salicylate (Sigma Aldrich) in water. Plants were inoculated with *F. graminearum* 24 h later, and disease severity was evaluated 5 dpi with the fungus. Plants irrigated and sprayed with water provided the controls.

Treatment of plants with 9-HOD and 9-HOT was initiated by infiltrating 1- μM solutions prepared in distilled water into the abaxial surface of *Arabidopsis* leaves with a needleless syringe 3 h prior to fungal inoculation. Leaves infiltrated with distilled water provided the controls.

SA quantification.

Quantification of SA content in *Arabidopsis* leaves was done using a modified biosensor strain of *Acinetobacter* sp. strain ADP1 (Huang et al. 2005, 2006).

Isolation and cloning of putative wheat lipoxygenases.

The isolation and cloning of *TaLpx-1* was carried out as previously described (Feng et al. 2010). Briefly, wheat expressed sequence tags (EST) identified as putative LOXs belonging to *TaLpx-1* were used as queries to search the wheat

genome database Dana-Farber Cancer Institute Wheat Gene Index. This resulted in the identification of a tentative contig representing the *TaLpx-1* locus. By comparing the sequences of EST covering the tentative contig, primers were designed to amplify putative full-length *TaLpx-1* cDNA from wheat tissue. cDNA generated from total RNA extracted from roots of germinating seedlings of the wheat cv. Bobwhite was used as the template. The PCR product (1.9 kb) was cloned and was sequenced for confirmation. The cloned product contained a fully functional lipoxygenase domain (PFAM00305) and corresponded to GenBank accession GQ166692, which was previously identified by Feng et al. (2010) as *Triticum aestivum* LOX1 (TaLOX1). However, the primers used in this study did not result in the cloning of the PLAT/LH2 domain that was identified by Feng et al. (2010) as being part of the TaLOX1.

LOX reaction and identification of LOX products.

The full-length coding region of *AtLOX1* and *TaLpx-1* were cloned in the pET28c vector (Novagen). BL21 (DE3) cells (Invitrogen) were used to express the recombinant proteins. To induce expression of the recombinant gene, the bacterial cultures were treated for 5 h with isopropyl β -D-1-thiogalactopyranoside (1 mM), after which the cells were harvested in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0, adjusted with NaOH) and were lysed by sonication. The soluble protein obtained after pelleting cell debris at 10,000 $\times$ g for 10 min was incubated with 2 mg of 18:2 (linoleic acid) or 18:3 (linolenic acid) (Cayman Chemicals) at room temperature for 1 h.

The extraction and identification of oxylipins was carried out as previously described (Keereetaweep et al. 2013). Lipids were extracted, were reduced with NaBH₄, and were resuspended in methanol. The separation of oxylipins was performed on an Agilent 1100 high-performance liquid chromatography (HPLC) system. The UV diode array detector was set at 234 nm to monitor the conjugated diene system. An EC 250/2 nucleosil 120-5 C18 column (250 $\times$ 25 mm, 5 μ m particle size, ID 2 mm) (Macherey and Nagel) was used for reverse phase HPLC with a binary gradient system (solvent A, methanol/water/acetic acid [80:20:0.1, vol/vol/vol]; solvent B, methanol/acetic acid [100:0.1, vol/vol]) at a flow rate 0.18 ml/min. The run was programmed for 3 min of 100% solvent A and a linear increase to 25% solvent B in 18 min, then to 100% solvent B in 2 min, followed by isocratic post-run of 100% solvent A for 10 min. Chiral phase HPLC (CHIRALCEL OD-H, 2.1 $\times$ 150 mm) (Diacel Chemical Industries) was also used to distinguish between enzymatic products and auto-oxidation products (Keereetaweep et al. 2013; Kilaru et al. 2011). An Agilent gas chromatography 7890A/MSD 5975C system with a capillary HP-5MS column (30 m $\times$ 0.250 mm, 0.25 mm coating thickness) (Agilent Technologies) was used to identify the trimethylsilyl derivatives of oxylipins in full mass scan mode. The oven temperature was programmed at 40°C for 1 min, from 40 to 200°C at 50°C/min, from 200 to 285°C at 5°C/min, followed by 300°C for 15 min. The flow rate was set at 0.5 ml/min, using ultrapure helium as a carrier gas, and the transfer line was kept at 280°C. Characteristic identification was based on molecular and fragment ions in comparison with commercial standards (Cayman Chemicals).

Generation of RNAi wheat transgenics.

To generate RNAi constructs for suppression of *TaLpx-1*, primers were developed for two regions in the lipoxygenase domain of *TaLpx-1*. The regions corresponded to 1,649 to 1,863 bp (*WhLOX2*) and 1,940 to 2,108 bp (*LpxB1.1*) on GQ166692. cDNA generated from total RNA isolated from 5-day-old roots of cv. Bobwhite served as the template. The

RT-PCR products so obtained were cloned in pANDA-Mini vectors for wheat transformation, which allows the cloning of the gene or PCR fragment of interest as inverted repeats that are separated by a GUS linker (Miki and Shimamoto 2004). The RNAi construct and the pAHC20 plasmid that contains the selectable *Ubi:bar* construct (Christensen and Quail 1996) were co-bombarded into immature wheat embryos and transformed plants were regenerated using a previously described protocol (Altpeter et al. 1996) as modified by Anand and associates (2003). The *bar* gene, which confers resistance to the herbicide glufosinate (trade name Liberty; Bayer Crop Sciences), was used to select transgenic calli. Two independent RNAi transgenic lines, 1097B and 1073A, were obtained for the region named *LpxB1.1* and one RNAi transgenic line 1218A was obtained for the region named *WhLOX2*. Genomic PCR was used to monitor the presence of RNAi constructs using the forward primers for the respective regions and a GUS linker primer in the T₁ generation. In the T₂ and T₃ generations, RT-PCR analysis using insert-specific primers was used to determine gene suppression.

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