An extracellular Zn-only superoxide dismutase from *Puccinia striiformis* confers enhanced resistance to host-derived oxidative stress

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Summary

Accumulation of reactive oxygen species (ROS) following plant-pathogen interactions can trigger plant defence responses and directly damage pathogens. Thus, it is essential for pathogens to scavenge host-derived ROS to establish a parasitic relationship. However, the mechanisms protecting pathogens from host-derived oxidative stress remain unclear. In this study, a superoxide dismutase (SOD) gene, *PsSOD1*, was cloned from a wheat—*Puccinia striiformis* f. sp. *tritici* (*Pst*) interaction cDNA library. Transcripts of *PsSOD1* were up-regulated in the early infection stage. Heterologous mutant complementation and biochemical characterization revealed that *PsSOD1* encoded a Zn-only SOD. The predicted signal peptide was functional in an invertase-mutated yeast strain. Furthermore, immunoblot analysis of apoplastic proteins in *Pst*-infected wheat leaves and bimolecular fluorescence complementation suggested that *PsSOD1* is a secreted protein that potentially forms a dimer during *Pst* infection. Overexpression of *PsSOD1* enhanced *Schizosaccharomyces pombe* resistance to exogenous superoxide. Transient expression of *PsSOD1* in *Nicotiana benthamiana* suppressed Bax-induced cell death. Knockdown of *PsSOD1* using a host-induced gene silencing (HIGS) system reduced the virulence of *Pst*, which was associated with ROS accumulation in HIGS plants. These results suggest that *PsSOD1* is an important pathogenicity factor that is secreted into the host-pathogen interface to contribute to *Pst* infection by scavenging host-derived ROS.

Introduction

Reactive oxygen species (ROS) are by-products of cellular respiration and include superoxide anion (O$_2^-$), hydroxyl radicals (·OH) and hydrogen peroxide (H$_2$O$_2$) (Mittler, 2002). These unstable molecules are produced both as a result of normal aerobic metabolic processes inside the plant cell and in response to abiotic stresses, including drought, heat, high salinity, highlight, osmotic stress and metal toxicity (Perez and Brown, 2014). ROS are also produced and play important roles during pathogenic invasion. ROS are key signalling molecules that trigger a variety of plant defence responses to control or clear pathogen infection (Apel and Hirt, 2004). Additionally, these species can cause direct damage to macromolecules, such as DNA, proteins, and lipids, on or in the pathogens, leading to pathogen death (Fridovich, 1978; Imlay, 2003). Because ROS are highly toxic to pathogens, it is necessary for effective pathogens to scavenge host-derived ROS to establish parasitic relationships.

Superoxide dismutases (SODs, EC 1.15.1.1) constitute the first line of cellular defences against ROS damages that catalyse the conversion of superoxide anions to molecular oxygen and hydrogen peroxide (Fridovich, 1995). These enzymes are widely expressed in prokaryotic and eukaryotic cells and generally utilize metal ions as cofactors. To date, four types of SODs with different metal cofactors have been reported: manganese SOD (Mn-SOD), iron SOD (Fe-SOD), copper/zinc SOD (Cu/Zn-SOD), and nickel SOD (Ni-SOD) (Abreu and Cabelli, 2010; Perry et al., 2010). The number, type, and localization of SOD isozymes vary with the species, developmental stage, and environmental stress conditions. Mn-SOD and Fe-SOD are closely related; their protein structures differ from the other two groups, and they were recently assigned to one family. Mn-SOD and Fe-SOD are prevalent in prokaryotes, mitochondria and chloroplasts.
of eukaryotes and likely evolved from a common ancestor (Miller, 2012). Cu/Zn-SODs are found in some bacteria and in the cytosol of eukaryotes, including yeast, mammals and plants (Pilon et al., 2011). In plants, Cu/Zn-SOD is also localized to the peroxisomes and plastids (Myouga et al., 2008). Ni-SOD has only been found in streptomycetes (Wuerges et al., 2004) and cyanobacteria thus far (Priya et al., 2007).

The fungal SODs identified to date have Cu/Zn or Mn cofactors and are predominantly found in the cytosol or mitochondria. Several Mn-SODs are involved in the survival and the resistance of aerobic fungi to multiple stresses. Knocking out a Mn-SOD gene from Schizosaccharomyces cerevisiae results in rapid death in the stationary phase (Longo et al., 1999) and increases susceptibility to oxidative stress (O'Brien et al., 2004). The Mn-SOD mutants of Schizosaccharomyces pombe are more sensitive to various stresses than wild-type strains (Jeong et al., 2001). In addition, Mn-SODs have also been identified as virulence factors of many fungal pathogens. For example, a mitochondrial Mn-SOD SOD2 is required for Cryptococcus neoformans virulence to mice (Narasipura et al., 2005). Xie et al. (2012) also found that BbSOD2, a cytosolic Mn-SOD, contributes significantly to the virulence and stress tolerance of Beauveria bassiana.

Cu/Zn-SODs are the main contributors to cytosolic SOD activity. Currently, Cu/Zn-SODs are divided into two classes. One type contains intracellular, largely cytosolic Cu/Zn-SODs, and the other group consists of extracellular Cu/Zn-SODs (EC-SODs). EC-SODs are either secreted from cells or attached to the cell wall by a glycophosphatidyl inositol (GPI) anchor with a putative omega site at specific residues (Gleason et al., 2014). Numerous studies have indicated that Cu/Zn-SODs play central roles in fungal growth, pathogenesis and resistance to oxidative stress. For example, Saccharomyces cerevisiae and Candida glabrata lacking SOD1 (a cytosolic Cu/Zn-SOD) both show obvious growth defects and increased sensitivity to redox-cycling drugs, such as menadione (Manfredini et al., 2004; Briones-Martindel-Campo et al. 2015). In Candida albicans, a SOD1 knockout (a cytosolic Cu/Zn-SOD) exhibits attenuated virulence in an animal model of systemic infection (Hwang et al., 2002; Chaves et al., 2007) and is more susceptible to killing by macrophages (Hwang et al., 2002) and polymorphonuclear neutrophils (PMNs) (Chaves et al., 2007). In addition, a GPI-anchored protein, SOD5 (an EC-SOD) from C. albicans, is essential for pathogen defence and has been shown to be critical for combating the oxidative burst of infection (Gleason et al., 2014). The fungal pathogen Histoplasma capsulatum EC-SOD SOD3 facilitates Histoplasma pathogenesis by detoxifying host-derived ROS, promoting Histoplasma survival (Youseff et al., 2012).

Wheat stripe rust is one of the most serious diseases of wheat worldwide. This disease can cause wheat yield losses and pose a threat to food security. Puccinia striiformis f. sp. tritici (Pst), the causal agent of wheat stripe rust, is an obligate biotrophic fungus with a complex life cycle and a series of highly specialized infection structures. The host-pathogen interaction is accompanied by changes in ROS. Previous studies have found an accumulation of O_2^- and H_2O_2 at infection sites in the incompatible interaction between wheat and Pst, but ROS could not be detected in the compatible interaction (Wang et al., 2007). However, little information is available regarding ROS clearance in the wheat-Pst interaction. In this study, a Pst SOD gene (PsSOD1) that was significantly up-regulated in the early infection stage was isolated, and characterized using heterologous expression. Secretion of PsSOD1 was determined during Pst infection of wheat. In addition, over-expression and knockdown of PsSOD1 were performed to identify its functions in the wheat-Pst interaction. Our results indicate that PsSOD1 contributed to Pst infection of wheat by protecting Pst from host-derived oxidative stress.

Results
PsSOD1 encodes a Zn-only SOD
A cDNA containing a full-length gene orthologous to SOD, designated PsSOD1, was isolated from a previously described cDNA library (Ma et al., 2009). Subsequently, the 1062-bp full-length cDNA was obtained using RT-PCR. The open reading frame (ORF) of PsSOD1 consists of 612 nucleotides and is predicted to encode a polypeptide of 203 amino acids with a calculated molecular mass of 21,802 Da, an isoelectric point (pI) of 8.94, and a Cu/Zn-SOD domain. Interestingly, PsSOD1 only contains Zn^{2+} binding sites (H111, T119, E135, and D138) and lacks Cu^{2+} binding sites, which indicates that PsSOD1s not a traditional Cu/Zn-SOD enzyme (Supporting Information Fig. S1B). In addition, signal peptide prediction using SignalP 4.1 revealed that PsSOD1 is a secreted protein (Supporting Information Fig. S1B).

The 203 amino acid sequence was used as a query sequence to search the most up-to-date databases. Homologous proteins from other fungi with the highest similarities to PsSOD1 were identified. The protein sequence showed 56% identity with Cu/Zn-SOD from Puccinia graminis f. sp. tritici CRL 75-36-700-3 (GenBank accession number XP_003326013), 45% identity with Cu/Zn-SOD from Melampsora larici-populina 98AG31 (GenBank accession number XP_007413252), and 34% identity with Cu/Zn-SOD from Puccinia sorghi (GenBank accession number KNZ59826). The phylogenetic analysis of PsSOD1 with homologous proteins from other fungi revealed that PsSOD1 displays greater similarity to Cu/Zn-SODs from basidiomycetous fungi, especially rust fungi, compared with those from ascomycetous fungi (Fig. 1A).

The alignment of PsSOD1 with the three aforementioned Cu/Zn-SODs from other rust fungi is shown in
Supporting Information Fig. S2, displaying significant interspecies variations in the amino acid sequences. Further analysis revealed that homologous proteins of PsSOD1 shown in Fig. 1A contained both Cu²⁺ and Zn²⁺ binding sites (Supporting Information Fig. S3A), or only had Cu²⁺ binding sites (Supporting Information Fig. S3B), which were different from PsSOD1. Thus, PsSOD1 might encode a novel SOD. In addition, genome databases of some representative rust fungi (including Uromyces, Puccinia, and Melampsora) were investigated. The results showed that PsSOD1-like proteins are only present in the stripe rust fungus genome, and other rust fungi do not contain Zn-only SODs. The phylogenetic relationships of PsSOD1 with homologous proteins (Cu/Zn-SODs and Cu-only SODs) from other rust fungi also revealed that PsSOD1 is separated into a new SOD branch (Fig. 1B). These results indicate that PsSOD1 may be a novel SOD that is specific to stripe rust fungi.

PsSOD1 is conserved between Pst isolates

To identify intraspecies polymorphisms in PsSOD1, we compared the coding regions of five sequenced Pst isolates, including a Chinese isolate (CYR32), three US isolates (PST21, PST78 and PST130) and a UK isolate (PST87-7). Compared with the PsSOD1 sequence from
The expression levels of PsSOD1 were calculated by the comparative Ct method with EF-1 as an internal standard. Relative quantifications are compared with the expression levels of non-germinated urediniospores. The means and standard errors were calculated from three replicates. US, urediniospores; Bars indicate the standard deviation of the mean; double asterisks indicate $P < 0.01$.

**PsSOD1 is up-regulated during Pst infection of wheat**

The $PsSOD1$ transcript levels were measured at different developmental stages during $Pst$ infection by quantitative reverse transcription PCR (qRT-PCR). $PsSOD1$ was expressed in ungerminated urediniospores and infected wheat tissues harvested from 6 to 264 h postinoculation (hpi). In ungerminated urediniospores, $PsSOD1$ transcripts were detected at relatively low levels. However, the expression of $PsSOD1$ was sharply up-regulated at 12 hpi and remained constant until 2 days postinoculation (dpi) (Fig. 2). During the later stages of infection in wheat, the expression level of $PsSOD1$ was extremely low (Fig. 2). These results indicate an in-planta induction of $PsSOD1$ in the early stage of $Pst$ infection.

**PsSOD1 largely complements the S. cerevisiae SOD1 mutant**

To identify the function of $PsSOD1$ in $S. cerevisiae$, pDR195 and pDR195-$PsSOD1$ were transformed into a $S. cerevisiae$ SOD1 mutant with obvious growth defects under aerobic conditions. Numerous transformants were obtained on synthetic complete (SC) media without uracil, and they were confirmed by PCR analysis and then sequenced. The transformants displayed identical phenotypes, although only data from transformant CS-1 are presented below.

The growth curves of the $S. cerevisiae$ SOD1 mutant carrying the empty pDR195 vector and the complemented strain CS-1 were monitored in SC media containing glucose or ethanol as the sole carbon source. The results showed a clear increase in the growth rate of the complemented strain CS-1 compared with the $S. cerevisiae$ SOD1 mutant carrying the empty pDR195 vector in SC media with glucose (Fig. 3A). In SC media with ethanol, similar results were observed (Fig. 3B). In addition, the sensitivity of the complemented strain CS-1 to menadione was assayed. As shown in Fig. 3C, the CS-1 strain displayed increased resistance to menadione compared with the control. These results indicate that the expression of the $PsSOD1$ gene in $S. cerevisiae$ largely complements the defects observed in the SOD1 mutant.

**Biochemical characterization of PsSOD1**

The recombinant PsSOD1 was expressed as a soluble protein in cultures of *Escherichia coli* BL21(DE3)plysS transformed with the plasmid pGEX4T-1-$PsSOD1$ following induction by 1 mM IPTG, as shown in the SDS-PAGE profiles (Fig. 4A). The GST-tagged protein was purified to a single protein band by one-step chromatography. The electrophoretic mobility of the expressed GST-PsSOD1 fusion protein was consistent with the predicted molecular weight of approximately 46 kDa (Fig. 4A).

Biochemical characterization of PsSOD1 was then performed. The optimum temperature was approximately 20°C. Higher temperatures resulted in a rapid loss of enzymatic activity (Fig. 4B). The pH optimum was determined to be approximately pH 9.0 (Fig. 4C). In addition, metal cations also showed differential effects on the enzymatic activity of PsSOD1. Inclusion of 0.5 mM Zn$^{2+}$ in the reaction enhanced the enzymatic activity by 85% (Fig. 4D). Addition of Cu$^{2+}$ did not result in obvious changes in enzymatic activity (Fig. 4D), which is consistent with the bioinformatics prediction of PsSOD1. Furthermore, the enzymatic activity of PsSOD1 was inhibited by Fe$^{3+}$ and Mn$^{2+}$ to some extent (Fig. 4D).

PsSOD1 is secreted into the apoplastic fluids of wheat and potentially forms dimers

To functionally validate the SignalP 4.1 predictions, the signal peptide of PsSOD1 was tested using a genetic assay based on the requirement of yeast cells for invertase secretion to grow on raffinose media. As a negative
control, we tested the N-terminus of the Magnaporthe oryzae homologue Mg87, which was not predicted to be secreted. The secretory leader of Avr1b was used as a positive control. The results showed that both the PsSOD1 and Avr1b fused constructs enabled the invertase mutated yeast strain to grow on CMD-W media (yeast can grow without invertase secretion) and YPRAA media (yeast can grow only when invertase is secreted) (Fig. 5A). However, when the Mg87 N-terminus was fused to the truncated invertase, the transformed yeast strains did not grow on YPRAA plates (Fig. 5A). These results confirmed that the signal peptide of PsSOD1 was functional, but the N-terminus of Mg87 was not, which was consistent with the predictions of SignalP 4.1 (Fig. 5A).

Cu/Zn-SOD generally exists as a polymer (Zeinali et al., 2015). To confirm secretion and polymerization of PsSOD1 during Pst infection of wheat, apoplastic proteins from uninfected and Pst-infected wheat leaves were separated by native-PAGE and immunoblotted with an anti-PsSOD1 antibody. The Western blot results for apoplastic proteins from Pst-infected leaves revealed clear differences. For apoplastic proteins from Pst-infected leaves, the specific band (approximately 40 kDa) was observed (Fig. 5B), and the molecular weight was approximately two times higher than that of the PsSOD1 monomer. However, the antibody did not bind to proteins in the apoplastic proteins from uninfected wheat leaves (Fig. 5B). For the positive control, consistent with the native-PAGE results shown in Supporting Information Fig. S5A, the GST-PsSOD1 fusion proteins were clearly observed by the presence of a band of approximately 46 kDa (Fig. 5B), indicating GST-PsSOD1 fusion proteins could not form dimers in spite of dimerization of GST itself (Supporting Information Fig. S5A). In addition, when apoplastic proteins from Pst-infected leaves were separated in the SDS-PAGE gels and immunoblotted, the specific band similar to the PsSOD1 monomer in molecular weight appeared (Supporting Information Fig. S5B). Thus, we concluded that PsSOD1 likely forms dimers when it is secreted into the host.

To confirm the oligomerization of PsSOD1, interactions between PsSOD1 were tested by in planta bimolecular fluorescence complementation (BiFC) experiments in transiently transformed Nicotiana benthamiana leaves. The results showed that strong fluorescence signals were...
observed when agrobacteria carrying pSPYNE(R)173-PsSOD1 or pSPYCE(M)-PsSOD1 were co-infiltrated into *N. benthamiana* leaves (Fig. 5C). However, no fluorescence was observed when agrobacteria containing pSPYNE(R)173-PsSOD1 or the empty vector pSPYCE(M) was co-infiltrated (Fig. 5C), indicating that PsSOD1 might interact with itself and form dimers.

**Overexpression of PsSOD1 enhances stress resistance**

To determine the role of *PsSOD1* in scavenging exogenous superoxide, *S. pombe* cells carrying the recombinant plasmid pREP41-PsSOD1 were challenged in vitro with superoxide. To generate superoxide in vitro, increasing amounts of xanthine oxidase were added to *S. pombe* suspensions in Tris buffer with excess hypoxanthine; the amount of superoxide proportionally increases with the concentration of xanthine oxidase enzyme (data not shown). The results showed that the survival of the *S. pombe* strain containing the recombinant plasmid pREP41-PsSOD1 following the superoxide challenge was significantly increased compared with the control strain carrying the empty pREP41 plasmid (Fig. 6A), indicating that extracellular PsSOD1 may protect *S. pombe* from exogenous superoxide.

In addition, to investigate whether *PsSOD1* functions in suppressing the host defence responses, *PsSOD1* was overexpressed in tobacco using potato virus X (PVX) delivery in combination with Bax, a pro-apoptotic protein from mouse that triggers a hypersensitive response (HR)-like cell death response in plants. When *N. benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* strains individually carrying PVX-PsSOD1, empty vector, or MgCl₂, no cell death was observed (Fig. 6B; circle 1, 4 and 6); tobacco leaves infiltrated with Bax + PsSOD1 (Fig. 6B; circle 2) or Bax only (Fig. 6B; circle 5) both showed a similar cell death phenotype (after 5 days). However, when PsSOD1 was infiltrated prior to Bax for 24 h, cell death was significantly suppressed (Fig. 6B; circle 3).

To confirm that *PsSOD1* and *Bax* were successfully expressed in *N. benthamiana* leaves infiltrated by *A. tumefaciens* carrying different vectors, RT-PCR was performed. As shown in Fig. 6C, *PsSOD1* and *Bax* were both detected after infiltration, either alone or in combination. However, they were not detected after treatment with the empty vector or MgCl₂. These results indicate that *PsSOD1* is related to cell death suppression.

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Fig. 4. Purification and biochemical characterization of PsSOD1 expressed in *E. coli* BL21(DE3)plysS.
A. The SDS-PAGE profiles of PsSOD1 expressed in *E. coli* BL21(DE3)plysS. Lane 1, uninduced *E. coli* cell lysates harbouring pGEX4T-1-PsSOD1; lane 2, *E. coli* cell lysates harbouring pGEX4T-1-PsSOD1 induced by IPTG; lane 3, soluble fractions from the cell culture expressing PsSOD1; lane 4, purified GST-PsSOD1 proteins; M, marker.
B and C. Thermal and pH stability of the purified PsSOD1.
D. Effects of metal ions on the activity of the purified PsSOD1. Asterisks indicate a significant difference (*P* < 0.05) compared with the control using Student’s *t*-test.
Silencing of PsSOD1 by HIGS reduces the virulence of Pst in wheat

Currently, there is no effective transformation system for rust fungi; thus, the HIGS technique mediated by BSMV was used to knockdown the expression of PsSOD1 in Pst. All BSMV-inoculated plants displayed mild chlorotic mosaic symptoms at 9 days postinoculation (dpi), and no obvious defects were observed during subsequent growth (Fig. 7A). In the TaPDS-silenced plants, a bleaching phenotype was observed at 15 dpi (Fig. 7A), indicating that the BSMV-HIGS system functioned correctly. The fourth leaves of the wheat plants were inoculated with CYR32, and the rust disease phenotypes were photographed at 15 dpi. Wheat leaves inoculated with BSMV:PsSOD1 showed...
PsSOD1 protects *S. pombe* cells from exogenous superoxide in vitro. Yeasts were incubated in increasing amounts of superoxide generated by addition of increasing amounts of xanthine oxidase to hypoxanthine. The *S. pombe* wild-type JM837 individually carrying pREP41 and pREP41-PsSOD1 was incubated for 4 h at 37°C, and viable colony forming units (cfu) were determined. The results are plotted as relative yeast survival compared with viable cfu of yeasts incubated in the absence of superoxide (0 mU/ml xanthine oxidase). The results represent the mean from three replicate challenges per strain. Asterisks indicate significant differences (**P < 0.01) compared with the control.

B. Transient expression of PsSOD1 in *N. benthamiana*. Tobacco leaves were infiltrated with *A. tumefaciens* cells carrying *PsSOD1*, an empty vector or Bax alone (circles 1, 4, 5), co-infiltrated with *A. tumefaciens* cells individually carrying *PsSOD1* and Bax (circles 2), or infiltrated with *A. tumefaciens* cells carrying *PsSOD1* and followed 24 h later by a second infiltration of *A. tumefaciens* cells carrying Bax (circles 3). Photos were taken from 3 to 6 days after the second infiltration. 1, *PsSOD1*; 2, *PsSOD1* + Bax (co-infiltration); 3, *PsSOD1* + Bax (infiltration 24 h later); 4, empty vector; 5, Bax; 6, MgCl₂.

C. RT-PCR analysis of *Bax* and *PsSOD1* expression levels in plant tissues treated as described in (B). Total RNA was extracted 60 h after the second infiltration.

**Fig. 6.** Overexpression of *PsSOD1* in different heterologous systems.

A. *PsSOD1* protects *S. pombe* cells from exogenous superoxide in vitro. Yeasts were incubated in increasing amounts of superoxide generated by addition of increasing amounts of xanthine oxidase to hypoxanthine. The *S. pombe* wild-type JM837 individually carrying pREP41 and pREP41-*PsSOD1* was incubated for 4 h at 37°C, and viable colony forming units (cfu) were determined. The results are plotted as relative yeast survival compared with viable cfu of yeasts incubated in the absence of superoxide (0 mU/ml xanthine oxidase). The results represent the mean from three replicate challenges per strain. Asterisks indicate significant differences (**P < 0.01) compared with the control.

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**HIGS of *PsSOD1* impairs fungal growth and increases *H₂O₂* accumulation in the wheat-*Pst* compatible interactions**

To determine how *PsSOD1* is involved in *Pst* pathogenicity, we assessed the fungal development and host response in HIGS plants inoculated with *Pst*. At 24 hpi, the number of hyphal branches, haustorial mother cells and haustoria in the wheat seedlings inoculated with BSMV:*PsSOD1*-as1, *PsSOD1* transcript was reduced by 45%, 80%, and 57% at 24, 48, and 120 hpi with CYR32, respectively; in leaves inoculated with BSMV:*PsSOD1*-as2, the *PsSOD1* transcript was reduced by 58%, 64%, and 41%, respectively, compared with BSMV:*γ*-infected wheat leaves (Fig. 7D). These results indicate that the expression of *PsSOD1* was significantly reduced via BSMV-HIGS.
large in the HIGS plants as the control at 120 hpi (Fig. 8C, H and M). However, uredia size was unchanged, so here we observed an unusual overgrowth of hyphae.

To analyse the host response, H$_2$O$_2$ accumulation was detected using DAB staining. The results showed that H$_2$O$_2$ accumulation was approximately 18-fold higher in the wheat seedlings inoculated with BSMV:PsSOD1-as1 and BSMV:PsSOD1-as2 than the control plants at 24 hpi in the compatible interaction (Fig. 8D and I; Supporting Information Fig. S8D). At 48 hpi, H$_2$O$_2$ accumulation in the HIGS plants was more than four times higher than that of the control plants. (Fig. 8E, J and N).

**Discussion**

Although SODs are known to play an important role in ROS scavenging, few studies have addressed the role of this gene in plant-pathogen interactions. In the present study, a Zn-only SOD gene from Pst (PsSOD1) was cloned for the first time, and its expression and secretion were characterized during Pst infection of wheat. Furthermore, the function of PsSOD1 was identified via heterologous expression and a BSMV-HIGS system in the wheat-Pst interaction. The results indicate that PsSOD1 is a novel SOD specific to stripe rust fungi and contributes to Pst protection against host-derived oxidative stress.

Cu/Zn-SOD was the first antioxidant enzyme to be characterized. Most Cu/Zn-SODs share a signature motif related to the active site (a copper-binding site and a zinc-binding site) (Zelko et al., 2002). Recently, a Cu-only SOD that lacks a zinc site was identified (Gleason et al., 2014). In the present study, PsSOD1 was cloned from Pst, and the protein encoded by PsSOD1 exhibited the greatest similarity to Cu/Zn-SODs from other fungi. It was determined that PsSOD1 is likely a Cu/Zn-SOD. Further domain analysis revealed that this protein only contains the zinc-binding site without the copper-binding site, which differs from the previously identified Cu/Zn-SODs in other species, suggesting that PsSOD1 potentially encodes a novel SOD.
Fig. 8. Histological observation of fungal growth and host response in wheat infected by BSMV-γ and recombinant BSMV after inoculation with CYR32.

A–E. Fungal growth at 24 hpi (A) or 48 hpi (B), infection unit area at 120 hpi (C), H$_2$O$_2$ accumulation at 24 hpi (D) or 48 hpi (E) in BSMV-γ-infected plants.

F–J. Fungal growth at 24 hpi (F) or 48 hpi (G), infection unit area at 120 hpi (H), H$_2$O$_2$ accumulation at 24 hpi (I) or 48 hpi (J) in BSMV:PsSOD1-infected plants. H$_2$O$_2$ accumulation was calculated using DAB staining.

K. The average number of HB, HMC and H decreased significantly in HIGS plants infected by CYR32.

L. Hyphal length, which is the average distance from the junction of the substomatal vesicle and the hypha to the tip of the hypha, was clearly decreased in HIGS plants infected by CYR32.

M. The infection unit area at 120 hpi per infection unit was significantly increased in HIGS plants infected by CYR32.

N. A significant increase in ROS accumulation was observed in CYR32-infected HIGS plants at 48 hpi. Values represent the means ± standard errors of three independent samples. Differences were assessed using Student's t-tests. Asterisks indicate $P < 0.05$. SV, substomatal vesicle; HMC, haustorial mother cell; IH, infection hypha; HB, hyphal branch; H, haustoria.
To confirm this finding, the function of PsSOD1 was identified through complementation of the S. cerevisiae SOD1 mutant. Compared with the control, the complemented strain CS-1 exhibited increased growth rates and higher resistance to menadione. These results indicate that the expression of PsSOD1 in S. cerevisiae could complement the defects observed in the SOD1 mutant. Thus, PsSOD1 and SOD1 display identical functions during this process, which confirmed that the coding product of PsSOD1 is a SOD. In addition, PsSOD1 was biochemically characterized. We found that Zn$^{2+}$ in the reaction significantly increased the enzymatic activity of PsSOD1, but addition of Cu$^{2+}$ had no effect on enzymatic activity, which is consistent with the bioinformatics prediction of PsSOD1. These results further indicate that PsSOD1 is a Zn-only SOD.

Cu/Zn-SODs are widely distributed in the cytoplasm (Fink and Scandalios, 2002), nucleus (Chang et al., 1988), the intermembrane space of mitochondria (Sturtz et al., 2001) and peroxisomes (Keller et al., 1991). In addition, many species have an extracellular Cu/Zn-SOD. Currently, extracellular Cu/Zn-SODs from yeast, nematodes (C. elegans, C. briggsae, and Haemonchus contortus), crustaceans (blue crab, crayfish), and vertebrates (human, rabbit, mouse, rat, pufferfish) have been identified (Landis and Tower, 2005). In this study, PsSOD1 was predicted to be a secreted protein. To confirm this hypothesis, the predicted signal peptide of PsSOD1 was identified using a yeast secretion system. The results showed that the signal peptide of PsSOD1 was functional, demonstrating that PsSOD1 is a secreted protein.

In addition, numerous studies have reported that Cu/Zn-SOD generally exists as a homodimer or homotetramer (Zeinali et al., 2015). To further confirm secretion and polymerization of PsSOD1 in vivo, apoplastic proteins from uninfected and Pst-infected wheat leaves were immunobotted with an anti-PsSOD1 antibody. Interestingly, the specific band (approximately two times higher than that of a PsSOD1 monomer in molecular weight) was observed when the apoplastic proteins from the Pst-infected leaves were separated using native PAGE, whereas the specific band similar to the PsSOD1 monomer in molecular weight appeared when apoplastic proteins from Pst-infected leaves were separated using native PAGE. Therefore, we speculate that the secreted PsSOD1 potentially forms a dimer in the apoplastic fluids of wheat leaves. Subsequent BIFC experiments confirmed that PsSOD1 could interact with itself, further validating the dimerization of PsSOD1. These results are consistent with previous studies in other species, such as E. coli (Wintjens et al., 2008), Salmonella enterica (Ammendola et al., 2008), C. albicans (Fréalle et al., 2005) and humans (McCord and Fridovich, 1969).

Previous reports have shown that the expression of Cu/Zn-SODs can be dramatically regulated by various physiological changes and stress conditions. Cu/Zn-SOD mRNA levels are elevated in response to a wide array of mechanical, chemical, and biological stressors, such as heat shock (Hass and Massaro, 1988), shear stress (Inoue et al., 1996), UVB and X-ray irradiation (Yamaoka et al., 1994; Isoherranen et al., 1997), nitric oxide (Frank et al., 2000), ozone (Rahman et al., 1991), and hydrogen peroxide (Yoo et al., 1999). In the present study, we found that PsSOD1 expression was significantly up-regulated from 12 to 48 hpi. During Pst infection of wheat, ROS accumulation generally peaks at 24 hpi (Wang et al., 2007). However, O$_2^-$ and H$_2$O$_2$ generation is seldom detected in the wheat-Pst compatible interactions (Wang et al., 2007), implying that Pst has a set of effective defence components to scavenge ROS. Thus, it is reasonable that the expression of PsSOD1 was up-regulated before 48 hpi because PsSOD1 is used for scavenging host-derived ROS in the early stage of infection, which promotes infection of Pst by removing ROS stress. In the later stage, ROS could still be detected (Wang et al., 2007; Cheng et al., 2015). These observations led to questions regarding ROS quenching during this period. Voegele et al. (2005) and Link et al. (2005) established the idea that rust fungi use the sugar alcohols mannitol and arabinol for ROS quenching because they found that mannitol and arabinol increased dramatically in Vicia faba leaves infected with Uromyces fabae in the later infection stage and that mannitol and arabinol were sufficient to suppress ROS. Therefore, it is a rational inference that ROS clearance during Pst infection is dependent on SOD in the early stage and later is attributed to sugar alcohols.

Numerous studies have demonstrated that up-regulation of Cu/Zn-SOD expression can enhance resistance to oxidative stress in Histoplasma (Youseff et al., 2012), S. cerevisiae (Fabrizio et al., 2003) and Drosophila melanogaster (Orr and Sohal, 1994). In the present study, PsSOD1 was overexpressed in S. pombe treated with exogenous superoxide. Consistent with previous findings, overexpression of PsSOD1 enhanced S. pombe resistance to exogenous superoxide, indicating that PsSOD1 can be secreted from the S. pombe cells and scavenge exogenous ROS. Similarly, PsSOD1 may play important roles in the wheat-Pst interactions by eliminating host-derived oxidative stress. In addition, PsSOD1 overexpression in tobacco significantly suppressed bax-induced cell death. Therefore, we speculate that overexpression and secretion of PsSOD1 favour Pst infection. On the one hand, ROS quenching in the host-pathogen interface could reduce damage to Pst itself. On the other hand, Pst is a biotrophic fungus that is dependent on the live host. Scavenging ROS may protect host cells from HR-like cell death.
which ensures the smooth progression of nutrient uptake in *Pst*.

SODs are important virulence factors in nearly all pathogenic fungi (Hwang et al., 2002; Cox et al., 2003; Narasipura et al., 2003; Lambou et al., 2010). For example, SOD1 from *C. albicans* and *C. neoformans* directly participates in fungal pathogenicity. Killing of mutant SOD1 cells by macrophages is enhanced in vitro, and fungal virulence is greatly attenuated in vivo (Cox et al., 2003; Narasipura et al., 2003). Disruption of *C. neoformans* SOD1 leads to decreased expression of many *Cryptococcus* specific virulence factors, including laccase, urease and phospholipase (Cox et al., 2003). In the present study, we used a BSMV-HIGS approach to determine the role of *PsSOD1* in the *Pst*-wildtype interaction. The reduced disease symptoms in HIGS wheat seedlings infected by CYR32 suggested that the suppression of *PsSOD1* could reduce the virulence of *Pst*. In addition, *H₂O₂* accumulation was significantly increased, and the fungal development was impeded. Previous studies have shown that ROS, especially *H₂O₂*, act as antimicrobial agents during the plant defence response (Shetty et al., 2008). For example, micromolar concentrations of *H₂O₂* inhibit spore germination of a number of fungal pathogens in vitro (Peng and Kuc, 1992). A concentration of 0.1 mM *H₂O₂* was shown to completely inhibit the growth of cultured bacteria *Pectobacterium carotovorum* subsp. *carotovorum* and result in >95% inhibition of *Phytophthora infestans* growth (Wu et al., 1995). Shetty et al. (2007) used in vitro experiments to show that 5 mM *H₂O₂* could inhibit the development of an inoculum from 4-day-old *Septoria tritici* cultures, whereas a concentration of approximately 50 mM was required to inhibit an inoculum from 16-day-old cultures. Thus, we concluded that *H₂O₂* accumulation at the host-pathogen interface in HIGS plants could restrict fungal development during *Pst* infection, resulting in a reduction in the number of uredia.

Although the spread of the secondary hyphae was limited in the early stage of *Pst* infection, the hyphae grew faster with the progression of *Pst* infection. The average infection area per infection site in the HIGS plants was more than twice as high as that of the control at 120 hpi. However, uredia did not grow better. Increasing evidence has suggested that oxidative damage may be a key factor of ageing in species ranging from *C. elegans* to *Drosophila* to humans (Stadtman, 1992; Wallace, 1999; Finkel and Holbrook, 2000; Hekimi and Guarente, 2003). Furthermore, oxidative stress can also cause fungal degeneration, resulting in several changes, including the overgrowth of hyphae, formation of sectors and reduced sporulation and virulence (Li et al., 2014). In the HIGS plants, ROS were significantly accumulated and caused damage to *Pst*. Thus, we infer that abnormal growth of hyphae may be due to *Pst* degeneration caused by ROS stress.

In conclusion, the present study revealed a key role of *PsSOD1* during *Pst* infection. In the wheat-*Pst* compatible interaction, highly expressed *PsSOD1* was secreted into the host-pathogen interface, contributing to *Pst* infection by scavenging ROS derived from the host and suppressing host cell death.

### Experimental Procedures

#### Plant materials, strains and culture conditions

The wheat cultivar Suwon 11 (Su11) and the *Pst* pathotype CYR32 (virulent to Su11) were used in the wheat-*Pst* interaction study. Plant cultivation and inoculation with *Pst* were performed as described previously (Kang et al., 2002). To study the *PsSOD1* expression levels in wheat leaves infected by CYR32, urediniospores of CYR32 and leaf tissues were sampled at 0, 6, 12, 18, 24, 36, 48, 72, 120, 168 and 264 h post-inoculation (hpi). *N. benthamiana*, which was used for transient expression, was grown at 25°C with a light regime of 16 h light/8 h darkness.

The strains used in this study are listed in Supporting Information Table S2. *A. tumefaciens* was cultured at 30°C, *E. coli* at 37°C, and *S. cerevisiae* and *S. pombe* at 28°C, all in growth chambers in the dark.

#### RNA extraction and qRT-PCR

Total RNA was extracted using RNAiso Reagent (TaKaRa, Tokyo, Japan) according to the manufacturer’s instructions. Potential genomic DNA was digested with DNase I. First-strand cDNA was synthesized using a GoScript™ Reverse Transcription System (Promega, Madison, WI) with an oligo(dT)18 primer.

The transcript levels of *PsSOD1* were measured at different developmental stages of the *Pst* infection process by qRT-PCR according to the procedure described by Cheng et al. (2015). Elongation factor-1 (EF-1) was used as the endogenous reference to normalize the gene expression in *Pst* (Yin et al., 2009). Each reaction was carried out in triplicate, and three non-template controls were included in the experiment. The specificity of the amplicon was verified at the end of the PCR run using dissociation curve analysis. Two parameters, i.e., a relative quantity of RNA at least twofold higher or lower than the controls and *P* ≤ 0.005, were used to assess the significance of the differences between time points.

The primers used for qRT-PCR are listed in Supporting Information Table S2.

#### Cloning of *PsSOD1*, domain prediction, sequence alignment and polymorphism analysis

To clone the *PsSOD1* gene, primers (Supporting Information Table S2) were designed based on the *PsSOD1* cDNA sequence containing the complete ORF from the wheat-*Pst* interaction cDNA library (Ma et al., 2009). The *PsSOD1* gene was PCR-amplified using a CYR32-infected Su11 cDNA sample as a template. The physicochemical properties of the amino acid sequence of *PsSOD1* were determined using the ProtParam tool of ExPaSy (http://www.expasy.org). The
Characterization of a Superoxide Dismutase PsSOD1  13

Plasmid construction

To identify the function of PsSOD1, the ORF of PsSOD1 without the signal peptide was amplified and inserted into the NotI/BamHI restriction sites in plasmid pDR195 to obtain the complementation construct pDR195-PsSOD1.

For biochemical characterization of PsSOD1, the coding region sequences without a signal peptide were amplified and inserted into the BamHI/NcoI restriction sites of vector pGEX-4T-1 to generate the recombinant plasmid pGEX-4T-1-PsSOD1.

Functional identification of the predicted signal peptide of PsSOD1 was performed with a yeast secretion system (Jacobs et al., 1997). The yeast secretory trap vector pSUC2T7-M13ORI (pSUC2), which carries a truncated invertase, SUC2, lacking both its initiation methionine and signal peptide, was used. DNA fragments encoding the predicted signal peptide of PsSOD1, the identified secretion protein Atr1b (the positive control) and Mg87 without a signal peptide (the negative control) (Gu et al., 2011) were inserted into the EcoRI/XhoI restriction sites of vector pSUC2, respectively.

For overexpression in S. pombe, the vector pREP41 (Craven et al., 1998) was used. The PsSOD1 amplicon and the pREP41 vector were digested with the restriction enzymes NdeI and BamHI and then ligated to generate the recombinant plasmid pREP41-PsSOD1.

To determine whether PsSOD1 forms a polymer, the ORF of PsSOD1 without the signal peptide was inserted into the BamHI/XhoI restriction sites in vector pSPYNE(R173) (Waadt et al., 2008) and the ClaI/XhoI restriction sites in plasmid pSPYCE(M) (Waadt et al., 2008) to generate the recombinant plasmids pSPYNE(R173-PsSOD1 and pSPYCE(M)-PsSOD1, respectively.

For overexpression of PsSOD1 in tobacco, the ORF of PsSOD1 without a signal peptide and the Bax gene were PCR-amplified and inserted into the ClaI/EcoRI restriction sites in vector potato virus X (PVX) to construct the recombinant plasmids PVX-PsSOD1 and PVX-Bax, respectively.

A BSMV γ RNA-based vector was constructed as previously described by Holzberg et al. (2002). To ensure the specificity of the gene silencing, the fragments that showed the highest polymorphism within this gene family and the lowest sequence similarity with other PsSOD1 and wheat genes in a BLASTN search of the National Center for Biotechnology Information database were chosen to construct the RNA-based derivative plasmids. Consequently, two cDNA fragments derived from the coding region and the 5′ untranslated region (UTR) (216 bp, nucleotides 217–432) and from the coding sequence and the 3′ UTR (202 bp, nucleotides 833–1034) were used to construct the recombinant plasmids PsSOD1-as1 and PsSOD1-as2, respectively, in an antisense orientation.

The primers for all plasmid constructions are listed in Table S2.

Complementation of the S. cerevisiae YO6913 mutant with PsSOD1

For the S. cerevisiae complementation assays, pDR195 and pDR195-PsSOD1 were transformed into the SOD1-deficient strain YO6913 using electroporation. The transformants were selected on SC media without uracil at 28°C. The putative transformants carrying the vector pDR195-PsSOD1 were confirmed using PCR analysis. The function of PsSOD1 was identified based on the growth rate of the positive transformants in SC media with the indicated carbon sources. Growth was monitored in SC with different carbon sources as previously described by Longo et al. (1996).

In addition, the complemented strain carrying the recombinant pDR195-PsSOD1 plasmid was used to assay sensitivity to menadione, (2-methylnaphthalene-1,4-dione), an endogenous superoxide-generating compound. Yeast cells grown in SC without uracil were standardized to 1 × 10⁷ cells/ml. Five microlitre volumes of a 10-fold dilution series prepared from this suspension were spotted on the surface of SC agar plates containing 0, 0.5, 1 and 2 mM menadione. The plates were incubated for 48 h at 28°C, and growth was observed during the incubation. The ΔSOD1 mutant carrying an empty pDR195 vector was used as the control.

Expression of GST-tagged fusion proteins and antibody generation

The recombinant plasmid pGEX-4T-1-PsSOD1 was used to transform E. coli BL21(DE3)plysS grown in LB medium supplemented with ampicillin (100 mg/l) at 37°C. Protein expression was induced at an A₆₀₀ of 0.6 by addition of 1.0 mM IPTG for 10 h. The harvested cells were suspended in Tris-HCl buffer (20 mM Tris-HCl; pH 7.9) and lysed by sonication. The supernatant containing the soluble proteins was collected by centrifugation at 12,000 rpm for 15 min and analysed by SDS-PAGE followed by staining with Coomassie brilliant blue.

Purification of the fusion proteins was performed using GSTrap 4B (GE). The GST tag was removed by thrombin
cleavage. Antibodies were obtained by repeated injection of rabbits with the PsSOD1 protein.

**SOD activity assay**

SOD activity was assayed using a photochemical method based on the reduction of nitroblue tetrazolium (NBT). The 3 ml reaction mixture contained 39 mM L-methionine, 225 μM nitroblue tetrazolium (NBT), 8 μM riboflavin, 30 μM EDTA- Na2, and 10 μl purified enzyme in 50 mM potassium phosphate buffer (pH 7.8). The reaction was initiated by illuminating the reaction mixture for 20 min, and photochemically produced superoxide reacted with NBT. Absorbance of formazan, the product of NBT reduction, was then recorded at 560 nm. One unit of SOD activity was defined as the amount of enzyme that caused 50% of the maximum inhibition of NBT reduction. All assays were performed in triplicate, and average values were reported. Total protein content was measured as described by Lowry et al. (1951) using bovine serum albumin as the standard.

The thermal and pH stabilities of the purified enzyme were assessed after the enzyme solution samples were incubated at 20 to 70°C and pH 5 to 13 for 30 min, respectively. The residual activity of each sample was determined after the 30 min reaction. The effects of four metal ions (0.5 mM Mn2+, Cu2+, Zn2+, or Fe3+) on the SOD activity of the purified PsSOD1 were measured. Enzyme solutions containing each of the tested agents were incubated in 50 mM phosphate buffer (pH 7.8) at 25°C for 30 min and then assayed for residual activity as described above. The metal content of PsSOD1 was determined using graphite furnace atomic absorption spectroscopy (Thermo Electron, USA) after the enzyme was diazoyzed extensively in 10 mM phosphate buffer (pH 7.8) containing 1 mM EDTA and then in buffer without EDTA. All assays were repeated three times.

**Signal peptide validation of PsSOD1**

The recombinant plasmid pSUC2-PsSOD1, the control plasmid pSUC2-Avr1b and pSUC2-Mg87 were transformed into the yeast strain YTK12 as previously described by Gietz et al. (1995), respectively. All transformants were confirmed by PCR using specific primers. Transformants were grown on yeast minimal medium with sucrose instead of glucose (CMD-W media: 0.67% yeast N base without amino acids, 0.075% tryptophan dropout supplement, 2% sucrose, 0.1% glucose, and 2% agar). To assess invertase secretion, colonies were transferred onto YPRAA plates containing raffinose and lacking glucose (1% yeast extract, 2% peptone, 2% raffinose, and 2 mg/ml ampicillin). The YTK12 strain transformed with the pSUC2 vector encoding the truncated invertase, and the untransformed YTK12 strain were used as negative controls.

**Preparation of vacuum infiltrate samples and immunoblot analysis**

For apoplast protein extraction, a vacuum infiltration procedure was used according to the method described by Dani et al. (2005). Uninfected and Pst-infected wheat leaf tissues at 48 hpi were excised, washed in chilled H2O (4°C) and then submerged in chilled vacuum infiltration buffer (50 mM phosphate buffer, 200 mM NaCl, pH 7.5) in a modified vacuum desiccator. A vacuum of 80 kPa was applied for 10 min using a vacuum pump (KNF Neuberger, Freiburg, Germany) to remove the gas from the apoplasmic spaces; during evacuation, the container was periodically shaken to dislodge air bubbles from the leaf surfaces. Following the gradual release of the vacuum, leaf strips were blotted dry. The extract was centrifuged in VentaSpin tubes with polypropylene mesh inserts (Whatman International, Maidstone, UK) at 900 × g for 10 min at 4°C. The vacuum infiltrate (VI) was collected in the bottom of the tubes, dialyzed against H2O overnight at 4°C, and then resuspended in a small volume of water or buffer before use.

For the western blot analysis, apoplastic proteins from Pst-infected wheat leaves were separated using native PAGE or SDS-PAGE and transferred onto nitrocellulose membranes. The blot was blocked with 5% skim milk in TBS buffer (10 mM Tris–HCl buffer containing 150 mM NaCl; pH, 7.5) for 2 h at room temperature. The samples were then incubated with the anti-PsSOD1 antibody (1:500) in 2% skim milk/TBS overnight at 4°C, washed with TBS containing 0.1% Tween-20 (TBST), and finally reacted with goat anti-rabbit IgG horseradish peroxidase (HRP) (1:500) for 2 h at 25°C. After incubation and washing, the nitrocellulose membrane was immunostained with 3,3′-diaminobenzidine (DAB) for 10 min in the dark. Apoplastic proteins from uninfected wheat leaves and purified GST-PsSOD1 fusion proteins were used as controls.

**Split GFP assay**

nYFP sequences were fused to the N-terminal sequences of PsSOD1 in the vector pSPYNE(R)173, and cYFP sequences were fused to the C-terminal sequences of PsSOD1 in the plasmid pSPYCE(M). The fusion proteins were introduced into N. benthamiana leaves using the Agrobacterium-mediated transient expression method (Xuan et al., 2013). Interactions of the co-expressed proteins were monitored by detecting YFP fluorescence using a Zeiss LSM 510 META confocal microscope. All assays were repeated independently at least three times with comparable results.

**Overexpression of PsSOD1 in S. Pombe**

To determine the role of PsSOD1 in resisting exogenous superoxide, the complete ORF of PsSOD1 was introduced into the overexpression vector pREP41, and the recombinant plasmid pREP41-PsSOD1 was transformed into the wild-type S. pombe JMB37 by electroporation. The transformants were selected on SC media without leucine at 28°C and validated using PCR. The positive transformant was cultured and collected from log-phase liquid cultures. Cells (1 × 10⁸) were incubated in a superoxide-generating system consisting of 50 mM Tris pH 8, 100 mM hypoxanthine, and increasing amounts of xanthine oxidase (X4500; Sigma) in a total volume of 500 ml. Yeast cultures were incubated for 4 h at 28°C with shaking (200 rpm) in a humidified chamber. After incubation, the yeast cultures were removed, and serial dilutions were plated on solid media to identify viable fungal colony forming units (cfu). The JMB37 strain carrying the empty pREP41
vector was used as the negative control. Survival was statistically compared between strains using one-tailed Student’s t-tests.

Overexpression of PsSOD1 in N. Benthamiana

The reconstructed vectors PVX-PsSOD1, PVX-Bax, and PVX empty vector were transformed individually into A. tumefaciens strain GV3101. Infiltration experiments were performed using 4- to 6-week-old tobacco plants. A. tumefaciens cell suspensions carrying the transgenes were infiltrated into tobacco leaves as described by Wang et al. (2011). At 24 h after the initial infiltration with A. tumefaciens carrying PVX-PsSOD1, the same infiltration site was challenged with A. tumefaciens cell suspensions carrying the Bax gene. Empty vector or MgCl₂ was infiltrated in parallel as controls. RT-PCR was used to evaluate the expression of the PsSOD1 and Bax genes in tobacco leaves exposed to different treatments. The infiltrated tobacco leaves were harvested 60 h after the second infiltration, and total RNA was extracted. The development of symptoms was monitored 3 to 6 days after the second infiltration.

The primers used for RT-PCR are listed in Supporting Information Table S2.

BSMV-mediated PsSOD1 gene silencing in the compatible wheat-Pst interaction

BSMV-VIGS was carried out as previously described by Cheng et al. (2015). To silence PsSOD1, two BSMV viruses (BSMV:PsSOD1-as1 and BSMV:PsSOD1-as2) were used to inoculate wheat seedlings. BSMV:TaPDS (TaPDS: wheat phytoene desaturase gene) and BSMV:Y were used as controls for the BSMV infection. Mock inoculations were performed with 1 × FES buffer as previously described (Cheng et al., 2015). At least 18 wheat seedlings were used for each assay. BSMV-infected wheat plants were kept in a growth chamber at 23 ± 2°C. The fourth leaves were further inoculated with fresh CYR32urediniospores at 9 d after virus inoculation, and the plants were then maintained at 18 ± 2°C and sampled at 0, 24, 48, and 120 hpi for qRT-PCR and histological observation (Wang et al., 2007). The silencing efficiency of PsSOD1 was confirmed using qRT-PCR as described above. The phenotypes of the fourth leaves were observed and photographed at 14 days after pathogen inoculation. Biological replicates were carried out in triplicate.

Quantification of Pst in inoculated leaves

To measure changes in fungal biomass, relative quantification of the single-copy target genes PsEF1 and TaEF1 was carried out (Panwar et al., 2013). Total genomic DNA of the wheat cultivar Su11 or the Pst pathotype CYR32 were used to prepare standard curves derived from at least six serial dilutions for each. The correlation coefficients for the analysis of the dilution curves were above 0.99. The relative quantities of the PCR products of PsEF1 and TaEF1 in mixed/infected samples were calculated using the gene-specific standard curves to quantify the Pst and wheat genomic DNA, respectively.

The primers used are listed in Supporting Information Table S2.

Characterization of a Superoxide Dismutase PsSOD1

Histological observation of fungal growth and host response

To characterize the cellular interaction between wheat and Pst, the fungal development and host response were observed microscopically. The leaf segments were fixed and stained as described previously (Wang et al., 2007). The number of hyphal branches, haustorial mother cells and haustoria, hyphal length and spread of hyphal growth were determined as previously described (Cheng et al., 2015). To obtain high-quality images of Pst infection structures in wheat leaves, wheat germ agglutinin conjugated to Alexa Fluor-488 (Invitrogen) was used as previously described (Ayliffe et al., 2010).

To detect plant responses, H₂O₂ accumulation was studied at 24 and 48 hpi using 3,3′-diaminobenzidine (DAB; Amresco, Solon, OH) staining (Wang et al., 2007). Fifty infection sites on five randomly selected leaf segments per treatment were assessed. Standard deviations were determined, and Tukey’s test was used for statistical analysis.

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Characterization of a Superoxide Dismutase PsSOD1 17


**Supporting information**

Additional supporting information may be found in the online version of this article at the publisher's web-site:

**Fig. S1.** Prediction of conserved domains (A) and signal peptides (B) of PsSOD1 from Pst. The protein domains and signal peptides of PsSOD1 were predicted using Pfam analysis (http://pfam.sanger.ac.uk/) and SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/), respectively.

**Fig. S2.** Alignment of the amino acid sequences of PsSOD1 with Cu/Zn-SODs from other rust fungi. Alignment was maximized by introducing gaps, which are indicated by dashes. Identical (*), highly similar (:), and similar (.) amino acids are also indicated. Abbreviations: CYR32, PsSOD1 from the Pst pathotype CYR32; Pg, Cu/Zn-SOD from P. graminis f. sp. tritici CRL 75-36-703 (GenBank accession number XP_003326013); Ps, Cu/Zn-SOD from Puccinia sorghi (GenBank 11 accession number KNZ59826); Mlp, Cu/Zn-SOD from Melampsora larici-populina 9AG31 (GenBank accession number XP_007413252).

**Fig. S3.** Analysis of conserved domains of Cu/Zn-SODs (A) and Cu-only SODs (B). The protein domains were predicted using Pfam analysis (http://pfam.sanger.ac.uk/).

**Fig. S4.** Multiple sequence alignment of PsSOD1 from different Pst isolates. Identical (*) and 16 highly similar amino acids are indicated. Red box: different amino acid substitutions; ◀: Zn binding site; blue line: conserved domain.

**Fig. S5.** A. The native PAGE profiles of the purified GST and GST-PsSOD1 proteins. Lane 1, purified GST proteins; lane 2, purified GST-PsSOD1 fusion proteins; M, marker. B. Western blot analysis of the apoplastic proteins separated in the SDS-PAGE gels. Lane 1, apoplastic proteins from Pst-infected wheat leaves; lane 2, apoplastic proteins from healthy wheat leaves.

**Fig. S6.** Sequence regions for HiGS 1 in this study.

**Fig. S7.** Standard curves generated for the absolute quantification of Pst (A) and wheat (B). Threshold cycles (Ct) are plotted against the initial copy number of template DNA (104, 105, 106, 107, 108, and 10104). Genomic DNA of the wheat cultivar Su11 or the Pst pathotype CYR32 ureidiospores was used to construct the standard curves.

**Fig. S8.** HiGS of PsSOD1 shows limited fungal development and increased ROS accumulation in the host cells at 24 hpi. Values represent the means ± standard errors of three independent samples. Differences were assessed using Student’s *t*-tests. Asterisks indicate *P* < 0.05. (A) The average number of hyphal branches (HB), haustorial mother cells (HMC) and haustoria (H) did not differ significantly in CYR32-infected HiGS plants compared with the control. (B) Hyphal length was clearly decreased in CYR32-infected HiGS plants. (C) The infection unit area at 48 hpi per infection unit was significantly decreased in CYR32-infected HiGS plants, whereas there was no difference in the infection unit area at 72 hpi. (D) ROS accumulation increased significantly in CYR32-infected HiGS plants at 24 hpi.

**Table S1.** Overview of intraspecies nucleotide polymorphism in *PsSOD1*.

**Table S2.** Oligonucleotides and strains in this study.