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Change of Defensive-related Enzyme in Wheat Crown Rot Seedlings Infected by *Fusarium graminearum*

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Crown rot caused by *Fusarium* pathogens is one of the most economically destructive diseases of wheat. The objective of this study was to investigate defense-related enzyme responses in wheat seedlings infected with *Fusarium graminearum.* Three wheat varieties were employed to the experiment. Physiological analysis of varieties indicated that defensive-related enzymes were induced by *F. graminearum* infection. The superoxide dismutase and peroxidase activities increased dramatically and the phenylalanine ammonia-lyase activity was stimulated to a higher level in resistant variety Hongyouzi than in susceptible variety Wo0102. However, the chitinase activity was higher in susceptible variety Wo0102 than in Hongyouzi after infection. These results suggest that the defense enzymes could be stimulated in wheat seedlings by *Fusarium* infection.

Keywords: crown rot, *Fusarium graminearum*, wheat, plant defense

Introduction

Fusarium pathogens cause two serious diseases in wheat: *Fusarium* head blight (FHB) and crown rot (CR). *Fusarium* head blight, causing severe losses in yield and grain quality worldwide (Bai et al. 2001), which has been intensively studied internationally, whereas crown rot is reported more restricted in its geographical distribution (Wildermuth et al. 2001). Crown rot occurs on seedling or older plants, with typical symptoms of brown to red-brown rot at a lower stem during seedling stage or white heads at maturity (Wildermuth et al. 2001), respectively. In Australia, crown rot is the second most economically devastating wheat disease and is distributed over a large geographical region covering most of the wheat belt (Mitter et al. 2006). Symptoms of crown rot have also been reported in other areas (Akinsanmi et al. 2004). These two diseases have been recorded in the same field in Australia and the Southern Cape Province of South Africa (Marasas et al. 1988). The expression and severity of the crown rot disease are promoted by the hot and dry weather at anthesis and crop maturation (Mitter et al. 2006). However, the mechanisms of infection and host resistance to crown rot are unknown.

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In plants, it was known that resistance to microbial pathogens was often associated with defense related enzymes (Bol et al. 1990). Resistance and defense enzymes expression could be induced at the site of pathogen infection (Jetiyanon 2007). Particularly, the involvement of the peroxidase (POD), phenylalanine ammonia-lyase (PAL) and chitinase in plant resistance against pathogens were extensively demonstrated (Joosten and Witt 1989; Jetiyanon 2007). It was also reported that the reactive oxygen species generated were usually rapidly dismutated via superoxide dismutase (SOD) catalysis to H_2O_2 during early plant response to pathogen infection (Brisson et al. 1994; Mittler 2002). Despite the severity of CR disease of wheat in some countries, very little is known about the defense responses activated following pathogen infection. Therefore, the objective of this study was to investigate the plant defense mechanisms against CR pathogen infection by analyzing the activity of defensive-related enzymes in wheat after the inoculation of *F. graminearum*.

Materials and Methods

Plant materials and preparation of spore suspensions

Three wheat varieties including Hongyouzi, Sumai3 and Wo0102, selected from 200 wheat varieties after CR test, and representing high resistant, moderate susceptible and susceptible materials respectively, were employed in this experiment. Among them, Hongyouzi and Wo0102 are landraces from Shanxi and Anhui in China, while Sumai 3 is a FHB resistant source derived from the cross of Funo/Taiwan wheat and released by Jiangsu Academy of Agricultural Sciences. The inoculum of *F. graminearum* F15 was isolated from the field in China and had been well characterized for its strong virulence (Zhang et al. 2004). The inoculum was according to Mitter et al. (2006). Each treatment was repeated three times and 10 replicates arranged in a randomized block design.

Evaluation of crown rot severities and statistical analysis

CR disease severity was evaluated by scoring individual seedling using a 0–5 scale modified from that of Wildermuth and McNamara (1994) at 35th days after inoculation. The scale was based on the length of leaf sheath affected by disease relative to the total seedling height, where 0 = no obvious symptom, 1 = 1–10% necrosis lesion; 2 = 11–25% necrosis lesion; 3 = leaf sheath 25–50% necrosis; 4 = leaf sheath 51–75% necrosis; 5 = leaf sheath necrosis greater than 75% with severe reduction of seedling height. Disease severity index expressed as a proportion (in percentage) of every plant was computed using the following formula:

Disease severity index = [Σsum of disease rating/(total number of ratings × maximum disease rating)] × 100. The scale of resistance to susceptible as follow: High resistance, disease severity index (DSI) 1%–10%; resistance, DSI 10%–25%; Middle susceptible, DSI 25%–50%; Susceptible, DSI 50%–75%. Data were subjected to one-way analyses of variance (ANOVA) and mean was performed using the least significant difference (LSD) test ($p = 0.05$).

Crude enzyme extraction and activity assay

In order to study the defensive related enzyme responses on crown rot seedling, two cultivars, Hongyouzi and Wo0102 were used in next experiments. Fresh wheat stems were flash-frozen in liquid nitrogen, crushed into a fine powder in a mortar with a pestle. They were homogenized in 2 ml of a pre-chilled 0.1 M Tris–HCl buffer, pH 7.0 containing 1% polyvinyl-polypyrrolidone. The homogenate was centrifuged at 12,000 *g* in a refrigerated tabletop centrifuge for 15 min at 4°C. The supernatant to be used for the enzymatic activity assay was transferred to a 1.5 ml vial and stored at –20°C. The standard Bradford assay was employed to test the protein concentration for plant extracts in each sample.

Superoxide dismutase activity (SOD)

All extracts were tested for SOD activity using the riboflavin/methionine system (Beauchamp and Fridovich 1971). Total SOD activity was assayed spectrophotometrically at 560 nm. One unit of SOD activity is defined as the amount that inhibits nitroblue tetrazolium photoreduction by 50% under the assay conditions.

Detection of peroxidase activity

All extracts were tested for peroxidase activity using guaiacol as the hydrogen donor. Procedures were modified from Hammerschmidt et al. (1982). The 1 ml reaction mixture in 1.5 ml cuvette contained 0.25% (v/v) guaiacol in 0.01M sodium phosphate buffer (pH 6.0), enzyme extract, and 0.1M H_2O_2. Enzyme extract was added last to initiate the reaction. The changes in absorbance at 470 nm were recorded at 30 s intervals for 3 min. The enzyme activity was expressed as changes in the absorbance min^{-1} mg $protein^{-1}$.

Peroxidase isoenzyme was also detected after native PAGE (polyacrylamide gel electrophoresis) performed on a 0.1 mm polyacrylamide gel. After electrophoresis, the gels were soaked for 10 min in 50 mM Tris buffer, pH 7.4, then incubated with 0.46% (v:v) guaiacol and 13 mM H_2O_2 in the same buffer at room temperature until red bands appeared.

Detection of phenylalanine ammonia-lyase (PAL) activity

Frozen roots (1 g) were ground in a mortar under liquid nitrogen and homogenized in 10 ml 0.1 M sodium borate buffer (SBB) (pH 8.8) containing 10 mM β-mercapto-ethanol and 1% PVPP. Samples were homogenized by shaking in an ice bath for 1 h. Crude extracts were then centrifuged at 12,000 rpm for 20 min at 4ºC. Reaction mixtures containing 0.75 ml enzyme extract, 1.25 ml SBB and 0.5 ml SBB plus 0.05 M L-phenylalanine were incubated in a 40ºC water bath for 1 h. The reaction was stopped with 0.1 ml 6 N HCl. Phenylalanine ammonia-lyase activity was determined by measuring absorbance at 290 nm. The production of *trans*-cinnamic acid formed from the substrate L-phenylalanine per hour at 40ºC was measured by the absorbance change at 290 nm. PAL activities were expressed as Δ absorbance (290 nm) min^{-1} gram fresh $weight^{-1}$.

Detection of chitinase activity after inoculation

Chitinase activity was assayed by measuring the amount of the reducing end group, GlcNAc (N-acetyl-β-D-glucosamine), produced from colloidal chitin (Shi et al. 2007). The mixture consisting 0.3 mL of enzyme extract; 0.2 mL of 1.0% colloidal chitin was incubated at 37ºC for 1 h, the mixture was then inactivated in boiling water for 5 min. After centrifugation at 5000 rpm for 10 min, 0.2 mL supernatant liquid were kept in boiling water for 3 min. Afterwards, 0.1 mL potassium borate solution (0.8 M) and 3 mL 1% DMAB were added. After incubation at 37ºC for 20 min, the value of absorbency for each treated sample was detected at 544 nm using a spectrophotometer. The activity was calculated from a standard curve based on known concentrations of N-acetyl-β-D glucosamine. The chitinase activity was defined as the amount of liberated GlcNAc hour^{-1} gram fresh weight^{-1} (Boller and Metraux 1988; Shi et al. 2007).

Statistical analysis

All data were analyzed by analysis of variance (ANOVA) and the treatment means were separated using Fisher's protected least significant difference (LSD) test using SAS software (SAS Institute, Gary, NC, USA).

Results

Crown rot severity after inoculation with F15

The severity of crown rot was evaluated by inoculated seedlings of *F. graminearum* on three genotypes, Hongyouzi, Sumai3 and Wo0102. Typical symptoms appeared two weeks after inoculation and increased lesion at different rates in different varieties. Differences in crown rot severities were obvious among the three genotypes (Fig. 1). The results

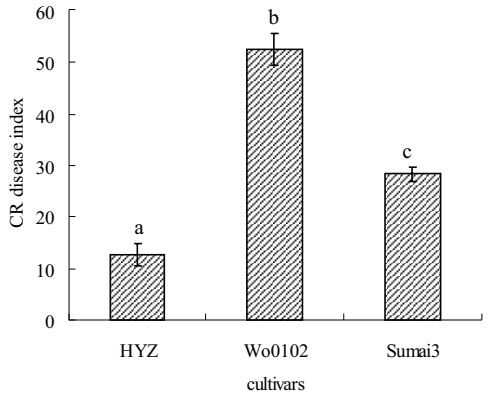

Figure 1. Crown rot disease index of wheat cultivars at 35 days after inoculation (DAI)

The assess cultivars were Hongyouzi (HYZ), Wo0102 and Sumai3. Vertical bars represent standard deviations from mean of 5 replications. Mean values followed by different letters in a column are significantly different

showed that the resistant variety had a small lesion after *F. graminearum* inoculation. Disease severity index for the resistant genotype Hongyouzi was only 12.6, whereas the susceptible genotype Wo0102 was 52.5. The FHB resistant genotype Sumai3 showed to be moderate susceptible to crown rot (Fig. 1).

Changes of SOD activity of infected wheat plant

The SOD activities of treated plants were not different significantly between the controls throughout the assays (Fig. 2). However, the total SOD activity was generally higher in Hongyouzi than in the susceptible genotype Wo0102 at 3 days after inoculation. Compared to the susceptible genotype Wo0102, SOD activity in Hongyouzi increased significantly from 24.4% to 38.5% (Fig. 2).

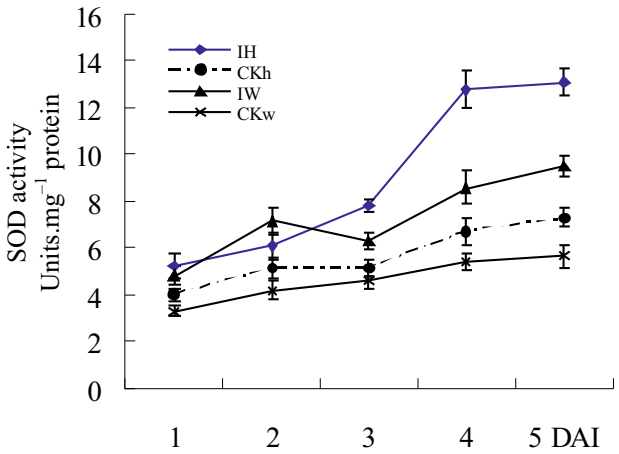

Figure 2. Influence of infected plants on superoxide dismutase activity before and after inoculation with F15 days after inoculation (DAI)

IH, infected Hongyouzi (♦), IW, infected Wo0102 (þ), CKh, non-infected healthy control of Hongyouzi (—) and CKw, non-infected control of Wo0102 (×), each value at particular sampling time is the mean from three independent experiments. Each value is the mean±S.E. for $n = 3$

Changes of peroxidase (POD) activity in infected wheat plant

The activities of POD were significantly ($P = 0.05$) increased at two days after inoculation with *F. graminearum* and reached to the highest value at four days in Wo0102. However, the activity of IH was still increasing after 4th day. In addition, the activity of the POD after inoculation with *F. graminearum* was always higher than control (Fig. 3A). Native PAGE assay detected seven isomers in IH and five isomers in IW at 5th day. The new isozymes (Rf = 0.32, 0.55) appeared in IH on 5th day. The intensities of band 1 (Rf = 0.11), and 2 (Rf = 0.19) were more stronger than those in non-inoculated plants and the activity of band 1 (Rf = 0.11) in IH increased significantly compared to others. It seems that the bands of

Rf = 0.19, 0.26, 0.48 and 0.51 existed in non-inoculated plants but the new bands Rf = 0.11 and 0.55 were induced from *F. graminearum* infection.

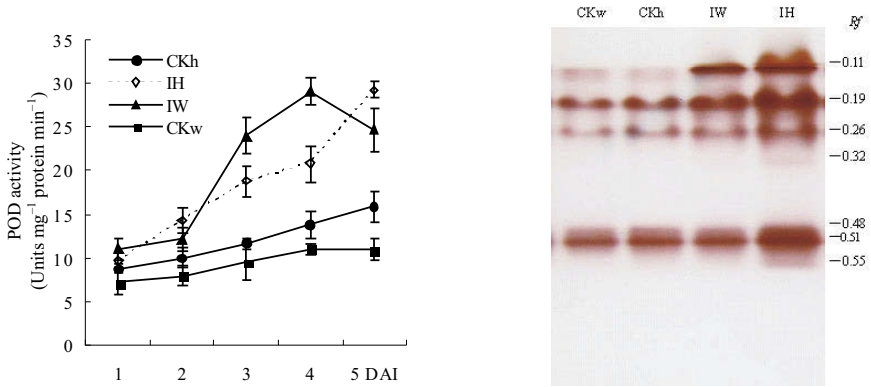

Figure 3. Influence of F15 on total peroxidase (POD) activity in wheat seedlings days after inoculation (DAI)

A, activity of POD in seedling, B, pattern of POD isozymes at five days after inoculation and control plants by native PAGE (12.5%).

IH, infected Hongyouzi (¯), IW, infected Wo0102 (ɒ), CKh, non-infected healthy control of Hongyouzi (—) and CKw, non-infected control of Wo0102 (¾), each value at particular sampling time is the mean from three independent experiments. Each value is the mean ± S.E. for $n = 3$.

Rf, distance of protein migration divided by distance of migration of the dye front

Change of PAL activity following F15 inoculation

The activation of PAL activity is an early, common and important response of plant to biotic and abiotic stresses. As shown in Figure 4, the activities of PAL in the two non-infected genotypes were low between different time-frames was not significant. However, after inoculation with *F. graminearum*, the PAL activity in two of the three varieties increased. The increase in IH from the second day was increased rapidly, and the levels of PAL activity remained higher than that in the susceptible genotype Wo0102. There was a significant difference ($P = 0.05$) between the two genotypes.

Change of chitinase activity following F15 inoculation

Chitinase activity was measured using colloidal chitin as substrates at five days after inoculation (Table 1). As shown in Table 1, the chitinase activities significantly enhanced in both Hongyouzi and Wo0102 after inoculation, and its activities in IW were significantly higher than those in IH (Table 1). However, the percent of increase of IW was higher than that of IH. This seems to suggest that the increase of chitinase activity might be a response to disease.

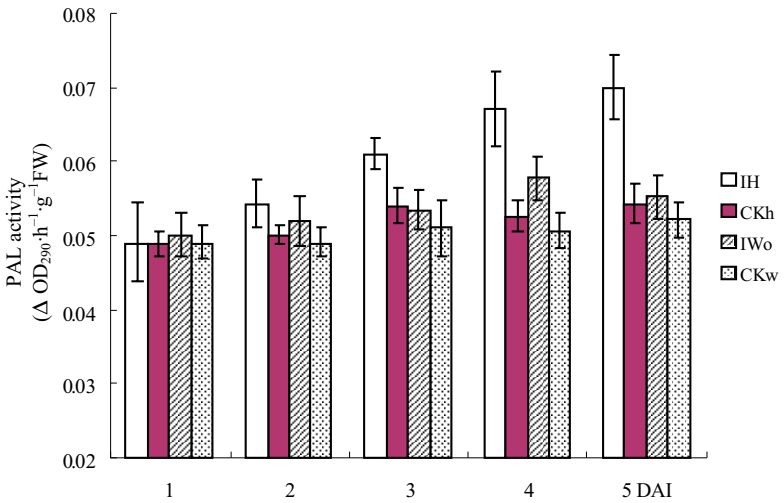

Figure 4. Change of PAL activity in wheat seedling days after inoculation (DAI) with F15

Infected Hongyouzi (IH), infected Wo0102 (IW_o), non-infected healthy control of Hongyouzi (CKh) and non-infected control of Wo0102 (CKw), each value at particular sampling time is the mean from three independent experiments. Vertical bars represent standard deviations from mean of 10 replications. Each value is the mean ± S.E. for $n = 3$

Table 1. Chitinase activity in wheat seedlings from Hongyouzi and Wo0102 five days after inoculation with F15

Treatment	Chitinase activity (mg D-GlcNAc·g^{-1} FW·h^{-1})	Percentage of increase (%)
CKh	1.32 ± 0.06 a	62.8 ± 1.7 a
IH	2.15 ± 0.12 b	
CKw	1.24 ± 0.08 a	144.3 ± 3.8 b
IW	3.03 ± 0.15 c	

Note: chitinase activity was carried out five days after the inoculation. For IH (Infected Hongyouzi), IW (infected Wo0102), CKh (non-infected healthy control of Hongyouzi) and non-infected control of Wo0102 (CKw), each value represents the mean from three independent experiments with three replicates each. Every enzymatic extract was measured three times. Mean values with standard deviation followed by different letters in a column are significantly different (a = 0.05).
Percentage of increase was determined from chitinase activity = [(Inoculation–Control)/Control × 100].

Discussion

The defensive response plays an important role in disease resistance. Identifying induced enzymes such as chitinase, peroxidase, PAL and chitinases could further elucidate plant defense responses (Desmond et al. 2006). Accumulations of some hydrolases have been reported in great detail in dicotyledonous plants, suggesting a direct role in plant defense

mechanisms against pathogens (Benhamou 1996; Desmond et al. 2006). On the other hand, the hydrolysis of fungal cell walls by chitinase releases small fragments that can act as elicitors. Elicitors can activate plant defense response such as induction of pathology related-proteins (Roldán Serrano et al. 2007). We found in this study that chitinase activities increased in both susceptible and resistant plants after *F. graminearum* inoculation (Table 1), that there was significant difference between the two genotypes used, and that the susceptible genotype Wo0102 had the higher activity (Table 1). This could indicate that the change of these enzymatic activities was a consequence of an altered metabolism of plants after infection. In addition, the high peroxidase activities are usually associated with later stages of the infection process and are linked to lignifications and generation of hydrogen peroxides that inhibit pathogens directly or generate other free radicals with antimicrobial effects (Benhamou 1996). We demonstrated in this study that SOD, POD and PAL activities increased in inoculated seedlings compared with controls (Figs 2–4), indicated that they play an important role in ameliorating damage to plant caused by pathogens in the partially resistance variety Hongyouzi. The novel POD isozymes appeared in IH on 5th day could also account for the observed delay in disease symptom development in Hongyouzi after infection with *F. graminearum* (Fig. 3B). It showed the biotic inducers may evoke transcriptional activation of plant defense and the triggering factor produced by inoculation is essential to further enhance synthesis and accumulation of defense products of SOD, POD and PAL (Benhamou 1996; Desmond et al. 2006).

Attention should be also paid to the differences among varieties to crown rot resistance. It is to be noticed that the wheat cultivar Sumai3, which is highly resistant to FHB, is widely used in the world and its resistance has been the best characterized, while results from the present study indicates that this cultivar was not highly resistant to crown rot. This implies that different mechanisms may be involved between FHB and CR resistance to *F. graminearum*. In addition, it has been reported that there were different resistant types to FHB among wheat germplasm, however, it is still unknown whether there are different resistant types to crown rot. In our study, we found that there are significant differences to crown rot resistance among wheat germplasm (data not shown). This is encouraging considering that there are some resistant resources in wheat germplasm by screening large-scale wheat germplasm, and the resistance types, if presence, should be studied in the future.

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