J. Amer. Soc. Hort. Sci. 148(2):74–82. 2023. https://doi.org/10.21273/JASHS05277-22

Physiological and Chitinase Gene Expression Responses of Male *Zizania latifolia* to *Ustilago esculenta* Infection

Wenjuan Xu
School of Horticulture, Anhui Agricultural University, Hefei 230036, China

NianNian Zhou
School of Horticulture, Anhui Agricultural University, Hefei 230036, China; and School of Horticulture, Nanjing Agricultural University, Nanjing 210095, China

Pengfei Guo, Huimin Zhou, and Shuangshuang Xu
School of Horticulture, Anhui Agricultural University, Hefei 230036, China

Xiaomei He
West Anhui University, Lu'an, Anhui, 237012, China

Defang Gan
School of Horticulture, Anhui Agricultural University, Hefei 230036, China

Keywords. antioxidant enzyme, biotic stress, pathogenesis-related protein, phenylalanine ammonia lyase, physiological response

Abstract. This study explored the effects of *Ustilago esculenta* inoculation on physiological responses (activities of defense and antioxidant enzymes) and chitinase gene expression in male *Zizania latifolia* "jiaobai" (without *U. esculenta* infection, with no galls, but normal flowering). Male jiaobai seedlings were injected at the five-leaf stage with *U. esculenta* suspension, and the impact on transcription of several genes encoding enzymes was examined. Compared with controls, expression of most enzymes was significantly different at 3 or 12 hours postinjection, and most *ZlChi* genes were involved in the response to *U. esculenta* inoculation. Fluorescence quantitative polymerase chain reaction results showed that *U. esculenta* was present in the roots of male jiaobai inoculated with *U. esculenta* at the shoot tips. Paraffin sections revealed many fungal hyphae in the roots at 15 d after inoculation, but few in controls. The results provide a basis for further study of the responses of male *Z. latifolia* to *U. esculenta* infection.

Zizania latifolia, also known as Manchurian wild rice, is an asexual perennial herb and the second most widely cultivated aquatic vegetable in China (Yu et al. 2020). The endophytic smut fungus *Ustilago esculenta* induces the formation of culm galls (jiaobai) in *Z. latifolia*. Normal jiaobai (edible swollen galls in stem bases filled with hyphae), gray jiaobai (inedible galls filled with dark teliospores, with an unacceptable taste), and male jiaobai (without *U. esculenta* infection, with no galls but normal flowering) are the three phenotypes of *Z. latifolia* found in their habitats (Wang et al. 2020). At present, the method of isolation and selection of *Z. latifolia* used in cultivation and conservation breeding requires considerable labor and material resources. Moreover, population diversity and physiological race differentiation of *U. esculenta* may exist among *Z. latifolia* populations (Tu et al. 2019). The molecular mechanisms of *Z. latifolia* responses to *U. esculenta* infection are not completely understood, although the mechanism of swelling and stem enlargement in infected *Z. latifolia* has received attention (Li et al. 2022; Zhang et al. 2021).

Research on the physiological and biochemical aspects of *Ustilago*-induced stem expansion of *Z. latifolia* has focused mainly on changes in antioxidant enzymes, including peroxidase (POD), superoxide dismutase (SOD), catalase (CAT), and polyphenol oxidase (PPO). The activities of SOD, CAT, and PPO decrease during the process of stem expansion, whereas POD activity increases during the middle stage of stem expansion, suggesting that POD might participate in the differentiation and expansion of *Zizania* cells and tissues (Jiang et al. 2007). Meanwhile, proteomic studies have explored the stem expansion process of *Z. latifolia.* The number and expression level of unique proteins in stems of *Z. latifolia* at different developmental stages varies significantly, and the formation of fleshy stem bases of *Z. latifolia* result from interactions between plants and endophytic fungi (Liu et al. 2011). During the process of fleshy stem expansion, 4389 upregulated and 3700 downregulated genes were detected in normal *Z. latifolia*. By comparison, in gray *Z. latifolia*, 3121 genes were upregulated and 2130 genes were downregulated (Wang et al. 2017). Jose et al. (2019) detected qualitatively and quantitatively differentially expressed proteins in response to local *U. esculenta* formation during the interaction between *Z. latifolia* and *U. esculenta* and found that *U. esculenta*

Received for publication 31 Oct 2022. Accepted for publication 16 Jan 2023.
Published online 20 Feb 2023.
This research was supported by grants from the Natural Science Foundation of Anhui Province (grant number 2008085MC80) and the Anhui Province Crop Variety Breeding Project. We thank Elixigen Corporation for editing this manuscript.
W.X. and N.N.Z. contributed equally to this work.
D.G. is the corresponding author. E-mail: dfgan1@163.com.

produced proteases and energy acquisition proteins that enhanced its defense and survival in the host.

Plants can be invaded by various pathogens. To cope with pathogen infection, a series of physiological and biochemical changes take place in challenged plants. Among these, changes in key enzyme activities related to plant disease resistance can effectively enable plants to withstand further infection by pathogens (Espinosa-Vellarino et al. 2020; Waszczak et al. 2018). These defense enzymes can improve the tolerance and resistance of plants to external abiotic and biotic stresses (Zhang 2020). Pathogenesis-related proteins (PRs), such as chitinases and β-1,3-glucanase, are a class of proteins induced by biotic or other external factors that play an important role in plant disease resistance (Tuan et al. 2019; Zhou et al. 2020). In the absence of pathogens, chitinase and β-1,3-glucanase activities in plants are very low, but when plants are infected by fungal phytopathogens, the activities of β-1,3-glucanase and chitinase increase within a short time, rapidly degrading chitin in the cell walls of phytopathogenic fungi and inhibiting the growth and reproduction of pathogenic fungi in resistant plants, thereby increasing the resistance of plants to pathogens (Bai et al. 2018; Hu et al. 2017; Khan et al. 2017).

In previous studies on interactions between *Z. latifolia* and *U. esculenta*, we established an in vitro *Z. latifolia* regeneration system, explored the *U. esculenta* inoculation system (Yang 2018), and studied the expression of *Z. latifolia* chitinase in response to abiotic stress (Zhou et al. 2020). However, the mechanism underpinning the interaction between *U. esculenta* and *Z. latifolia* that leads to enlargement of swollen stem galls remains unclear. To explore the physiological basis and molecular mechanisms of the *Z. latifolia* responses to *U. esculenta* infection, male (uninfected) *Z. latifolia* plants at the five-leaf stage were used as the experimental material to explore the effects of artificial inoculation on the responses of disease resistance-associated enzymes, phenylpropanoid metabolism-related enzymes and antioxidant enzymes, as well as the responses of membrane lipid peroxidation. In addition, paraffin sections of roots and stems of *Z. latifolia* plants at 15 d after *U. esculenta* inoculation were collected to probe changes in *U. esculenta* fungus development. This work provides an important basis for future studies on the mechanism of culm gall formation in *Z. latifolia*.

Materials and Methods

***U. esculenta* preparation and inoculation techniques.** Single colonies (mycelium) of *U. esculenta* were selected and cultured in potato dextrose liquid medium at 28 °C with shaking at 220 rpm on an orbital shaker. When the optical density at 600 nm (OD_{600}) reached 0.6 to 0.8, cultures were expanded at a dilution of 1:50 overnight. The suspension was then centrifuged at 2632 g_n for 20 min to collect cells, and the pellet was resuspended in potato dextrose liquid medium and diluted to OD_{600} = 2.5 (Geng 2018; Yang 2018).

Male jiaobai plants at the five-leaf stage (Fig. 1A) were injected at the stem tip growth point with the resulting *U. esculenta* cell suspension, 0.5 cm from the base of the bolting tube (Fig. 1B, red arrow). Male jiaobai plants injected with potato dextrose liquid medium served as controls (CK). Experiments were repeated three times with six plants per replicate.

Measuring the physiological and biochemical responses of *Z. latifolia* to *U. esculenta* injection. Crude extracts of leaves of *Z. latifolia* plants were prepared at 3, 6, 12, 18, and 24 h after inoculation, and the activities of defense enzymes and antioxidant enzymes were assayed. The activities of ascorbate peroxidase (APX) and phenylalanine ammonia lyase (PAL) were determined according to the methods of Gao and Cai (2018); the activity of POD was assayed according to the method of Wu (2018); the activities of SOD and CAT were determined according to the methods of Gao and Cai (2018); the activity of PPO was determined using the methodology described by Dalmadi et al. (2006). Extraction of chitinase and β-1,3-glucanase was performed according to the methods of Liu (2013) and Boller et al. (1983), respectively. The activity of β-1,3-glucanase was determined using the method of Jiang et al. (2015), and a glucose standard curve was prepared according to the method of Li (1998). Determination of chitinase activity and preparation of an *N*-acetylglucosamine standard curve were conducted according to the methods of Liu (2013) and Zhang et al. (2013), respectively. Three biological replicates were performed for each treatment, with six plants per replicate.

RNA extraction and quantitative reverse transcription polymerase chain reaction. Leaves at 3, 6, 12, 18, and 24 h, and 3 and 5 d after inoculation, were ground with liquid nitrogen, total RNA was extracted using TRIzol reagent (RNAiso Plus; Takara, Dalian City, China), and treated using a PrimeScript RT Reagent Kit and gDNA Eraser (Takara) to remove genomic DNA contamination. The quality and purity of samples were monitored using a spectrophotometer (NanoDrop 2000C; Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (1 μg) was reverse-transcribed to first-strand cDNA using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara) following the manufacturer's instructions. The resulting cDNA was diluted 10-fold with sterile water. Gene-specific primers (Supplemental Table 1) used for quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of the *Z. latifolia* chitinase gene were designed using Primer Premier 5.0 software (Kakhki et al. 2019). The expression level of the *Z. latifolia Actin*2 gene (primer sequences listed in Supplemental Table 1) (Wang et al. 2017), which is consistent in different organs and under different treatments, was used as an internal control (Supplemental Fig. 1). qRT-PCR was carried out according to the instructions of the PrimeScript RT Reagent Kit with gDNA Eraser (Takara). Three biological replicates were performed for each treatment, and each cDNA sample was tested using four technical replicates. Relative expression levels were calculated as $2^{-\Delta\Delta Cq}$ where ΔCq = Cq (gene of interest) – Cq (*Actin2*), ΔΔCq = ΔCq (treatment, inoculated with *U. esculenta*) − ΔCq (CK, inoculated with potato dextrose broth).

Detection of *U. esculenta* in inoculated plants. Genomic DNA was extracted from roots of plants at 15 d after inoculation, and expression of the *actin* gene of *U. esculenta* was measured by qRT-PCR (Ge et al. 2021). Male jiaobai plants inoculated with potato dextrose liquid medium served as a negative control, and *U. esculenta* DNA served as a positive control. Primers P1 (CAATGGTTCGGGAATGTGC) and P2 (GGGATACTTGAGCGTGAGGA) were used to amplify the *actin* gene sequence (KU302684.1). qRT-PCR was performed according to the preceding instructions, and products were detected by electrophoresis on a 2.0% agarose gel and purified. The obtained target fragments were ligated into the pMD19-T vector, transformed, and sequenced (Sangon Biotech Shanghai Co. Ltd., Shanghai, China).

At 15 d after inoculation, roots of male *Z. latifolia* were quickly fixed in formaldehyde-acetic acid-ethanol fixative comprising 5% formalin (37% to 40% formaldehyde) + 5% glacial

Fig. 1. Images of the five-leaf stage of male *Zizania latifolia* seedlings: (**A**) seedlings and (**B**) enlarged view of partial longitudinal cutting.

acetic acid + 90% alcohol (70%). Using qRT-PCR detection, roots that only amplified the *Ue-actin* gene of *U. esculenta* and roots without any amplified band were analyzed by paraffin section, including tissue cleaning, fluorescent staining of fungal cell walls with fluorescein isothiocyanate-conjugated wheat germ agglutinin (FITC-WGA), and counterstaining of plant cell walls with propidium iodide (Redkar et al. 2018), performed by Wuhan Seville Biotechnology Co., Ltd. (Wuhan, China).

Data processing. Spreadsheet software (Microsoft Excel 2016; Microsoft Corp., Redmond, WA, USA) was used for basic processing of test data, statistical analysis software (IBM SPSS statistics ver. 25; IBM Corp., Armonk, NY, USA) was used for analysis of variance ($P \leq 0.05$ indicates statistical significance), and data analysis/scientific graphing software (SigmaPlot 14.0, Systat International Software, San Jose, CA, USA); Origin 2019b 32-bit (OriginLab, Northampton, MA, USA) were used for mapping.

Results

Activities of antioxidant and disease-related enzymes. APX activity in leaves of male *Z. latifolia* inoculated with *U. esculenta* was significantly higher than in control plants at 12 h after inoculation, indicating that 12 h was a suitable timepoint for assessing APX involvement in the response to *U. esculenta* injection (Fig. 2). CAT activity in the treated group was also higher than that in the control group, with the highest level of 73.67 U/g fresh weight per minute occurring at 3 h after inoculation, and the difference was significant ($P \leq 0.05$; Fig. 2). The same trend was observed for POD activity, indicating that the response of POD activity to *U. esculenta* mainly occurred shortly after inoculation (Fig. 2). There was no significant difference in SOD activity and malondialdehyde (MDA) content between treated and control plants within 24 h after inoculation (Fig. 2).

PPO activity of the treated group was significantly lower than that of the control at 6 h after inoculation ($P \leq 0.05$), whereas PAL activity in the treated group was insignificant compared with that of the control group ($P \leq 0.05$; Fig. 3). Chitinase in leaves of male *Z. latifolia* showed a pattern of decreasing activity over time, and there was a significant difference between treated and control groups only at 12 h after inoculation ($P \leq 0.05$; Fig. 4). The activity of β-1,3-glucanase in the treated group was higher than that of the control group at 3, 6, and 18 h after inoculation, with a significant difference at 3 h after inoculation ($P \leq 0.05$; Fig. 4).

Expression profiling of *ZlChi* genes in response to *U. esculenta* infection. Expression of individual *ZlChi* genes was detected at 3, 6, 12, 18, and 24 h, and 3 and 5 d after treatment. The expression patterns of *ZlChi* genes were different, among which expression of three *ZlChi* genes (*ZlChi2*, *ZlChi5*, and *ZlChi9*) could not be detected, whereas that of the other eight responded to *U. esculenta* inoculation (Fig. 5). *ZlChi1* expression was upregulated, and the difference was significant ($P \leq 0.05$) at 18 h and 3 d after inoculation. Expression of *ZlChi6* was upregulated at 3, 6, and 12 h after inoculation, and the difference was significant ($P \leq 0.05$). The relative expression level of *ZlChi8* was highest at 5 d after inoculation, and the difference was significant ($P \leq 0.05$), whereas the relative expression level of *ZlChi10* was also upregulated at 3, 6, and 18 h after inoculation. The relative expression level of *ZlChi11* reached a peak (10.50) at 3 h after injection, but expression of the other *ZlChi* genes was low or downregulated in response to *U. esculenta* inoculation (Fig. 5).

Detection of *U. esculenta* in inoculated plants. The root DNA templates at 15 d after inoculation were amplified using primers targeting *Ue-Actin*. Agarose gel electrophoresis showed that only one band was detected in some roots of male jiaobai plants inoculated with *U. esculenta*, and sequencing results showed that the fragment was 243 bp in size, with 100% homology to *Ue-Actin* (KU302684.1). In addition, two bands of 243 bp (*Ue-Actin*t) and 139 bp (86% identical to the *NIH2624 actin* gene XM_001209659.1 of *Aspergillus terreus*) were detected in other inoculated roots. Meanwhile, some roots were not infected at all by any fungi, and some were only infected with one fungal species (Fig. 6). This indicated that some *Z. latifolia* plants had been successfully infected by *U. esculenta* 15 d after inoculation.

Roots that only amplified bands (243 bp) of *U. esculenta* at 15 d after inoculation and control roots were selected for paraffin sectioning. The root tissue of male *Z. latifolia* plants was quickly fixed in formaldehyde-acetic acid-ethanol (FAA) fixative, and paraffin sections were stained with FITC-WGA and counter-stained with propidium iodide. In the same field of vision (10× field of view), numerous fungal hyphae could be observed in the roots of male *Z. latifolia* plants inoculated with *U. esculenta*, whereas few fungal hyphae of *A. terreus* could be seen in the roots of control

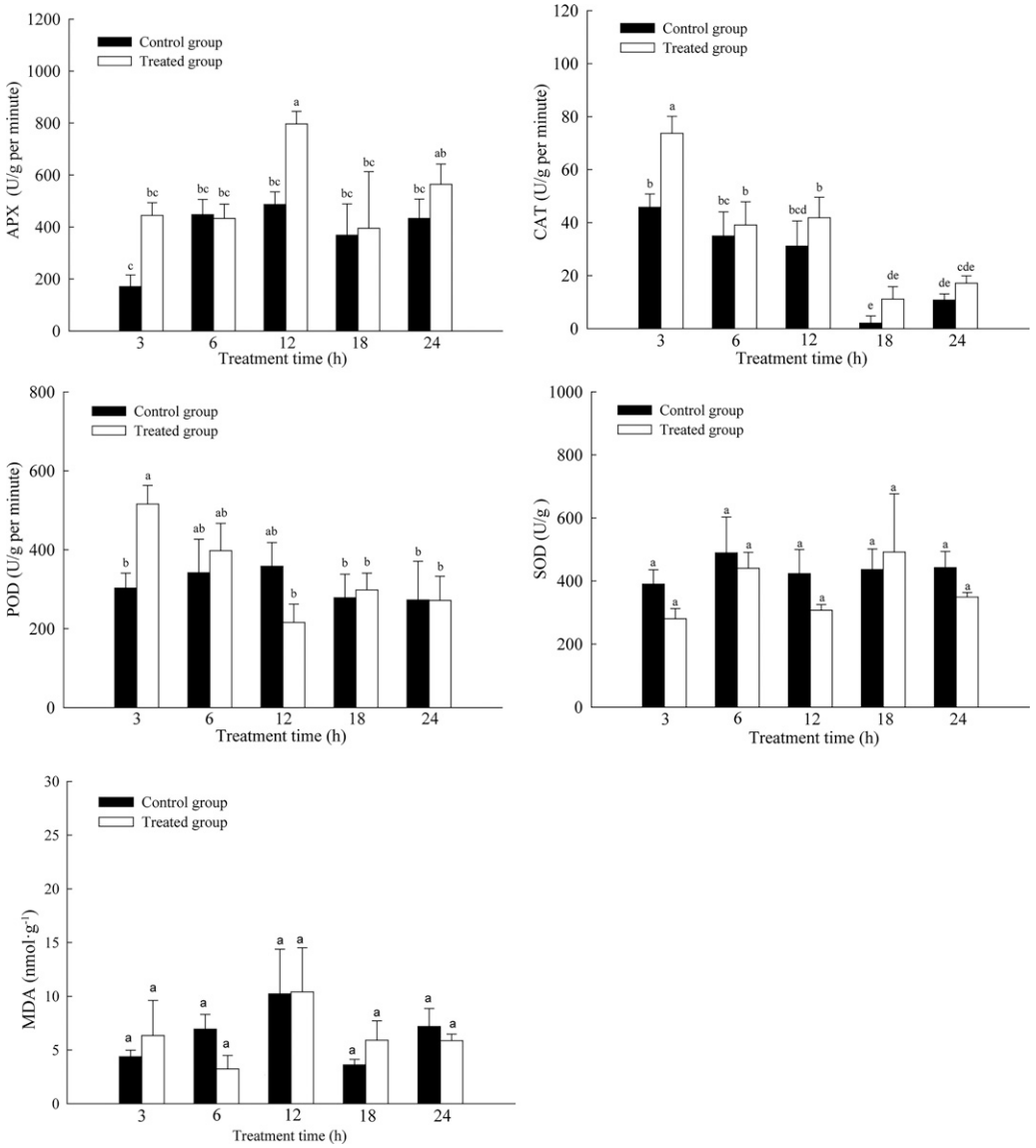


Fig. 2. Changes in the activities of antioxidant-related enzymes ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and malondialdehyde (MDA) content following infection of male *Zizania latifolia* by *Ustilago esculenta*. Error bars represent standard deviations of mean values from three biological replicates. Shared lowercase letters indicate no significant differences in activities of a particular enzyme between different treatments via analysis of variance ($P \leq 0.05$).

male *Z. latifolia* plants (Fig. 7). This indicated that *U. esculenta* had successfully infected male *Z. latifolia* plants.

Discussion

Physiological and biochemical responses of *Z. latifolia* to *U. esculenta* inoculation. When plants encounter stress, it can regulate various antioxidant enzymes (e.g., APX, POD, SOD, and CAT) and disease resistance-related enzymes (chitinase and β-1, 3-glucanase) to reduce damage to cells and tissues (Tyagi et al. 2020; Wang et al. 2021a). Key enzymes of the phenylpropane metabolic pathway (e.g., PAL and PPO) can also respond quickly to invasion of plant pathogens (Sakhuja et al. 2021; Syed et al. 2021). Yan et al. (2013) reported that *U. esculenta* infection led to an increase in APX, CAT, POD, glutathione reductase, and SOD activities in *Z. latifolia* leaves, and a decrease in the concentrations of superoxide free radicals and MDA. Our results showed that inoculation of *Z. latifolia* with *U. esculenta* significantly increased the activities of APX and CAT enzymes in leaves. The activities of CAT and POD were highest at 3 h after inoculation, whereas SOD activity was highest at 18 h after inoculation. Changes in antioxidant enzyme activity were consistent with previous reports by Yan et al. (2013).

Chitinase (EC 3.2.1.14) is a type of glycosidase that degrades chitin from fungi and responds to fungal infection (Zhou et al. 2020). β-1,3-glucanase (EC 3.2.1.39) inhibits the growth of fungi by catalyzing the hydrolysis of β-1,3-glucan in fungal cell walls, participates in the defense of plants against pathogenic fungi (Ali et al. 2021). When crops are infected by phytopathogenic fungi, chitinase and β-1,3-glucanase can inhibit the growth of pathogenic fungi, thus mediating resistance to fungal pathogens (Toufiq et al. 2018). Our results showed that chitinase activity in male *Z. latifolia* leaves was higher than in controls, and the gradual decrease in activities of chitinase and β-1,3-glucanase was similar to that reported by Wang et al. (2021b).

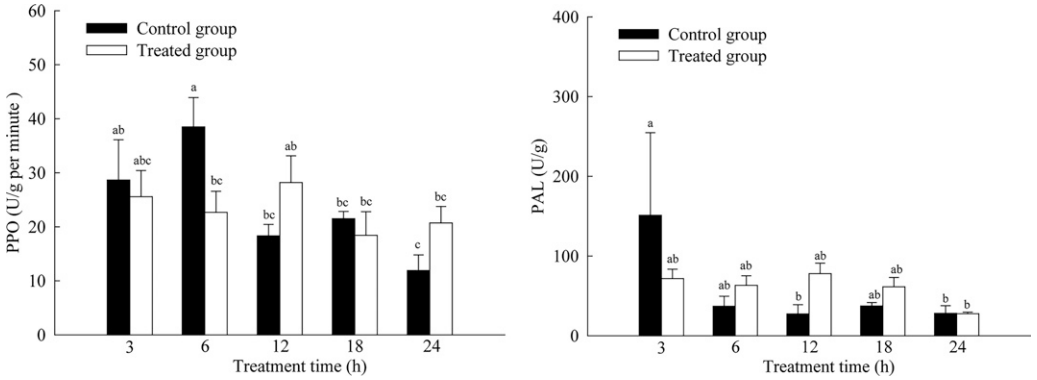


Fig. 3. Changes in the activities of enzymes related to the phenylpropanoid metabolism pathway, namely polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL), after infection of *Zizania latifolia* by *Ustilago esculenta*. Error bars represent standard deviations of the mean values of three biological replicates. Shared lowercase letters indicate no significant differences in activities of a particular enzyme between different treatments via analysis of variance ($P \leq 0.05$).

PPO (EC 1.10.3.1) is associated with disease resistance, pest resistance, and stress tolerance (Kanjanapongkul and Baibua 2021; Zhao et al. 2021), and PAL (EC 4.3.1.24) regulates the biosynthesis of secondary metabolites associated with plant disease resistance (Dong and Lin 2021). In the current study, PPO activity in treated plants was significantly lower than that in controls at 6 h post-injection. PAL activity in treated plants was also higher than that in controls. These results revealed that the increases in PPO and PAL activities in treated plants mainly occurred in the early stages of the colonization process. Previous research also showed that PAL activity was higher during the early stages of *U. esculenta* colonization in *Z. latifolia* (Cheng 2002). However, in the present study, PAL activity reached the highest level at 12 h after inoculation. Thus, infection by *U. esculenta* altered antioxidant enzyme activity in *Z. latifolia* plants. Endophytic fungi and their hosts need to adapt and evolve together over a long period of time to form a mutually beneficial symbiotic relationship (Delavaux et al. 2019; Saikkonen et al. 2004). The observed changes in the activities of enzymes in *Z. latifolia* plants may be related to the formation of a mutually beneficial symbiosis between *Z. latifolia* and *U. esculenta*. However, infection of male jiaobai plants by *U. esculenta* and the resulting symbiotic relationship require further investigation.

Expression patterns of *ZlChi* genes in response to inoculation with *U. esculenta*. Plant chitinases are pathogenesis-related proteins that degrade chitin, damage the fungal cytoskeleton, inhibit the growth of pathogenic fungi, and elicit antifungal effects (Bartholomew et al. 2019; Chen et al. 2018). Previous research showed that chitinase genes regulate the responses of *Morus laevigata* to *Sclerotinia sclerotiorum* infection (Jiang et al. 2020). Similarly, upregulated expression of *GH19* (TEA028279 and TEA019397) indicated that chitinase genes play defensive roles in responses to fungal infection (Bordoloi et al. 2021). The current study showed that, apart from *ZlChi2*, *ZlChi5*, and *ZlChi9* genes, expression of the other eight *ZlChi* genes responded to *U. esculenta* infection. Thus, we speculated that these genes may be involved in the infection process of male *Z. latifolia* by *U. esculenta*. Previous research revealed time differences in the expression patterns of different *CmCHT* genes in *Cucumis melo* induced by pathogen infection (Wang et al. 2021b). Similarly, in the present study, expression of most *ZlChis* also showed temporal differences. Expression of most chitinase genes peaked during the early stages after inoculation, indicating an involvement in the colonization of *U. esculenta*. However, expression of *ZlChi1*, *ZlChi4*, and *ZlChi8* peaked at 3 or 5 d after inoculation, indicating that these genes might play a key role in the later stages of *U. esculenta* infection.

Detection of *U. esculenta* in male *Z. latifolia* 15 d after inoculation. In the current study, fluorescence quantitative PCR was used to detect fungal genomic DNA in male *Z. latifolia* roots at 15 d after inoculation, and the results showed that only the *Ue-Actin* gene, or two fragments (the *Ue-Actin* gene fragment and the

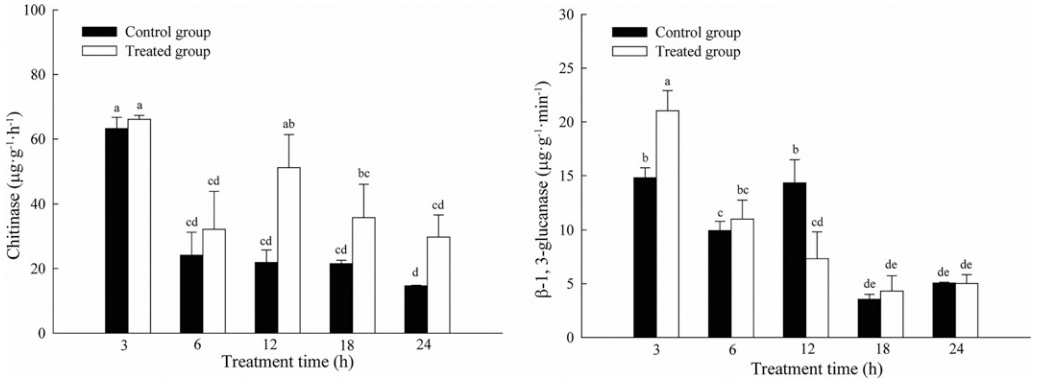


Fig. 4. Changes in chitinase and β-1,3-glucanase activities in *Zizania latifolia* following infection by *Ustilago esculenta*. Error bars represent standard deviations of the mean values of three biological replicates. Shared lowercase letters indicate no significant differences in activities of a particular enzyme between different treatments via analysis of variance ($P \leq 0.05$).

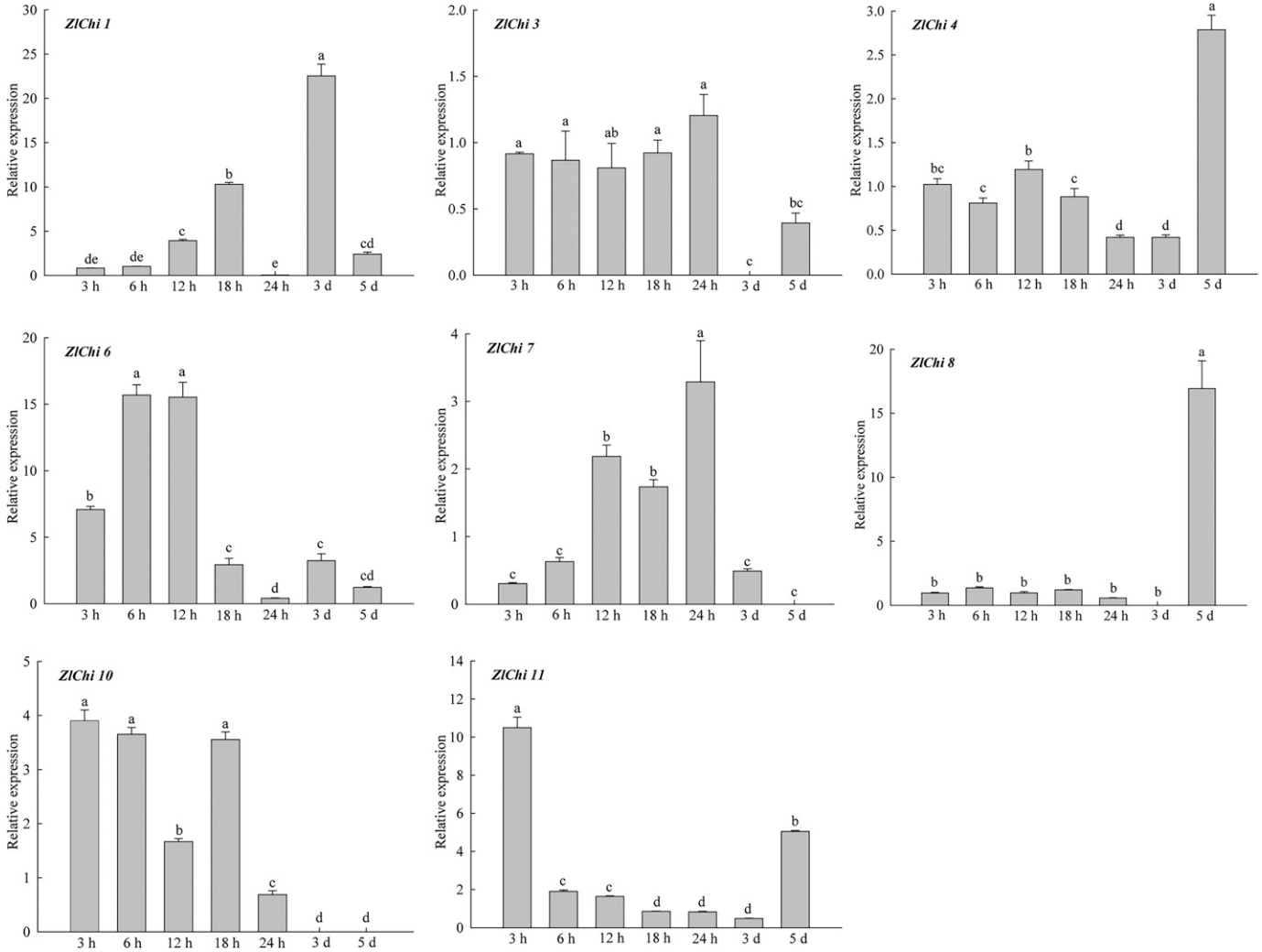


Fig. 5. Patterns of relative gene expression analysis of *ZlChi* genes in *Zizania latifolia* leaves revealed by real-time fluorescence quantitative polymerase chain reaction in response to *Ustilago esculenta* infection. *ZlActin2* served as an internal control to normalize data. The y-axes, indicating relative expression levels, are drawn to different scales depending on the magnitude and direction of change. Upregulated expression is represented by whole-number y-axis values, whereas downregulated gene expression is indicated by decimal fractions on the y-axis scale. Error bars represent standard deviations of the mean values of three biological replicates. Shared lowercase letters indicate no significant differences in activities of a particular enzyme between different treatments via analysis of variance ($P \leq 0.05$).

NIH2624 actin gene fragment), were amplified from the roots of some inoculated plants, indicating that *U. esculenta* successfully infected and reached the roots of male jiaobai plants at 15 d after inoculation. Meanwhile, the *NIH2624 actin* gene fragment was amplified from other treated plants, which indicates that there were other fungi (*A. terreus*) within male *Z. latifolia* plants.

Li (2016) found that *U. esculenta* in normal *Z. latifolia* (with swollen stem bases) was mainly distributed in the root and stem

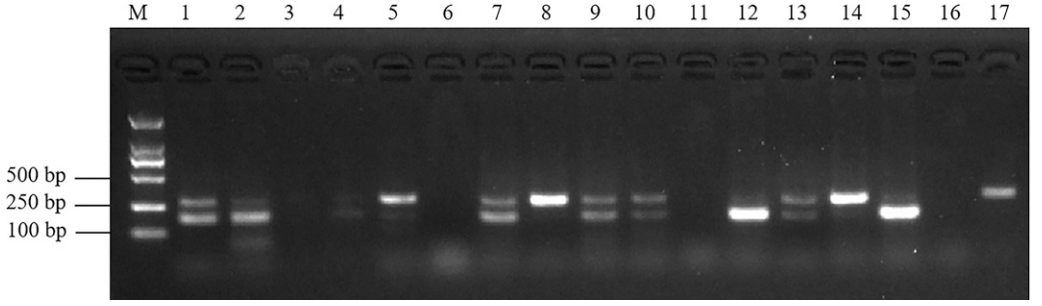


Fig. 6. Electrophoretic image (2.0% agarose gel) of real-time quantitative polymerase chain reaction products of *Ue-Actin* from roots of male *Zizania latifolia* 15 d after inoculation with *Ustilago esculenta*; M = DNA markers; lanes 1–15 = extracts of male *Z. latifolia* plants inoculated with *U. esculenta*; lane 16 = negative control; lane 17 = positive control.

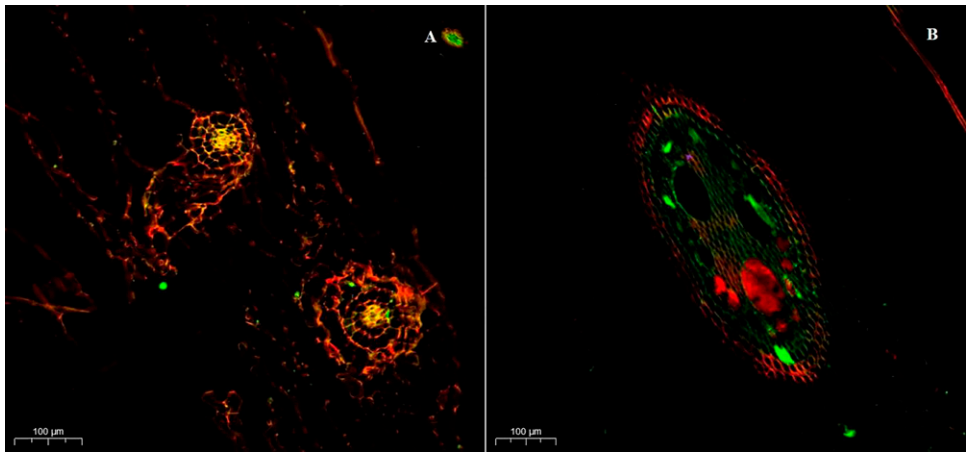


Fig. 7. Paraffin sections of roots from male *Zizania latifolia* 15 d after inoculation with *Ustilago esculenta* [10× field of view, fluorescein isothiocyanate-conjugated wheat germ agglutinin staining; green fluorescence represents fungal hyphae and red fluorescence is the cell wall of root tissue]. (**A**) Paraffin section of roots of male *Z. latifolia* plants inoculated with potato dextrose liquid medium (CK = control). (**B**) Paraffin section of roots of male *Z. latifolia* plants inoculated with *U. esculenta*.

in the form of hyphae, with the number of hyphae in the stem being closely related to the supply of nutrients to the stem. Zhou (2018) found that mycelium of *U. esculenta* were present in the stem of normal *Z. latifolia* at the five-leaf stage, with the growth and distribution of *U. esculenta* in the stem of normal *Z. latifolia* being closely related to the growth and development state of the plant. In the present study, paraffin sections revealed multiple fungal hyphae in the roots of inoculated plants, although only a small number of fungal hyphae were detected in the roots of control plants, indicating that there might be other fungi within *Z. latifolia* plants. The existence of microbial diversity in *Z. latifolia* is consistent with the results of Zhan (2017). Similarly, Tu et al. (2019) isolated several strains of *U. esculenta* as well as six other species of fungi and 10 species of bacteria from five samples of galls harvested at different stages of swelling. In the present study, *U. esculenta* was detected in the roots of some inoculated plants, indicating that *U. esculenta* had successfully colonized male *Z. latifolia* plants. However, a small number of other fungi were also detected in roots, which suggests that there might be a variety of fungi in *Z. latifolia* plants. Whether these fungal hyphae in inoculated plants can cause expansion into fleshy stem galls needs to be investigated in future studies.

Conclusions

We injected male jiaobai plants at the five-leaf stage with *U. esculenta* suspension and examined the activities of related enzymes. Compared with controls, the activities of most enzymes were significantly different at 3 or 12 h after infection. Most *ZlChi*s were involved in the response to *U. esculenta* inoculation. Fluorescence quantitative PCR results showed that *U. esculenta* was present in the roots of male jiaobai, and paraffin sections revealed many fungal hyphae in roots at 15 d after inoculation. These results provide a basis for further studies on the interactions between *U. esculenta* and *Z. latifolia*, and the stem expansion mechanism of *Z. latifolia*.

References Cited

Ali M, Lamin-Samu AT, Muhammad I, Farghal M, Khattak AM, Jan I, Haq SU, Khan A, Gong ZH, and Lu G. 2021. Melatonin mitigates the infection of *Colletotrichum gloeosporioides* via modulation of the chitinase gene and antioxidant activity in *Capsicum annuum* L. Antioxidants. 10(1):7. https://doi.org/10.3390/antiox10010007.

Bai YJ, Zhang PL, Huang W, Feng ZS, Li M. 2018. Analysis of expression profiles of chitinase and β-1,3-glucanase genes in muskmelon fruit tissue inoculated with *Alternaria alternata.* Shipin Kexue. 39(2):185–191. https://doi.org/10.7506/spkx1002-6630-201802029.

Bartholomew ES, Black K, Feng ZX, Liu W, Shan N, Zhang X, Wu LC, Bailey L, Zhu N, Qi CH, Ren HZ, Liu XW. 2019. Comprehensive analysis of the chitinase gene family in cucumber (*Cucumis sativus* L.): From gene identification and evolution to expression in response to *Fusarium oxysporum.* Int J Mol Sci. 20(21):5309. https://doi.org/10.3390/ijms20215309.

Boller T, Gehri A, Mauch F, Vögeli U. 1983. Chitinase in bean leaves: Induction by ethylene, purification, properties, and possible function. Planta. 157(1):22–31. https://doi.org/10.1007/BF00394536.

Bordoloi KS, Krishnatreya DB, Baruah PM, Borah AK, Agarwala N. 2021. Genome-wide identification and expression profiling of chitinase genes in tea [*Camellia sinensis* (L.) O. Kuntze] under biotic stress conditions. Physiol Mol Biol Plants. 27(2):369–385. https://doi.org/10.1007/s12298-021-00947-x.

Chen JJ, Piao YL, Liu YM, Li XN, Piao ZY. 2018. Genome-wide identification and expression analysis of chitinase gene family in *Brassica rapa* reveals its role in clubroot resistance. Plant Sci. 270:257–267. https://doi.org/10.1016/j.plantsci.2018.02.017.

Cheng LJ. 2002. Study on physiological and biochemical changes of *Zizania latifolia* during pregnancy (Master's Diss) [in Chinese]. Zhejiang University, Hangzhou, Zhejiang, China.

Dalmadi I, Rapeanu G, Van Loey A, Smout C, Hendrickx M. 2006. Characterization and inactivation by thermal and pressure processing of strawberry (*Fragaria* × *ananassa*) polyphenol oxidase: A kinetic study. J Food Biochem. 30(1):56–76. https://doi.org/10.1111/j.1745-4514.2005.00045.x.

Delavaux CS, Weigelt P, Dawson W, Duchicela J, Essl F, Kleunen MV, König C, Pergl J, Pyšek P, Stein A, Winter M, Schultz P, Kreft H, Bever JD. 2019. Mycorrhizal fungi influence global plant biogeography. Nat Ecol Evol. 3(3):424–429. https://doi.org/10.1038/s41559-019-0823-4.

Dong NQ, Lin HX. 2021. Contribution of phenylpropanoid metabolism to plant development and plant–environment interactions. J Integr Plant Biol. 63(1):180–209. https://doi.org/10.1111/jipb.13054.

Espinosa-Vellarino FL, Garrido I, Ortega A, Casimiro I, Espinosa F. 2020. Effects of antimony on reactive oxygen and nitrogen species (ROS and RNS) and antioxidant mechanisms in tomato plants. Front Plant Sci. 11:674. https://doi.org/10.3389/fpls.2020.00674.

Gao JS, Cai YP. 2018. Experimental guidance of plant physiology [in Chinese]. China Agricultural University Press, Beijing, Beijing, China.

Ge XT, Yin YM, Gao LD, Hu YL, Xia WQ, Ye ZH, Tang JT, Zhang YF. 2021. Selection and application of reference genes for qRT-PCR in *Ustilago esculenta* (in Chinese). J Agric Biotechnol. 29(2): 402–412. https://doi.org/10.3969/j.issn.1674-7968.2021.02.020.

Geng Y. 2018. A preliminary study on the technical system of *Ustilago esculenta* artificial inoculation of unpregnant water bamboo (Master's Diss) [in Chinese]. Yangzhou University, Yangzhou, Jiangsu, China.

Hu N, Tu XR, Li KT, Ding H, Li H, Zhang HW, Tu GQ, Huang L. 2017. Changes in protein content and chitinase and β-1,3-glucanase activities of rice with blast resistance induced by Ag-antibiotic 702. Plant Dis Pests. 8(4):33–36. https://doi.org/CNKI:SUN:PDSP.0.2017-04-009.

Jiang HH, Jin XY, Shi XF, Xue YF, Jiang JY, Yuan CL, Du YJ, Liu XD, Xie RF, Liu XM, Li LJ, Wei LJ, Zhang CX, Tong LJ, Chai YR. 2020. Transcriptomic analysis reveals candidate genes responsive to *Sclerotinia sclerotiorum* and cloning of the Ss-inducible chitinase genes in *Morus laevigata.* Int J Mol Sci. 21(21):8358. https://doi.org/10.3390/ijms21218358.

Jiang JZ, Zhang Q, Cao PS. 2007. Changes of protective enzyme activity during the expansion of succulent stems of *Zizania latifolia* [in Chinese]. Jiangsu Agric Sci. 3:93–95.

Jiang L, Jin P, Wang L, Yu X, Wang HY, Zheng YH. 2015. Methyl jasmonate primes defense responses against *Botrytis cinerea* and reduces disease development in harvested table grapes. Scientia Hortic. 192:218–223. https://doi.org/10.1016/j.scienta.2015.06.015.

Jose RC, Bengyella L, Handique PJ, Talukdar NC. 2019. Cellular and proteomic events associated with localized formation of smut-gall during *Zizania latifolia—Ustilago esculenta* interaction. Microb Pathog. 126:79–84. https://doi.org/10.1016/j.micpath.2018.10.028.

Kakhki RK, Neshani A, Sankian M, Ghazvini K, Hooshyar A, Sayadi M. 2019. The short-chain dehydrogenases/reductases (SDR) gene: A new specific target for rapid detection of *Mycobacterium tuberculosis* complex by modified comparative genomic analysis. Infect Genet Evol. 70:158–164. https://doi.org/10.1016/j.meegid.2019.01.012.

Kanjanapongkul K, Baibua V. 2021. Effects of ohmic pasteurization of coconut water on polyphenol oxidase and peroxidase inactivation and pink discoloration prevention. J Food Eng. 292:110268. https://doi.org/10.1016/j.jfoodeng.2020.110268.

Khan A, Nasir IA, Tabassum B, Aaliya K, Tariq M, Rao AQ. 2017. Expression studies of chitinase gene in transgenic potato against *Alternaria solani.* Plant Cell Tissue Organ Cult. 128(3):563–576. https://doi.org/10.1007/s11240-016-1134-y.

Li F, Zhang JF, Zhong HY, Chen JM. 2022. Germicide fenaminosulf promots gall formation of *Zizania latifolia* without directly affecting the growth of endophytic fungus *Ustilago esculenta.* BMC Plant Biol. 22(1):418. https://doi.org/10.1186/S12870-022-03803-6.

Li RL. 1998. Biochemistry experiment [in Chinese]. Wuhan University Press, Wuhan, Hubei, China.

Li S. 2016. Cloning and expression analysis of resistance genes *Zl-RPM1* and *Zl-ADR* in *Zizania latifolia* (Master's Diss) [in Chinese]. China Jiliang University, Hangzhou, Zhejiang, China.

Liu Q, You WY, Yu XP, Ruan SL, Cui HF, Ma HS, Ye ZH. 2011. A preliminary study on proteome variations associated with gall formation in *Zizania latifolia* Turcz. Plant Mol Biol Rpt. 29(2):360–368. https://doi.org/10.1007/s11105-010-0236-7.

Liu YR. 2013. Germplasm screening for high chitinase activity in common wheat (*Triticum aestivum* L.) (Master's Diss) [in Chinese]. Anhui Agricultural University, Hefei, Anhui, China.

Redkar A, Jaeger E, Doehlemann G. 2018. Visualization of growth and morphology of fungal hyphae in planta using wga-af488 and propidium iodide co-staining. Bio. 101:e2942. https://doi.org/10.21769/BioProtoc.2942.

Saikkonen K, Wäli P, Helander M, Faeth SH. 2004. Evolution of endophyte–plant symbioses. Trends Plant Sci. 9(6):275–280. https://doi.org/10.1016/j.tplants.2004.04.005.

Sakhuja M, Zhawar VK, Pannu PPS. 2021. Regulation of antioxidant enzymes by abscisic acid and salicylic acid under biotic stress caused by *Fusarium fujikuroi* in rice. Indian Phytopathol. 74:823–830. https://doi.org/10.1016/j.tplants.2004.04.005.

Syed BA, Patel M, Patel A, Gami B, Patel B. 2021. Regulation of antioxidant enzymes and osmo-protectant molecules by salt and drought responsive genes in *Bambusa balcooa.* J Plant Res. 134(1):165–175. https://doi.org/10.1007/s10265-020-01242-8.

Toufiq N, Tabassum B, Bhatti MU, Khan A, Tariq M, Shahid N, Nasir IA, Husnain T. 2018. Improved antifungal activity of barley-derived chitinase I gene that overexpress a 32kDa recombinant chitinase in *Escherichia coli* host. Braz J Microbiol. 49(2):414–421. https://doi.org/10.1016/j.bjm.2017.05.007.

Tu ZH, Yamada S, Hu D, Ito Y, Iwasaki T, Yamaguchi A. 2019. Microbial diversity in the edible gall on white bamboo formed by the interaction between *Ustilago esculenta* and *Zizania latifolia.* Curr Microbiol. 76(7):824–834. https://doi.org/10.1007/s00284-019-01693-w.

Tuan LNA, Du BD, Ha LDT, Dzung LTK, Phu DV, Hien NQ. 2019. Induction of chitinase and brown spot disease resistance by oligochitosan and nanosilica–oligochitosan in dragon fruit plants. Agric Res. 8(2):184–190. https://doi.org/10.1007/s40003-018-0384-9.

Tyagi S, Shumayla, Verma PC, Singh K, Upadhyay SK. 2020. Molecular characterization of *ascorbate peroxidase* (APX) and *APX-related* (*APX-R*) genes in *Triticum aestivum* L. Genomics. 112(6): 4208–4223. https://doi.org/10.1016/j.ygeno.2020.07.023.

Wang F, Yang S, Wang YS, Zhang B, Zhang F, Xue H, Jiang Q, Ma Y. 2021a. Overexpression of chitinase gene enhances resistance to *Colletotrichum gloeosporioides* and *Alternaria alternata* in apple (*Malus* × *domestica*). Scientia Hortic. 277(3):109779. https://doi.org/10.1016/j.scienta.2020.109779.

Wang J, Bai YJ, Feng ZS, Zhu WT, Zeng YRK, Jiang LY. 2021b. Gene expression and activities of pathogenesis-related enzymes in postharvest muskmelons infected with *Alternaria alternata.* Shipin Kexue. 42(14):94–102. https://doi.org/10.7506/spkx1002-6630-20200228-313.

Wang ZD, Yan N, Wang ZH, Zhang XH, Zhang JZ, Xue HM, Wang LX, Zhan Q, Xu YP, Guo DP. 2017. RNA-seq analysis provides insight into reprogramming of culm development in *Zizania latifolia* induced by *Ustilago esculenta.* Plant Mol Biol. 95(6):533–547. https://doi.org/10.1007/s11103-017-0658-9.

Wang ZH, Yan N, Luo X, Guo SS, Xue SQ, Liu JQ, Zhang JZ, Guo DP. 2020. Gene expression in the smut fungus *Ustilago esculenta* governs swollen gall metamorphosis in *Zizania latifolia.* Microb Pathog. 143:104107. https://doi.org/10.1016/j.micpath.2020.104107.

Waszczak C, Carmody M, Kangasjärvi J. 2018. Reactive oxygen species in plant signaling. Annu Rev Plant Biol. 69(1):209–236. https://doi.org/10.1146/annurev-arplant-042817-040322.

Wu XY. 2018. The effect of different temperature treatments on seed germination and seedling physiological and biochemical indexes of *Ulmus parvifolia* (Master's Diss) [in Chinese]. Shandong Agricultural University, Tai'an, Shandong, China.

Yan N, Wang XQ, Wang ZD, Zhang YL, Xue HM, Guo DP. 2013. Antioxidative system and chlorophyll fluorescence of *Zizania latifolia* Turcz. Plants affected by *Ustilago esculenta* infection (in Chinese). Acta Ecol Sin. 33(5):1584–1593. https://doi.org/10.5846/stxb201208151148.

Yang F. 2018. Establishment of regeneration system of *Zizania latifolia in vitro* and study on inoculation of *Ustilago esculenta* (Master's Diss) [in Chinese]. Anhui Agricultural University, Hefei, Anhui, China.

Yu XT, Chu MJ, Chu C, Du YM, Shi J, Liu XM, Liu YH, Zhang HB, Zhang ZF, Yan N. 2020. Wild rice (*Zizania* spp.): A review of its nutritional constituents, phytochemicals, antioxidant activities, and health-promoting effects. Food Chem. 331:127293. https://doi.org/10.1016/j.foodchem.2020.127293.

Zhan MD. 2017. Isolation and purification of two endophytic fungi in *Zizania latifolia* and optimization of culture conditions (Master's Diss) [in Chinese]. Anhui Agricultural University, Hefei, Anhui, China.

Zhang DD, Yun XF, Bao YY, Qian C, Gao XM. 2013. Effect of extracts celery fresh root and rhizosphere area on the chitinase and beta-1.3-glucan enzymes activity of cucumber leaf (in Chinese). Neimenggu Nongye Daxue Xuebao Ziran Kexue Ban. 34(4):21–27. https://doi.org/10.16853/j.cnki.1009-3575.2013.04.005.

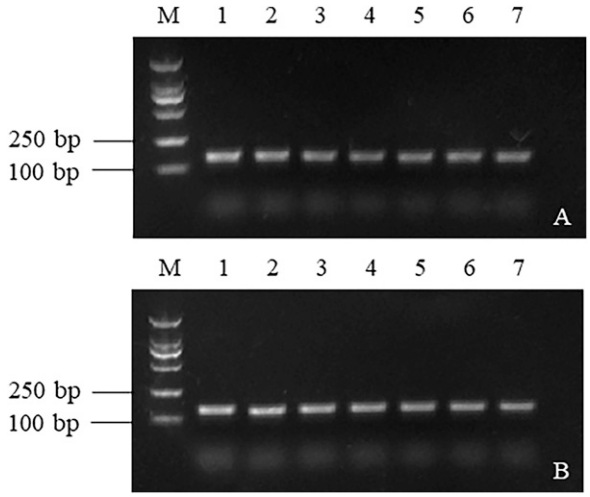
Zhang JP. 2020. Early molecular detection and identification of differential expressed proteins of adzuki bean infected with *Uromyces vignae* (Master's Diss) [in Chinese]. Heilongjiang Bayi Agricultural University, Daqing, Heilongjiang, China.

Zhang ZP, Song SX, Liu YC, Zhu XR, Jiang YF, Shi LT, Jiang JZ, Miao MM. 2021. Mixed transcriptome analysis revealed the possible interaction mechanisms between *Zizania latifolia* and *Ustilago esculenta* inducing jiaobai stem-gall formation. Int J Mol Sci. 22(22): 12258–12258. https://doi.org/10.3390/IJMS222212258.

Zhao KH, Xiao ZP, Zeng JG, Xie HQ. 2021. Effects of different storage conditions on the browning degree, PPO activity, and content of chemical components in fresh *Lilium* bulbs (*Lilium brownii* F. E. Brown var. *viridulum* Baker.). Agriculture. 11(2):184. https://doi.org/10.3390/agriculture11020184.

Zhou MQ. 2018. Cloning and regulation of PIN1 gene among different development stages of swollen stem in *Zizania latifolia* (Master's Diss) [in Chinese]. China Jiliang University, Hangzhou, Zhejiang, China.

Zhou NN, An YL, Gui ZC, Xu SS, He XM, Gao J, Zeng DL, Gan DF, Xu WJ. 2020. Identification and expression analysis of chitinase genes in *Zizania latifolia* in response to abiotic stress. Scientia Hortic. 261(4):108952. https://doi.org/10.1016/j.scienta.2019.108952.



Supplemental Fig. 1. Electrophoretic image (2.0% agarose gel) of polymerase chain reaction amplification of *ZlActin* (primer sequences listed in Supplemental Table 1) from treated and control samples of *Zizania latifolia.* M = DNA markers. (**A**) Male *Z. latifolia* inoculated with potato dextrose liquid medium; (**B**) male *Z. latifolia* plants inoculated with *U. esculenta*; lanes 1 – 7 = 3, 6, 12, 18, and 24 h, and 3 and 5 d after treatment.

Supplemental Table 1. Basic information on *ZlChi*s in *Zizania latifolia*, and primer sequences used for real-time fluorescence quantitative polymerase chain reaction.

		Primers	
Gene name	Sequence ID	Forward primer	Reverse primer
ZlChi1	Zlat_10003182	GGGCATATCCAACAATGGCGAACT	CCGGAGATGAGGAGCACTAA
ZlChi2	Zlat_10047807	GGAAAGCTCAGCCATCGAGA	GAGGAGTGGGAGAAAGCGAAGT
ZlChi3	Zlat_10012767	GGTTCTGCTGCAACGAGAC	GGTGATGAAGGCCTGGTAGT
ZlChi4	Zlat_10016842	CTCCGAAGACGACTCCAAG	CGATCTCCTCGATGTAGCAG
ZlChi5	Zlat_10017446	GCTGCACCGATGTACAGATTG	CTTGTGCAGGAACAGCGAC
ZlChi6	Zlat_10048858	CGCTCTGGTTCTGGATGA	ATCTGTTGCCGTTGCACT
ZlChi7	Zlat_10029194	GACCGCATCGGCTACTACAAG	CTGGTTGTAGCAGTCGAGGTT
ZlChi8	Zlat_10031220	TTACTGCGACGCCACTGACA	CCCGTAGTTGAAGTTCCATGAG
ZlChi9	Zlat_10048857	ATGATGTCGTTCCTGGCTC	CTTGAAGAACGCCTCAGTG
ZlChi10	Zlat_10004398	CCCTGTCGAACAAACCACTGGAAAC	CCCCTGCGATCATCATGCCAAAT
ZlChi11	Zlat_10041954	ATGGTTCATCGCACTTCC	GGTGAGACACGTTCGTGAACAT
ZlActin 2	LOC102711684	TAACCGGCCACGTGTATTTA	AGAGCAGAGGCATTCCAAGT
Ue-actin	KU302684.1	CAATGGTTCGGGAATGTGC	GGGATACTTGAGCGTGAGGA