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RESEARCH ARTICLE

Chitin and laminarin additively trigger wheat reactive oxygen species but not resistance to Fusarium head blight

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Abstract

Plants respond to fungal infections by activating defense genes including producing reactive oxygen species (ROS). The fungus *Fusarium graminearum* causes Fusarium head blight (FHB), a serious disease of wheat and barley. FHB results in crop yield loss and contaminates grain with mycotoxins. In a prior study, we discovered that chitin induces tissue-specific ROS burst in wheat. However, it is unknown whether other fungal cell wall components could induce defense response in wheat. Therefore, we evaluated ROS and defense gene responses in different wheat tissues that had been treated with chitin, laminarin, or both. Different ROS patterns were induced in wheat treated with laminarin or chitin. Furthermore, we found that ROS were enhanced in wheat tissues treated with both chitin and laminarin. This study provides novel information for enhancing plat immunity to increase plant resistance.

KEYWORDS
chitin, Fusarium head blight, gene expression, laminarin, MAMPs, priming, ROS

Dear Editor,

Plants perceive microbe derived molecular patterns (MAMPs) and initiate a complex cascade of defense responses during plant and pathogen interactions. When sensing the presence of MAMPs, a variety of early defense responses occurs in the plants, including Ca^{2+} fluxes, reactive oxygen species (ROS) bursts, and defense gene activation (Bigeard et al., 2015). Cell walls of filamentous fungi consist of two main components, chitin and β-glucans, which can trigger plant immunity as MAMPs. Although chitin-triggered plant immunity is well studied, chitin represents only a small fraction of cell walls in fungi, with β-glucan being the most abundant polysaccharide in the fungal cell wall (Fesel & Zuccaro, 2016). The function of glucan is well characterized in fungus–animal interactions (Brown & Gordon, 2003); however, limited information is available on the perception and signaling of β-glucan during plant-pathogen interactions (Fesel & Zuccaro, 2016). The fungal pathogen *Fusarium graminearum* infects wheat floral tissues and causes Fusarium head blight (FHB), which results in grain contaminated with trichothecene mycotoxins including deoxynivalenol (DON). Our previous study discovered that chitin induces tissue-specific ROS bursts in wheat tissues, especially rachises and rachis nodes (Hao et al., 2022). A recent study showed that leaves of the monocots, *Hordeum vulgare* and *Brachypodium distachyon*, can recognize laminarin, a long chain β-1,3-glucan from the marine brown algae *Laminaria digitata* (Wanke et al., 2020). Here, we investigated defense responses in different wheat tissues treated with chitin, laminarin or co-treated with these two MAMPs. Using luminol-based chemiluminescent assays, we examined ROS induction in different wheat tissues. Two wheat varieties, Norm and Alsen, were used in our studies. Norm is a variety susceptible to FHB, whereas Alsen is moderately resistant to FHB and contains quantitative trait loci (QTLs) resistant to *F. graminearum* spread and initial infection (Bokore et al., 2017). As we reported previously, we did not detect ROS

triggered by chitin in wheat leaves (Hao et al., 2022); however, we observed elevated ROS production but no typical ROS peak in wheat leaves treated with laminarin (Figure S1A). To assess if wheat head tissues respond to laminarin, we examined ROS inductions in wheat head tissues treated with laminarin. In contrast to chitin triggered tissue-specific ROS responses in wheat head tissues, laminarin induced ROS response in all tested wheat head tissues including lemmas, paleae, rachises, and rachis nodes (Figure S1A). To investigate further, we treated wheat leaf and head tissues using chitin, laminarin, or chitin and laminarin simultaneously. We observed different ROS induction patterns in wheat tissues triggered by chitin, laminarin, or co-treatments. In the leaf tissues from both Norm and Alsen, no ROS

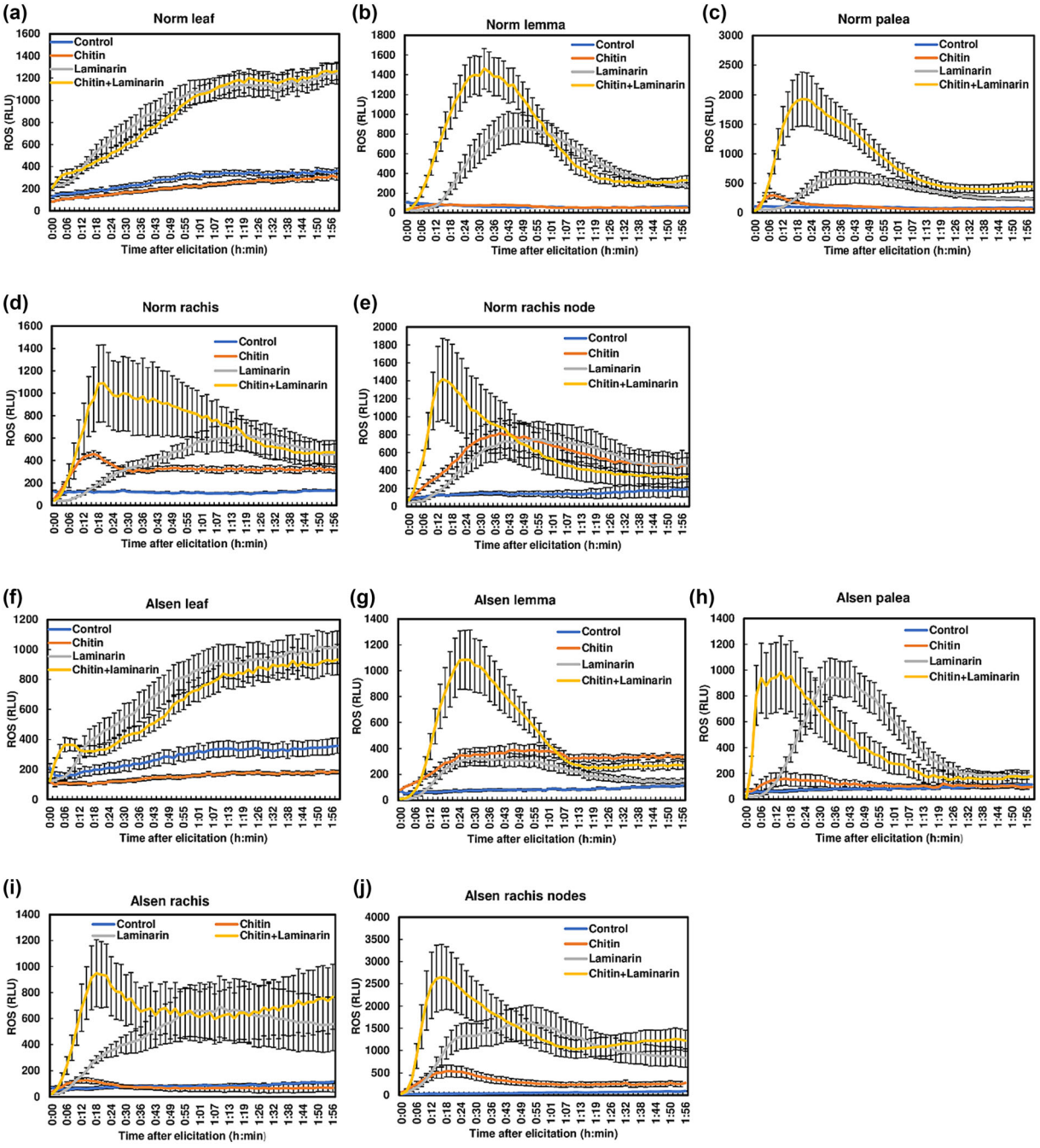

FIGURE 1 Chitin, laminarin and chitin plus laminarin-triggered different reactive oxygen species (ROS) patterns in tissues from wheat varieties, Norm and Alsen. (a,f) Leaf fragments were collected from 1- to 2-week-old leaves. (b–e,g–j) Flowering heads were dissected to paleae, lemmas, rachises, and rachis nodes. All tissues were treated with 200 μg/mL crab chitin, 3 mg/mL laminarin, or the combination of the two elicitors. Tissues without treatments served as negative controls. Production of ROS was measured using luminol-based chemiluminescence with L012 as a substrate. The plates were run on a 96-well plate reader (Synergy HT) and the signals (RLU, relative light unit) were recorded for about 120 min. The data represent means of replicates with standard error ($n = 12$). Experiments were repeated at least three times with similar results.

were induced by chitin, whereas elevated ROS were observed in both laminarin, and chitin plus laminarin treatments (Figure 1a,f). A small ROS peak was observed in Alsen leaves co-treated with chitin and laminarin (Figure 1f). ROS accumulation in leaves treated with laminarin or chitin plus laminarin was significantly higher than the control or those treated with chitin (Figure 2a,f). In Norm lemmas, no ROS

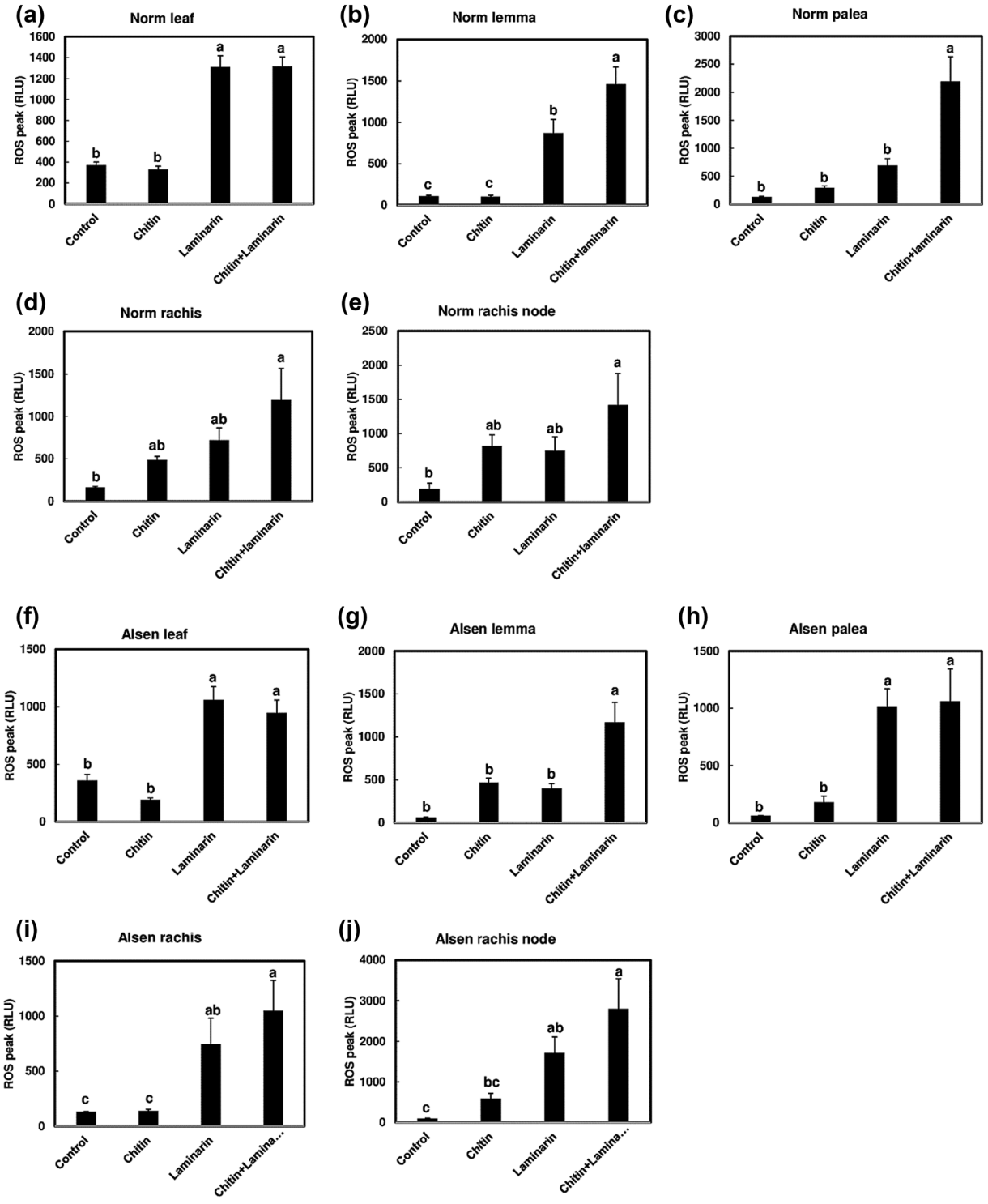

FIGURE 2 Comparison of ROS peaks induced by chitin, laminarin and chitin plus laminarin in different tissues from wheat varieties, Norm and Alsen. Tissues were collected, treated, and ROS were measured as described in Figure 1. (a–f) Leaf; (b–g) paleae; (c–h), lemmas; (d–i), rachises, and (e–j), rachis nodes. RLU, relative light unit. The data represent means of ROS peaks with standard error ($n = 12$). Statistical analyses were performed by one-way ANOVA and Tukey–Karmer honest significant difference (HSD) test using JMP15 ($n = 12$). Different letters indicate significant difference.

were induced by chitin, whereas a slow and long-lasting ROS peak (from 12 to over 100 min) was induced by laminarin (Figure 1b). Interestingly, a stronger ROS peak was initiated immediately in Norm lemmas when treated with chitin and laminarin simultaneously (Figure 1b). In wheat paleae, rachises, and rachis nodes, laminarin treatments led to long-lasting ROS peaks compared with chitin. Overall, ROS peaks induced by chitin plus laminarin were significantly higher than those induced by either chitin or laminarin in wheat lemmas and paleae (Figure 2b,c). A similar ROS induction pattern was observed in Alsen head tissues (Figure 1g,i,j) except in paleae (laminarin induced a slower ROS peak compared with chitin plus laminarin) (Figure 1h). Statistically significantly higher ROS peaks were induced by chitin plus laminarin than chitin in paleae, rachises, and rachis nodes in Alsen (Figure 2h–j), whereas a significant peak was observed in Alsen lemmas co-treated with chitin and laminarin (Figure 2h). A prior study demonstrated that MAMPs, including flg22 and chitin, enhance ROS triggered by *At*Pep (a family of peptides from Arabidopsis) (Klauser et al., 2013). To the best of our knowledge, this is the first observation of the enhanced ROS response triggered by chitin and laminarin simultaneously in wheat. It will be interesting to determine if this is a common phenomenon in other plant species.

Previous studies demonstrated that defense marker genes were transiently induced in wheat leaf tissues treated with chitin (Hao et al., 2022; Schoonbeek et al., 2015). A recent study showed that laminarin treatment modulated the expression of several wheat leaf defense genes, such as PAL, CHS, LOX, AOS, PR1, and PR2 (de Borba et al., 2022). Since faster and stronger ROS were induced in wheat head tissues treated with chitin plus laminarin than chitin or laminarin alone, we compared defense gene induction in wheat heads treated with chitin, laminarin, or chitin plus laminarin. Wheat heads from Norm were treated with chitin, laminarin or both, and collected at different time points. The defense genes affected by chitin or laminarin treatments in previous studies were screened in wheat head tissues treated with laminarin (Figure S1B). A subset of genes was selected, and the expression of these genes was compared in wheat heads treated with chitin, laminarin, or chitin plus laminarin. Our data revealed that the induction of *TaCEBiP* was significantly higher in heads treated with chitin plus laminarin than chitin or laminarin alone at 1, 3, and 6 h after treatment (Figure 3). A similar induction pattern was observed for *TaRbohD* at 1 and 3 h (Figure 3). Although ROS induction was observed in wheat head tissue treated with laminarin, the expression of *TaRbohD* was not induced. RBOHD activity can be triggered by various kinases and calcium-based signaling (Hu et al., 2020), therefore, the expression of *TaRbohD* may not be an accurate measure of laminarin-induced ROS. In our screening, *TaCERK1* was not significantly induced by laminarin (Figure S1B). Studies reported that perception of laminarin is independent of CERK1; neither CERK1 nor BAK1 is involved in laminarin recognition and signaling in *Nicotiana benthamiana* (Wanke et al., 2020). On the other hand, both chitin and laminarin recognitions are dependent on the chain length of the oligosaccharides. CERK1 is observed to recognize short chain β-glucan in Arabidopsis, and Arabidopsis plants deficient in CERK1 are impaired in β-glucan recognition (Melida et al., 2018). *Tacupredoxin-like* gene and *TaPDR2* were significantly induced at 3 h in the treated wheat heads; both genes appeared to be expressed higher when treated with chitin plus laminarin (Figure 3). The expression of *TaPR1* gene was highly induced by chitin and chitin plus laminarin at 3, 6, and 24 h; in contrast, it was not induced in wheat head tissue treated with laminarin at any time point (Figure 3). However, PR1 protein was detected in tobacco leaves infiltrated with laminarin within 48 h

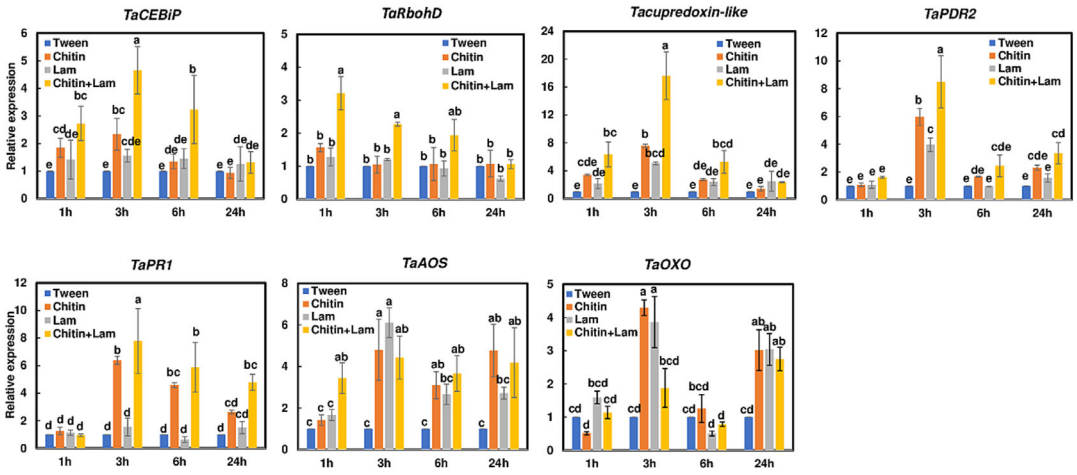

FIGURE 3 Chitin plus laminarin induce stronger defense gene expression in wheat heads than chitin or laminarin alone. Wheat heads (Norm) were dipped into 0.02% Tween containing 200 μg/mL chitin, 3 mg/mL laminarin (lam), or the combination of both. Heads treated with 0.02% Tween served as controls. Three heads from each treatment were collected at each time point (1, 3, 6, and 24 h) for RNA isolation and cDNA synthesis. Gene expression was determined using reverse transcriptase real-time PCR (RT-PCR). Wheat gene glyceraldehyde-3-phosphate dehydrogenase (*TaGAPDH*) was used as an internal control for transcript normalization. Gene induction fold was calculated relative to Tween control from 1, 3, 6, and 24 h respectively. The means of fold for each gene were calculated and compared by one-way ANOVA and Tukey–Karmer HSD test using JMP15 ($n = 3$). Different letters indicate significant difference.

(Klarzynski et al., 2000). In contrast to a few genes induced by laminarin, the expression of most of the defense genes was transiently induced and peaked at 3 or 6 h in wheat heads treated with chitin or chitin plus laminarin. Surprisingly, the expression of *TaAOS* and *TaOXO*, which are ROS-response marker genes, was significantly induced at 3 h in wheat heads treated with chitin or laminarin alone, but neither of them was induced when co-treated with both MAMPs at 3 h. At 24 h, both *TaAOS* and *TaOXO* were highly induced by MAMP treatments except *TaAOS* by laminarin (Figure 5). Overall, the expression of most tested genes was significantly upregulated in the treatments with chitin and laminarin simultaneously. On the contrary, the treatment of MAMPs only enhanced ROS triggered by *At*Pep but did not affect other defense responses such as MAPK activation, ethylene production or defense gene expression (Klauser et al., 2013).

Chitin and its modified form chitosan can enhance plant resistance against diseases by inducing host defense responses in both monocotyledons and dicotyledons (Pusztahelyi, 2018). Recent studies demonstrated that chitosan treatments triggered wheat resistance against *F. graminearum* infection and led to reduced FHB severity (Deshaies et al., 2022; Francesconi et al., 2020). To examine if chitin, laminarin or the combined treatment can enhance FHB resistance, we treated wheat heads as described above. After 24 h, the treated wheat heads were inoculated (single- floret inoculation) with *F. graminearum* strain PH-1 (Hao et al., 2019). Although chitin plus laminarin treatment enhanced ROS and defense gene expression, our data showed that only chitin treatments alone significantly reduced FHB severity at 7 days after inoculation, whereas chitin or chitin and laminarin co-treatments similarly reduced mycotoxin content (Figure 4a,b). The enhanced ROS and defense gene expression in wheat heads co-treated with chitin and laminarin did not lead to enhanced FHB resistance. This may be due to *F. graminearum* being a hemi-biotrophic pathogen; the treatments may inhibit FHB during the biotrophic stage but could promote FHB development during the necrotrophic stage. The interaction between *F. graminearum* and wheat is very complicated. In addition to the involvement of MAMPs, multiple effectors have been shown to affect FHB development by interfering with plant immunity (Hao et al., 2019, 2020; Jiang et al., 2020). Furthermore, *F. graminearum* produces DON, a mycotoxin that functions as a virulence factor to facilitate FHB spread in wheat heads (Proctor et al., 1995). DON production is triggered by a variety of host and environmental factors, including ROS component hydrogen peroxide, further complicating this plant-pathogen interaction (Audenaert et al., 2014). Therefore, more investigations are needed to elucidate how these treatments affect mechanisms or processes of FHB infection. In addition, it will be interesting to determine whether the co-application of chitin and laminarin more effectively increase plant resistance against biotrophic pathogens.

In summary, we demonstrated that chitin and laminarin trigger different ROS production patterns in wheat tissues. We discovered that ROS induction is enhanced in wheat head tissues treated with chitin and laminarin simultaneously compared with treatments of chitin or laminarin alone. We found that the expression of multiple plant defense genes was highly upregulated in wheat heads co-treated with chitin and laminarin. Furthermore, we showed that chitin treatments reduced FHB spread and mycotoxin contamination in wheat but treatments with laminarin plus chitin did not enhance the effect of chitin treatment alone.

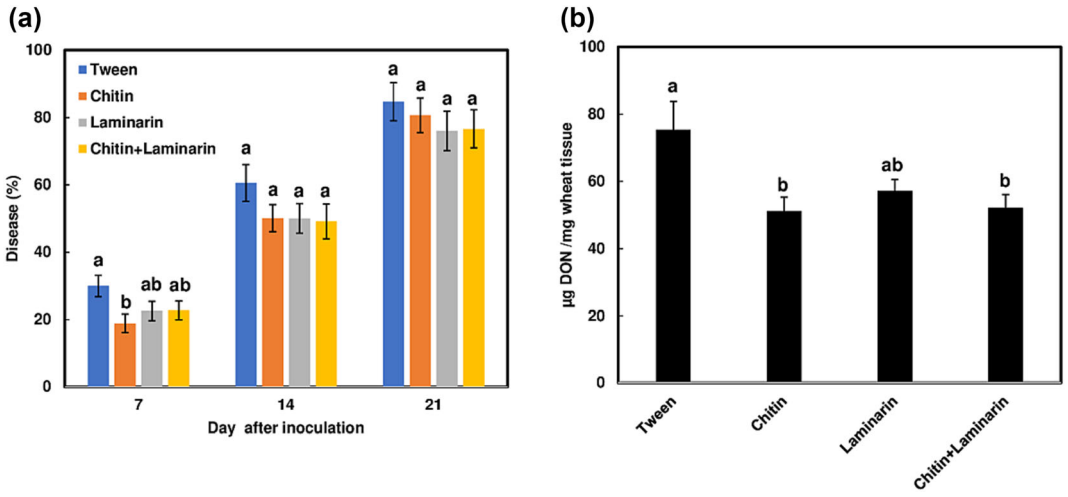

FIGURE 4 Chitin treatments enhance FHB resistance and reduce mycotoxin contamination. Wheat heads were treated with 200 µg/mL crab chitin, 3 mg/mL laminarin, or the combination of both. Following 24-h treatment, single floret inoculation (10 µL spore suspension containing 1000 conidia) was performed on wheat florets with *F. graminearum* strain PH-1. (a) FHB progression was evaluated and calculated by the percentage of infected florets at 7-, 14-, and 21-days post inoculation (dpi); the means of the percentage was compared by one-way ANOVA and Tukey–Karmer HSD test using JMP15 ($n = 24$). Different letters indicate significant difference at the $P < .05$. (b) Chitin treatments reduce mycotoxin contamination. Heads were collected at 21 dpi, and three heads were combined as a group. DON was extracted and analyzed by GC-MS. Bars represent the average and standard error of inoculated head with each treatment ($n = 8$). Statistical analyses were performed with one-way ANOVA and Tukey–Karmer HSD test using JMP15. Different letters indicate significant difference at $P < .05$.

AUTHOR CONTRIBUTIONS

Guixia Hao designed the study. Guixia Hao, Nicholas A. Rhoades, and Susan McCormick performed the experiments. Guixia Hao wrote the original draft. Susan McCormick, Nicholas A. Rhoades, and Guixia Hao edited the manuscript and all authors read and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests.

PEER REVIEW

The peer review history for this article is available in the Supporting Information for this article.

DATA AVAILABILITY STATEMENT

Data that support the findings of this work are included in the article and its Supplemental Information files.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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