Thioredoxins are involved in the activation of the PMK1 MAP kinase pathway during appressorium penetration and invasive growth in *Magnaporthe oryzae*

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**Summary**

In *Magnaporthe oryzae*, the Mst11-Mst7-Pmk1 MAP kinase pathway is essential for appressorium formation and invasive growth. To determine their roles in Pmk1 activation and plant infection, we characterized the two thioredoxin genes, TRX1 and TRX2, in *M. oryzae*. Whereas the Δtrx1 mutants had no detectable phenotypes, deletion of TRX2 caused pleiotropic defects in growth, conidiation, light sensing, responses to stresses and plant infection progresses. The Δtrx1 Δtrx2 double mutant had more severe defects than the Δtrx2 mutant and was non-pathogenic in infection assays. The Δtrx2 and Δtrx1 Δtrx2 mutant rarely formed appressoria on hyphal tips and were defective in invasive growth after penetration. Pmk1 phosphorylation was barely detectable in the Δtrx2 and Δtrx1 Δtrx2 mutants. Deletion of TRX2 affected proper folding or intra-/inter-molecular interaction of Mst7 and expression of the dominant active MST7 allele partially rescued the defects of the Δtrx1 Δtrx2 mutant. Furthermore, Cys305 is important for Mst7 function and Trx2 directly interacts with Mst7 in co-IP assays. Our data indicated that thioredoxins play important roles in intra-cellular ROS signalling and pathogenesis in *M. oryzae*. As the predominant thioredoxin gene, TRX2 may regulate the activation of Pmk1 MAPK via its effects on Mst7.

**Introduction**

Rice blast caused by *Magnaporthe oryzae* is one of the most destructive fungal diseases of rice worldwide. Although root infection has been observed under laboratory conditions, *M. oryzae* is a typical foliar pathogen that initiates plant infection by the formation of a highly specialized infection structure known as an appressorium. The pathogen then uses turgor pressure generated inside heavily melanized appressoria for the penetration of plant cuticle and cell wall (de Jong *et al.*, 1997). After penetration, highly vacuolated, bulbous invasive hyphae are enveloped by the extra-invasive-hyphal membrane (EIHM) produced by the plant cells (Kankanala *et al.*, 2007). During this biotrophic phase, *M. oryzae* delivers various effectors into plant cells via the conventional ER to Golgi secretory pathway or the Golgi-independent system involving the biotrophic interfacial complex (BIC) to avoid or suppress plant defense responses (Khang *et al.*, 2010; Zhang and Xu, 2014). At later infection stages, fungal invasive growth results in plant cell death, and conidia are produced by *M. oryzae* on blast lesions to re-initiate the infection cycle for disease spreading in the field.

In the past two decades, *M. oryzae* has been extensively studied for infection-related morphogenesis and fungal-plant interactions (Dean *et al.*, 2005). Over a 100 genes with diverse functions have been shown to be required for plant infection in this model plant pathogen, including various genes important for appressorium penetration and key components of well-conserved signal transduction pathways (Jeon *et al.*, 2007; Li *et al.*, 2012). Whereas the cAMP signalling pathway controls surface recognition and appressorium initiation, late stages of appressorium formation is regulated by the Pmk1 MAP kinase (MAPK) pathway, which also is required for appressorium penetration and invasive growth after penetration, two other critical infection processes in the infection cycle of *M. oryzae* (Zhao and Xu, 2007). Pmk1 is orthologous to Kss1 and

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the pmk1 deletion mutant is non-pathogenic (Xu and Hamer, 1996). A number of genes functioning upstream from Pmk1, including the MAPK kinase (MEK) Mst7 and MEK kinase (MEKK) Mst11, and an adaptor protein Mst50 have been identified (Zhao et al., 2005; Park et al., 2006). Several downstream targets regulated by Pmk1 also have been functionally characterized, including MST12, SLF1, GAS1, and GAS2 (Park et al., 2002; Xue et al., 2002; Li et al., 2011). Mutants deleted of MST12, one of the downstream transcription factors of the PMK1 pathway, still form melanized appressoria but are defective in appressorium penetration and invasive growth (Park et al., 2004). In addition, the cell wall integrity Mps1 MAPK and calcium signalling pathways also are known to be important for appressorium morphogenesis, penetration, and invasive growth in M. oryzae (Xu et al., 1998).

Reactive oxygen species (ROS) produced by NADPH oxidases or in mitochondria serve as critical signalling molecules or secondary messengers in cell proliferation and survival in eukaryotic organisms. In mammalian cells, ROS can induce or mediate activation of the MAPK pathways (McCubrey et al., 2006). The oxidative modification of signalling proteins by ROS (Thannickal and Fanburg, 2000) is one of the possible mechanisms for the activation of MAPK pathways. Apoptosis signal-regulated kinase 1 (ASK-1), an upstream MEK kinase that regulates the JNK and p38 MAPK pathways (Ichijo et al., 1997), binds to reduced thioredoxin in non-stressed cells. Under oxidative stress, thioredoxins become oxidized and disassociated from ASK-1, leading to activation of JNK and p38 pathways through oligomerization of ASK-1 (Nagai et al., 2007). In mammalian cells, protein kinase A (PKA) was also shown to be activated by ROS through formation of intramolecular disulfide bonds. Cys199 in the catalytic subunit of PKA was involved in the formation of a disulfide bond when treated with H2O2, leading to dephosphorylation of Thr197 and thus resulted in PKA inactivation (Humphries et al., 2005). Inhibition of phosphatases by ROS also has been shown to regulate JNK (Kamata et al., 2005) and p38 signalling (Liu et al., 2010a). In Arabidopsis, H2O2 activates the MAP kinases MPK3 and MPK6 via MEK or MEK kinase ANP1 (Kovtun et al., 2000). ROS-induced activation of MAPKs appears to be central for mediating cellular responses to multiple stresses, although the mechanism for its activation of MAPK pathways is not clear (Apel and Hirt, 2004).

An important redox system is formed by the thioredoxin (TRX) system. Thioredoxins are a family of small, ubiquitous proteins with a conserved active site sequence (Trp-Cys-Gly-Pro-Cys) that catalyzes a variety of redox reactions via reversible redox-active disulfide/dithiol. Due to the redox-active cysteine pair at the active site, thioredoxins can cycle between the oxidized disulfide (Trx-S2) and reduced dithiol [Trx-(SH)2] forms. They alter the redox state of target proteins via the reversible oxidation of an active site dithiol present in a CXXC motif. Thioredoxins are divided into two groups. Whereas group I thioredoxins exclusively contain the TRX domain, group II members have other domains in addition to TRX (Sadek et al., 2003). The budding yeast Saccharomyces cerevisiae contains three group I thioredoxins. Two cytoplasmic ones, Trx1 and Trx2, regulate cellular redox homeostasis. The mitochondrial thioredoxin Trx3 protects against the oxidative stress generated during respiratory metabolism. In the fission yeast Schizosaccharomyces pombe, the cytosolic Trx1 and mitochondrial Trx2 thioredoxins have similar amino acid sequences except in the C-terminal region (Cho et al., 2001). Trx1 plays a major role in protecting S. pombe against various external stresses and is required for sulfur metabolism (Song and Roe, 2008).

In living cells, TRX genes may be involved in the activation of MAPKs via regulating intracellular ROS levels or directly modifying the structure or redox status of key signalling components (Laloi et al., 2004; Fujino et al., 2006). However, to date, the role of thioredoxins in MAP kinase signalling has not been determined in the two model yeasts and Neurospora crassa, a model filamentous fungus. In Aspergillus nidulans, deletion of the trxA thioredoxin gene results in decreased growth, increased catalase activities, and inability to form reproductive structures such as conidiophores and cleistothecia (Thön et al., 2007). In Candida albicans, the thioredoxin Trx1 regulates three distinct regulatory proteins that are activated in responses to H2O2 stress, which are the Cap1 transcription factor, the Hog1 SAPK, and the Rad53 DNA checkpoint kinase (da Silva Dantas et al., 2010). In Podospora anserine, the PaMpk1 MAPK cascade was not affected in the Patrx1 and Patrx3 mutants, although both PaTrx1 and PaTrx3 are necessary for sexual reproduction and development of the crippled growth cell degeneration, which are two processes that involve the PaMpk1 pathway (Malagnac et al., 2007).

To understand the role of TRX genes in activating the PMK1 MAPK pathway, in this study we identified and characterized TRX1 and TRX2 in M. oryzae. Whereas the Δtrx1 mutants had no detectable phenotypes, deletion of TRX2 caused pleiotropic defects in growth, differentiation and pathogenesis. The Δtrx1 Δtrx2 double mutant had more severe defects than the Δtrx2 mutant and rarely formed appressoria on hyphal tips. Deletion of TRX2 affected Pmk1 activation and proper folding or intra-/inter-molecular interactions of the Mst7 MEK kinase. We further showed that Cys305 is important for Mst7 function, Trx2 directly interacts with Mst7 in co-IP assays, and expression of the dominant active MST7 allele partially rescued the defects of the Δtrx1 Δtrx2
mutant. Taken together, our data indicated that TRX2 may affect invasive growth via the Mst11-Mst7-Pmk1 pathway. It is likely that thioredoxins are important for intra-cellular ROS signalling and pathogenesis in M. oryzae.

Results

One of the M. oryzae TRX genes, TRX2, partially rescues the yeast Δtrx2 mutant

The M. oryzae genome has two group I TRX genes, MGG_15004 and MGG_04236, which were named TRX1 and TRX2, respectively, in this study. Both of them have an N-terminal TRX domain and share the same active site and catalytic residues (WCGPC) but the predicted Trx1 protein is twice the size of Trx2 (Supporting Information Fig. S1). In S. cerevisiae, Trx2 is the dominant cytoplasmic thioredoxin involved in environmental stress responses and the Δtrx2 mutant has increased sensitivity to hydrogen peroxide (Garrido and Grant, 2002).

To determine their functions in S. cerevisiae, the full-length ORFs of TRX1 and TRX2 were amplified from cDNA and cloned into pYES2. The resulting constructs were transformed into the yeast Δtrx2 mutant in the BY4741 background (Giaever et al., 2002). Expression of TRX2 but not TRX1 of M. oryzae partially complemented the increased sensitivity of Δtrx2 mutant to 6 mM H2O2 and 0.8 mM tert-butyl hydroperoxide (t-BOOH) (Garrido and Grant, 2002), although only data with the later was shown (Fig. 1A).

TRX2 is important for vegetative growth

The TRX1 and TRX2 gene replacement constructs were generated by double-joint PCR with the neomycin-resistant and hygromycin-resistant selectable markers, respectively, and transformed into protoplasts of Guy11. The resulting neomycin-resistant Δtrx1 and hygromycin-resistant Δtrx2 deletion mutants (Table 1) were identified by PCR and confirmed by Southern blot analysis (Supporting Information Fig. S2). In comparison with Guy11, the Δtrx1 mutant had no obvious defects in colony morphology (Fig. 1B) and growth rate (Table 2). However, the Δtrx2 mutant was reduced in growth rate (Table 2). It rarely produced aerial hyphae but was increased in colony pigmentation in comparison with Guy11 (Fig. 1B).

We also generated the Δtrx1 and Δtrx2 deletion mutants in the wild-type strain 70-15 (Table 1). The resulting mutants had the same phenotypes with those of Guy11, although only data from the Guy11 mutants were presented below.

Deletion of TRX2 but not TRX1 results in reduced conidiation

Conidiation was assayed with 10-day-old oatmeal agar (OTA) cultures. On average, the Δtrx1 mutant produced 3 x 10⁷ conidia per plate (9 cm²), which was similar with Guy11 (Table 2). Under the same conditions, the Δtrx2 mutant B9G was reduced approximately 15-fold in conidiation (Table 2).

To determine whether the two TRX genes have overlapping functions, we transformed the TRX2 knockout construct into the Δtrx1 mutant A52G (Table 1). Transformants resistant to both hygromycin and neomycin were screened by PCR and analysed by Southern blot hybridization (Supporting Information Fig. S2). The resulting Δtrx1 Δtrx2 double mutant D65 (Table 1) had similar growth rate and colony morphology with the Δtrx2 mutant (Fig. 1B). However, the double mutant was reduced approximately 70% in conidiation in comparison with the Δtrx2 mutant (Table 2).

To characterize the function of TRX2 deletion in conidiogenesis, we assayed the expression levels in vegetative hyphae of four transcription factor genes, CON1, CON7, HTF1 and COM1 that are known to be related to different stages of conidiation in M. oryzae (Odenbach et al., 2007; Zhou et al., 2009; Liu et al., 2010b). The expression level of COM1 that is important for conidiophore development (Zhou et al., 2009) was reduced approximately eightfold in the Δtrx2 mutant (Fig. 1C). Although CON1 expression was not significantly affected, the trx2 mutant also was reduced in the expression of the HTF1 and CON7 transcription factor genes (Fig. 1C). Whereas the htf1 mutant produces conidiophores but not conidia, the con7 mutant forms conidia with defective morphology (Odenbach et al., 2007; Liu et al., 2010b). TRX2 may affect conidiation by altering the expression levels of these transcription factors in M. oryzae.

TRX2 plays an important role in responses to oxidative and cell wall stresses

To determine the functions of thioredoxin genes in stress responses, we assayed growth rates of the Δtrx1, Δtrx2, and Δtrx1 Δtrx2 mutants (referred to as Δtrx mutants below) on complete medium (CM) with 0.7 M KCl, 0.5 M sorbitol, 300 µg ml⁻¹ Congo red, 200 µg ml⁻¹ Calcofluor white, or 6 mM H2O2. Whereas deletion of TRX1 and/or TRX2 had no obvious effect on tolerance to hyperosmotic stress, the Δtrx2 and Δtrx1 Δtrx2 mutants had increased sensitivity to Calcofluor white (CFW) and Congo red (Fig. 2A). Calcofluor white and Congo red are known to bind with beta-glucans and nascent chitin chains. Reduced growth in the presence of these compounds may be related to the defect of the Δtrx2 mutants in cell wall integrity. These results suggest that thioredoxins are not
involved in osmoregulation but TRX2 is important for responses to cell wall stress.

In the presence of H$_2$O$_2$, the $\Delta$trx2 and $\Delta$trx1 $\Delta$trx2 mutants but not $\Delta$trx1 mutant formed compact colonies with limited growth (Fig. 2A), indicating that only TRX2 plays a critical role in response to oxidative stress. Because the yeast $\Delta$trx2 mutant is hypersensitive to H$_2$O$_2$ but has increased tolerance to diamide (Muller, 1996), we also assayed the effects of diamide on $\Delta$trx mutants. In M. oryzae, the $\Delta$trx2 but not $\Delta$trx1 mutant was more tolerant to diamide than the wild type (Fig. 2A). On CM plates treated with or without 1.5 mM H$_2$O$_2$, colonies of Guy11 and $\Delta$trx1 were stained dark-purple with 2, 2'-azino-di-3-ethylbenzthiazoline-6-sulfonate (ABTS), one indicator of peroxidase/laccase activity (Guo et al., 2011). ABTS staining was not observed in the $\Delta$trx2 and $\Delta$trx1 $\Delta$trx2 mutants (Fig. 2B), indicating that extracellular peroxidase/laccase activities were significantly reduced in $\Delta$trx2 and $\Delta$trx1 $\Delta$trx2 mutants, which may be related to their increased sensitivity to H$_2$O$_2$.

The $\Delta$trx2 mutants are defective in response to light/dark transitions

When incubated under 12 h light/dark cycles, zones with dark pigmentation were visible on CM cultures of Guy11 and $\Delta$trx1 mutant cultures (Fig. 2C). Race tube cultures of the $\Delta$trx2 and $\Delta$trx1 $\Delta$trx2 mutants grown under the same conditions lacked these daily zones (Fig. 2C). When incubated under constant dark, daily zones were not observed in any of these strains (Fig. 2C). These results indicate that the TRX2 gene may play a role in light sensing or light/dark transitions. Because conidiation is induced by light in M. oryzae, failure of $\Delta$trx2 mutants in response to light/dark may be related to their defects in conidiation.

TRX2 is important for appressorium formation on hyphal tips

On artificial hydrophobic surfaces, the $\Delta$trx1 mutant was normal in appressorium formation (Table 2). In contrast, appressorium formation was slightly reduced in the $\Delta$trx2 and $\Delta$trx1 $\Delta$trx2 mutants compared with the wild type (Table 2). Nevertheless, appressoria formed by the $\Delta$trx2 mutants were normal in morphology (Fig. 3A). Interestingly, appressoria formed at hyphal tips were rarely observed in the $\Delta$trx2 and $\Delta$trx1 $\Delta$trx2 mutants after incubation for 48 h (Fig. 3B). Less than 1% of hyphal tips of the $\Delta$trx2 mutants formed appressoria on hyphal tips. These results showed that $\Delta$trx2 mutants were defective in appressorium formation on hyphal tips but not by germ
tubes, which is similar to the con7 mutant (Kong et al., 2013).

The Δtrx2 and Δtrx1 Δtrx2 mutants are defective in plant infection

When 2-week-old rice seedlings of cultivar CO-39 were sprayed with conidium suspensions of the wild type and Δtrx1 mutant, numerous lesions were observed on inoculated leaves at 7 dpi (Fig. 4A). Under the same conditions, only few, small black or brownish spots (not typical blast lesions) were observed on leaves inoculated with the Δtrx2 mutant (Fig. 4A). On leaves sprayed with conidia of the double mutant, small black or brownish spots were rarely observed in over five independent repeats (Fig. 4A). These results indicate that TRX2 plays a more critical role than TRX1 in plant infection. Nevertheless, the Δtrx1 Δtrx2 mutant had more severe defects than the Δtrx2 mutant in virulence, suggesting that TRX1 plays a minor role, possibly overlapping with TRX2, during plant infection.
To further characterize the Δtrx2 mutants, leaves of 10-day-old barley seedlings were wounded with a needle and inoculated with culture blocks. On leaves inoculated with the Δtrx2 and Δtrx1 Δtrx2 mutants, only limited darkening at the wound site but not extensive spreading of the necrosis zones was observed (Fig. 4B). When the diseased segments of barley leaves were surface sterilized and incubated for 2 days in a moisture chamber under constant light, abundant conidia and conidiophores were formed on lesions caused by Guy11 and the Δtrx1 mutant. However, no conidia or hyphal growth were observed on the sites inoculated with the Δtrx2 and Δtrx1 Δtrx2 mutants (Fig. 4C), suggesting that TRX2 is not only important for appressorium formation on hyphal tips but also plays an important role in invasive growth after penetration.

**Thioredoxins are important for invasive growth**

In penetration assays with onion epidermal cells, appressoria formed by Guy11 and the Δtrx1 mutant penetrated and produced invasive hyphae inside plant cells by 48 h post-inoculation (hpi) (Fig. 5A). Under the same conditions, the Δtrx2 mutant had only limited invasive hyphae in underlying plant cells (Fig. 5A), indicating that TRX2 is important for invasive growth in *M. oryzae*. Moreover, less than 20% of appressoria formed by the Δtrx1 Δtrx2 mutant could penetrate underlying plant cells. Even for those Δtrx1 Δtrx2 appressoria that were successful in penetration, they had only limited infectious growth and failed to differentiate branching invasive hyphae in underlying plant cells (Fig. 5A), indicating that thioredoxins are important for invasive growth in *M. oryzae*. 

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**Fig. 2.** Defects of the Δtrx1 and Δtrx2 mutants in responses to oxidative, hyperosmotic, and cell wall stresses.

A. The wild type (Guy11) and the Δtrx1, Δtrx2, and Δtrx1 Δtrx2 mutants were cultured for 7 days on CM with or without 200 μg ml⁻¹ Calcofluor white (CFW), 300 μg ml⁻¹ Congo red (CR), 0.7 M NaCl, 0.5 M Sorbitol, 6mM H₂O₂ or 2.0 mM diamide.

B. ABTS staining with CM cultures of Guy11 and the Δtrx mutants treated with or without 1.5 mM H₂O₂.

C. Race tube cultures of Guy11 and the Δtrx1 Δtrx2 mutants cultured on CM for 30 days under a 12 h light/dark (L/D) cycle or constant darkness (D/D).
which is consistent with virulence defects of the \( \Delta trx2 \) and \( \Delta trx1 \Delta trx2 \) mutants.

We also conducted penetration assays with barley leaves inoculated with culture blocks. On barley leaves inoculated with Guy11 and \( \Delta trx1 \) mutant, \( \Delta trx2 \) mutant, and \( \Delta trx1 \Delta trx2 \) mutant were incubated on hydrophobic surfaces for appressorium formation.

B. Differentiation of appressoria at hyphal tips on hydrophobic surfaces was observed in Guy11 and \( \Delta trx1 \) mutant but not in the \( \Delta trx2 \) and \( \Delta trx1 \Delta trx2 \) mutants after 48 h. Bar = 10 \( \mu m \).

Fig. 3. Appressorium formation assays with conidia and hyphal blocks.
A. Conidia from Guy11, \( \Delta trx1 \) mutant, \( \Deltatrx2 \) mutant, and \( \Delta trx1 \Delta trx2 \) mutant were incubated on hydrophobic surfaces for appressorium formation.

TRX2 expression is up-regulated in the \( \Delta trx1 \) mutant
Consistent with strong GFP signals observed in the TRX2-GFP transformant, \( TRX2 \) had similar expression levels in conidia, appressoria, and infected rice leaves (Supporting Information Fig. S4A). In contrast, \( TRX1 \) differed significantly in expression levels at different stages and had the highest expression level during appressorium formation (Supporting Information Fig. S4A). In comparison, \( TRX2 \) had a higher expression level than \( TRX1 \) in different cell types examined (Supporting Information Fig. S4B). These results indicate that \( TRX2 \) is constitutively expressed in \( M. oryzae \) and has a higher expression level than \( TRX1 \). We also assayed the effect of oxidative stress on thioredoxin gene expression. In vegetative hyphae harvested from CM cultures, the expression levels of \( TRX1 \) and \( TRX2 \) were not
significantly affected in samples treated with or without 6 mM H$_2$O$_2$ (Supporting Information Fig. S5).

To determine the effect of TRX2 deletion on TRX1 expression or vice versa, we assayed their expression levels in vegetative hyphae of Guy11 and Δtrx1 or Δtrx2 mutants.

In the Δtrx1 mutant, TRX2 expression had a fourfold increase (Supporting Information Fig. S6). In contrast, TRX1 expression was unaffected by deletion of TRX2. Therefore, it is possible that increased expression of TRX2 may suppress the defects caused by TRX1 deletion.
TRX2 is involved in the activation of the Pmk1 MAP kinase

Because Pmk1 regulates appressorium formation, penetration, and invasive growth (Xu and Hamer, 1996), we assayed its activation in the hyphae of \(\Delta trx1\) mutants. When detected with an anti-Pmk1 antibody, the 42 kD Pmk1-band had similar intensities in the wild type and \(\Delta trx1\) or \(\Delta trx2\) mutants (Fig. 7A). However, when detected with an anti-PTEY antibody, an antibody specific for detection of dual-phosphorylation of the TEY motif (Zhao et al., 2005; Liu et al., 2011; Zheng et al., 2012), the levels of Pmk1 phosphorylation were significantly reduced in the \(\Delta trx1\) and \(\Delta trx2\) mutants (Fig. 7A). In the \(\Delta trx1\ \Delta trx2\) mutant, phosphorylation of Pmk1 was not detectable (Fig. 7A). These results indicate that Pmk1 phosphorylation was significantly reduced in the double mutants. Meanwhile, the 46 kD Mps1-band had no obvious difference between the wild type and mutants, suggesting that the activation of Mps1 MAP kinase may be not affected by the deletion of thioredoxins. Therefore, thioredoxins, especially TRX2, may be involved in the activation of the Pmk1 MAPK kinase pathway in \(M.\ oryzae\).

Trx2 is required for the proper folding or dimerization of Mst7

In \(M.\ oryzae\), ROS signalling affects appressorium morphogenesis and fungal-plant interactions (Ryder et al., 2013). Because the Mst7 MEK that activates the downstream Pmk1 for appressorium formation (Zhao et al., 2005) is known to interact with itself in yeast two-hybrid assays (Zhao and Xu, 2007), thioredoxins may play a role in Mst7 activation by affecting its possible dimerization or formation of intramolecular disulfide bonds. To test this hypothesis, we firstly generated \(\text{P}_{\text{ppzT}}\text{:MST7-GFP}\) construct and co-transformed it into Guy11 with \(\text{P}_{\text{ppzT}}\text{:MST7}\text{–3xFLAG}\) construct. Transformants resistant to both hygromycin and neomycin were confirmed by PCR assays and western blot analysis to contain both transforming constructs. In western blot analysis with the resulting MST7-GFP MST7-3xFLAG transformant (Table 1), the 84-kD Mst7-GFP band was detected with an anti-GFP antibody in total proteins and proteins eluted from anti-FLAG beads (Fig. 7B), indicating that Mst7-GFP may interact with Mst7-3xFLAG to form dimers in vivo.

We then transformed the MST7-3xFLAG construct into Guy11 and the \(\Delta trx2\) mutant B9G. The resulting transformants GM7 (WT MST7-3xFLAG) and TM7 (\(\Delta trx2\ MST7\text{–3xFLAG}\)) were confirmed by PCR and western blot analysis with total proteins isolated from 2-day-old cultures was separated on SDS-PAGE gels (Fig. 7C). On western blots of the same proteins separated on native gels, both monomers and likely homodimers of Mst7-3xFLAG proteins were detected with an anti-FLAG antibody in total proteins and proteins eluted from anti-FLAG beads (Fig. 7B), indicating that Mst7-GFP may interact with Mst7-3xFLAG to form dimers in vivo.

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formed them into the 3xFLAG alleles by site-directed mutagenesis and trans-
fide bonds. To test this hypothesis, we generated the involved in the formation of intra- or inter-molecular disul-
and may be
interactions of Mst7, which may block the detection of the FLAG-epitope tag.

The Cys305 residue is essential for Mst7 function

Mst7 contains 4 cysteine residues that are conserved among its orthologs from Sordariomyces and may be involved in the formation of intra- or inter-molecular disulfide bonds. To test this hypothesis, we generated the MSTR114A, MSTR136A, MSTR201A, and MSTR305A, 3xFLAG alleles by site-directed mutagenesis and transformed them into the mst7 deletion mutant ZH32 (Zhao et al., 2005) respectively. The resulting MSTR114A, MSTR136A, and MSTR201A transformants (Table 1) were normal in appressorium formation (Fig. 8A) and plant infection (Fig. 8B). However, the mst7/MST7305A transformant MG2, similar to the original mst7 mutant, failed to form appressorium on hydrophobic surfaces (Fig. 8A) and was non-pathogenic (Fig. 8B). Therefore, the C305A residue is essential for Mst7 function.

We then conducted western blot analysis with total proteins isolated from the MST7305A-3xFLAG transformant. When separated on SDS-PAGE gels, the 47-kD band of the expected Mst7-3xFLAG fusion was detected with an anti-3xFLAG antibody (Fig. 8C). However, when separated on native gels, no Mst7-3xFLAG fusion bands were detected in the mst7/MST7305A-3xFLAG transformant (Fig. 8C). In the MST7WT-3xFLAG transformant, 3xFLAG fusion bands were detected in both native and SDS-PAGE gels (Fig. 8C). These data indicate that residue C305 is likely critical for the function and proper folding or intra-/inter-molecular interaction of Mst7.

Expression of MST7DA partially rescues the defects of the Δtrx1 Δtrx2 deletion mutant

To further investigate the relationship between Mst7 and Trx2, we transformed the dominant active MST7DA allele (Zhao et al., 2005) into the Δtrx1 Δtrx2 double mutant. Although the resulting MST7DA transformants MDD1 had similar colony morphology with the double mutant, it could form appressorium on hyphal tips as efficiently as the wild type (Fig. 9A), indicating a partial rescue of the Δtrx1 Δtrx2 mutant.

On barley leaves inoculated with culture blocks of the Δtrx1 Δtrx2/MST7DA transformant, appressorium formed on hyphal tips could penetrate and differentiate into invasive hyphae in epidermal cells (Fig. 9B). Moreover, the Δtrx1 Δtrx2/MST7DA transformant caused typical blast lesions on barley leaves (Fig. 9C). These results suggested that expression of the dominant active MST7DA allele can partially rescue the defects of the Δtrx1 Δtrx2 mutant.

To assay whether Trx2 regulates Mst7 through direct interaction, the MST7-GFP and TRX2–3xFLAG constructs expressed under the control of their native promoters were co-transformed into Guy11. In the resulting transformants confirmed to express both trans-
form fusion constructs, western blot analysis with proteins isolated from 2-day-old CM/5xYEG cultures showed that the 84-kD Mst7-GFP band was detectable with an anti-GFP antibody in total proteins or proteins eluted from anti-FLAG M2 beads (Fig. 9D). These results indicate that Mst7 and Trx2 may interact with each other in vegetative hyphae.

Discussion

Thioredoxins have the ability to reduce the disulfide bonds in many other proteins and, together with glutathione (GSH), to maintain a reduced cytoplasmic thiol redox balance. The rice blast fungus M. oryzae has two type I thioredoxins. Unlike that two type I thioredoxins in the

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budding yeast are similar in size, Trx1 is twice as large as Trx2 in *M. oryzae*. Based on complementation assays and mutant phenotype analyses, *TRX1* is likely the ortholog of yeast Trx2 and major cytoplasmic thioredoxin. For *TRX1*, GFP signals did not overlap with mitochondria stained with MitoTracker Red in the *TRX1-GFP* transformant and the *D trx1D trx2* mutant had more severe defects than the *D trx1* mutant. Therefore, Trx1 may not function as a mitochondrial thioredoxin but plays a minor role as a cytoplasmic thioredoxin in *M. oryzae*.

In *M. oryzae*, the *D trx2* mutant had increased tolerance to diamide but increased sensitivity to H$_2$O$_2$ or t-BOOH, which is similar to the yeast *trx2* mutant (Muller, 1996). Like the *trxA* deletion mutant of *A. nidulans* (Thon et al., 2007), the *D trx2* mutant of *M. oryzae* had increased catalase activities. In the budding yeast, Yap1 is important for regulating many oxidative stress response (OXR) genes, including *TRX2*, *TSA1*, *GPX2*, and *CTT1* (He and Fassler, 2005; Mulford and Fassler, 2011). Yap1 is activated upon ROS treatment by the formation of intramolecular disulfide bonds between C303 and C598 that disrupts the nuclear export signal (NES) (Delaunay et al., 2000; Kuge et al., 2001). Nuclear export of Yap1 is restored when disulfide bonds are reduced by thioredoxin Trx2, whose expression is controlled by Yap1. Therefore, *YAP1* and *TRX2* form a negative feedback loop (Marinho et al., 2014). The reduced form of Yap1 interacts with the nuclear export protein Crm1 in the nucleus, and with Ybp1 and peroxiredoxin Hyr1 in the cytoplasm. In *M. oryzae*, MoAP1 may be negatively regulated by Trx2 through some of the Cys residues, such as C494, C518, and C527. Another major regulator of responses to oxidative stress in *S. cerevisiae* is Skn7 (Raït et al., 2000). Whereas the yap1 mutant is sensitive to different ROS, the skn7 mutant has increased sensitivity to t-BOOH and H$_2$O$_2$ but not superoxide-generating reagents such as the thiol-oxidizing agent diamide.
(Morgan et al., 1997). Therefore, the defects of the yeast skn7 mutant in response to different oxidants are similar to that of the Δtx2 mutant in M. oryzae.

In M. oryzae, deletion of TRX1 and/or TRX2 had no obvious effects on tolerance to hyperosmotic stress or the activation of the Mps1 MAP kinase that regulates cell wall integrity in M. oryzae and other fungi (Xu et al., 1998; Zeng et al., 2012). However, the Δtx2 deletion mutants had increased sensitivity to Calcofluor white and Congo red. Recently, it has been shown that deletion of the thioredoxin reductase or peroxidase gene also resulted in an increased sensitivity to Congo red (Fernandez and Wilson, 2014). Therefore, TRX2 or intracellular ROS signalling also is important for responses to cell wall stress, although it is dispensable for osmoregulation. In M. oryzae, ROS signalling plays important roles in both development and virulence, and the CATB catalase gene is known to be essential for both cell wall integrity and virulence (Skamnioti et al., 2007).

Although they were only slightly reduced in growth rate, the Δtx2 and the Δtx1 Δtx2 mutants were reduced in conidiation. In A. nidulans, the txA deletion mutant also was reduced in growth and failed to form reproductive structures (Thön et al., 2007). However, the conidiation process differs between A. nidulans and M. oryzae. In M. oryzae, TRX2 appeared to affect the expression of several genes related to conidiation, including COM1 and HTF1, that are important for conidiophore development and differentiation of conidia (Liu et al., 2010b; Yang et al., 2010). CON1 and CON7 are involved in the later stages of conidiogenesis (Shi et al., 1998; Odenbach et al., 2007). Expression of CON7 also was reduced by deletion of TRX2. For some of these transcription factors, thioredoxins may be involved in keeping cysteine residues reduced, which may be important for their DNA binding activities or nuclear localization. Com1 (Cys328) and Htf1 (cys426) have only one cysteine residue that may form intermolecular disulfide bonds. For three cysteine residues of Con7 (Cys72, 258, and 263), two of them flank the predicted NLS (237GAQQHKPRRRYE250).

In M. oryzae, the Pmk1 MAP kinase pathway regulates three related but different infection processes: appressorium formation, penetration, and invasive growth (Zhao et al., 2007; Ding et al., 2010). Although the Δtx2 and Δtx1 Δtx2 mutant still formed appressoria by germ tubes, thioredoxins were essential for appressorium penetration and development of invasive hyphae in plant cells. In addition, the phosphorylation level of Pmk1 in vegetative hyphae was significantly reduced in the Δtx2 mutants and barely detectable in the Δtx1 Δtx2 mutant. Therefore, Trx2 may play a critical role in the activation of the Pmk1 pathway, which in turn may be responsible for the defects of the Δtx2 mutant in plant infection. Unfortunately, due to the defects of Δfortune and Δnd 1 Δ t mutants in plant infection, it is impossible to determine whether the phosphorylation level of Pmk1 was reduced in invasive hyphae. We attempted to determine the phosphorylation level of Pmk1 during appressorium formation but the Δfoatt and Δnd 1 Δ m mutants were significantly reduced in conidiation and we failed to isolate sufficient high quality proteins suitable for TEY phosphorylation assays from appressoria formed by germ tubes. Mst12, one of the downstream transcription factors of the Pmk1 pathway, is essential for the regulation of appressorium penetration and invasive growth by Pmk1 but it is dispensable for appressorium formation (Park et al., 2002; Park et al., 2004). Our data suggest that the involvement of Trx2 in the Pmk1 pathway as an upstream component is only important for appressorium penetration and invasive growth, which is similar to that of Mst12 as a downstream transcription factor. It is likely that phosphorylation of Mst12 may be affected during appressorium penetration and invasive growth in the Δfect and Δnd 1 Δ t mutants. However, the Δoveve and Δnd 1 Δ e mutants were defective in invasive growth and there is no commercially available antibody to detect the phosphorylation of Mst12. Nevertheless, it will be important to assay the phosphorylation status of Mst12 in the Δn the and Δnd 1 Δ h mutants and further characterize the functional relationship between Tx2 and Mst12 in the future when the phosphorylation sites of Mst12 by Pmk1 are clear and suitable detection methods become available. The Pmk1 and its orthologous MAPKs have been shown to be important for various plant infection processes (Zhao et al., 2007; Li et al., 2012). However, to our knowledge, there are no reports on the involvement of thioredoxins and redox status in the activation of this well-conserved MAPK pathway in plant pathogenic fungi. In C. albicans, Tx1 is involved in regulating the H2O2-induced activation of the Hog1 SAPK pathway (da Silva Dantas et al., 2010).

Due to a redox-active cysteine pair in their conserved active sites, thioredoxins can alter the redox state of target proteins. In plants and animals, many cellular proteins are directly regulated by the redox state via thioredoxins. In Arabidopsis, MKK6 is a member of the MAP2K subfamily that activates the p38 MAPKs. Upon oxidation, two cysteine residues of Cys109 and Cys196 in MKK6 form an intramolecular disulfide bond, which inhibits ATP binding and inactivates its kinase activity (Diao et al., 2010). Moreover, PKGIx and MEKK1 are also regulated by cysteine modifications (Cross and Templeton, 2006; Pantano et al., 2006; Burgoyne et. al., 2007; Leonberg and Chai, 2007). In M. oryzae, Pmk1 is activated by the upstream MEK Mst7 and MEKK Mst11. In yeast two-hybrid assays, Mst7 has been shown to interact with itself (Zhao et al., 2005). In this study, we showed that Mst7 may form homodimers in the wild type but not in the Δtx2 mutant. In addition, proper folding of Mst7 may be affected by TRX2 deletion because
Mst7-3xFLAG or Mst7-GFP fusion proteins were detectable on western blots of SDS-PAGE gels but not western blots of native gels. These results indicate that Trx2 plays a critical role in the proper folding or protein-protein interactions of Mst7 proteins, possibly by affecting the intra- or inter-molecular disulfide bonds. However, it remains possible that deletion of TRX1 and TRX2 may affect Ap1 or redox homeostasis in general, which may in turn indirectly affect MAP kinase signalling in M. oryzae. Site-directed mutagenesis showed that Cys305 of Mst7 is required for its function. The C305A mutation had no impact on the expression of Mst7-3xFLAG fusion proteins but affected the detection of Mst7 on western blots of native gels. Therefore, post-translational modifications that affected the detection of Mst7-3xFLAG should be relatively specific to the formation of disulfide bonds involving residue Cys305.

Interestingly, our data showed that the Δtrx1 Δtrx2 mutant still formed appressoria by germ tubes although it was defective in appressorium penetration and differentiation of invasive hyphae in plant cells. In M. oryzae, the Mst11-Mst7-Pmk1 MAPK cascade regulates appressorium penetration and invasive growth but it also plays an essential role in appressorium formation (Zhao et al., 2005; Ding et al., 2009). These results indicate that Trx2 may be more important for its role in the Pmk1 pathway during plant penetration and invasive hyphal growth. Nevertheless, appressorium formation on hyphal tips was affected in the Δtrx2 and Δtrx1 Δtrx2 mutants, suggesting that thioredoxins displayed a hyphal-specific role in infection-related morphogenesis. We noticed that the con7 and Δtrx2 mutants had the same defect in appressorium formation on hyphal tips although both of them were normal in appressorium formation by germ tubes on hydrophobic surfaces (Kong et al., 2013). Therefore, it will be important to further characterize the functional relationship between CON7 and TRX2.

In S. cerevisiae, the cytosolic thioredoxin system consists of the thioredoxin reductase Trr1 that catalyzes the transfer of reducing equivalents from NADPH to thioredoxin (Yang and Ma, 2010). Thioredoxin reductase is essential for viability in Cryptococcus neoformans and affects the circadian conidiation rhythm in N. crassa (Onai and Nakashima, 1997; Missall and Lodge, 2005; Thön et al., 2007). In M. oryzae, mutants with the thioredoxin reductase or thioredoxin peroxidase-encoding gene deletion were severely attenuated in their ability to grow in rice cells and failed to produce spreading necrotic lesions on leaf surfaces (Fernandez and Wilson, 2014). In this study, we also generated the ΔMotrr1 deletion mutant. The ΔMotrr1 mutant had less severe defects than the Δtrx2 or Δtrx1 Δtrx2 mutants, suggesting that the ΔMotrr1 mutant still had low levels of thioredoxin activities. It is possible that other closely related enzymes have low capacity to reduce thioredoxins in the presence of MoTrr1. On the other hand, it could simply be because some of the thioredoxin functions are solely dependent on proteins but not related to the reduced diithiol-form (Trx-SH). Overall, our results showed that Trx2 is the major thioredoxin in M. oryzae. The Δtrx2 mutant had a more severe phenotype than the Δtrx1 mutant in conidiation, appressorium formation, appressorium penetration, and plant infection. Based on the phenotypes of the Δtrx2 and Δtrx1 Δtrx2 mutants and transformants of Δtrx1 Δtrx2 expressing the dominant active MST7 allele, TRX2 may regulate the activation of Pmk1 MAPK via its effects on Mst7. In M. oryzae, thioredoxins may be important for intracellular ROS signalling and pathogenesis.

**Experimental procedures**

**Strains and culture conditions**

The wild-type, mutant strains, and different transformants of M. oryzae used in this study are listed in Table 1. Conidiation and growth rate were assayed with OTA cultures as previously described (Xue et al., 2002; Li et al., 2004). Vegetative hyphae harvested from 2-day-old 5xYEG (0.5% yeast extract and 2% glucose) or CM were used for isolation of DNA, RNA, and proteins (Zhou et al., 2011b). To assay for defects in stress responses, growth rate was measured with cultures grown on CM with 0.7 M KCl, 0.5 M sorbitol, 300 µg ml⁻¹ Congo red, 200 µg ml⁻¹ Calcofluor, 6 mM H₂O₂, or 2.0 mM diamide (Zheng et al., 2012).

**Generation of the TRX gene replacement constructs and mutants**

The double-joint PCR approach (Kim et al., 2012) was used to generate the TRX1 and TRX2 gene replacement constructs. The upstream and downstream flanking sequences of TRX1 were amplified with the primer pairs F1/R2 and F3/R4 (Supporting Information Table S1) respectively. The full-length neomycin-resistance gene was amplified from pFL2 vector (Zhou et al., 2011a). The resulting PCR products were used as the templates for double-joint PCR to generate the TRX1 gene replacement construct, which was transformed into protoplasts of wild-type strains Guy11 and 70-15. Putative Δtrx1 deletion mutants were identified by PCR and further confirmed by Southern blot analysis (Supporting Information Fig. S2).

Similar strategies were used to generate the trx2 deletion construct, with the hygromycin-phosphotransferase (hph) gene as the selectable marker. Products from double-joint PCR were then transformed into protoplasts of Guy11, 70-15, or the Δtrx1 mutant A52G. Putative Δtrx2 deletion or Δtrx1 Δtrx2 double mutants identified by PCR were confirmed by Southern analysis (Supporting Information Fig. S2).

**Generation of the TRX1-, TRX2- and PMK1-GFP fusion constructs**

The full-length TRX1 gene, along with its 1.5-kb promoter region was cloned into pDL2 that carries the hph cassette by yeast gap repair as described (Zhou et al., 2011a). The
resulting TRX1-GFP fusion construct was confirmed by sequencing analysis and transformed into protoplasts of mutant A52G. Transformants resistant to both neomycin and hygromycin were screened by PCR and examined for GFP signals. Similar strategies were used to generate the TRX2-GFP and PMK1-GFP construct with the neomycin-resistance gene amplified from pFL2 (Zhou et al., 2011a) and transformant of the Δtx2 mutant B9G. The PMK1-GFP construct also was transformed into protoplasts of Guy11 as a control.

Appressorium formation, penetration, and plant infection assays

Conidia were harvested from 12-day-old OTA cultures and re-suspended in sterile distilled water to 10^6 conidia ml^-1 (Liu et al., 2011). For appressorium formation assays, drops of 50 μl of conidial suspensions were placed on cover slips or GelBond membranes (Cambrex, East Rutherford, NJ) and incubated at 25°C (Liu et al., 2011). Appressorium formation assays with hyphal tips were performed as described (Liu et al., 2010b). Two-week-old seedlings of rice cultivar LTH or CO-39 and 8-day-old seedlings of barley cultivar Golden Promise were used for infection assays with conidia (5 x 10^5 conidia ml^-1) re-suspended in 0.25% gelatin (Park et al., 2002; Tucker et al., 2004; Kong et al., 2012). For assaying hyphal growth and conidiation with wound-inoculated leaves, segments of barley leaves with the wound sites were excised, surface sterilized and incubated in a moisture chamber under constant light as described (Xu and Hamer, 1996; Ding et al., 2010). Penetration assays with onion epidermis and barley or Brachypodium distachyon leaves were performed as described (Tucker et al., 2004; Kankanala et al., 2007).

Complementation of the yeast Δtx2 mutant

The S. cerevisiae Δtx2 deletion mutant was derived from strain BY4741 (Open Biosystems, Huntsville, AL). The full-length TRX1 and TRX2 genes were amplified from first strand cDNA of strain Guy11 and cloned into pYES2 (Invitrogen) respectively. The resulting constructs were transformed into the Δtx2 mutant with the alkali-cation yeast transformation kit (MP Biomedicals, Solon, OH). Ura3 markers transformed were confirmed by PCR and assayed for sensitivity to 0.8 mM tert-butyl hydroperoxide as described (Garrido and Grant, 2002).

Generation of the MST7-3xFLAG and MST7-GFP fusion constructs and mutant alleles of MST7

The MST7-3xFLAG fusion construct (pM7F) was generated by cloning the MST7 gene into pFL7 by yeast gap repair (Zhou et al., 2011a). The same approach was used to generate the MST7-GFP construct. The QuickChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) was used to introduce the C114A, C136A, C201A, and C305A mutations in the MST7-3xFLAG vector pM7F. After transformation of protoplasts of the mst7 mutant ZH32 (Zhao et al., 2005), wild-type Guy11, or Δtx2 mutant B9G, transformants expressing individual MST7 fusion or mutant constructs were identified by PCR and confirmed by western blot analysis.

References


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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. Trx1 and Trx2 of Magnaporthe oryzae. A schematic drawing of the Trx1, Trx2, and MGG_08147 with the TRX1, DUFX682, PUL (PLAP, Ufd3p and Lub1p), and PPPDE putative peptidase domains labelled. B. Phylogenetic relationship of Trx1, Trx2, and their orthologs. The amino acid sequences of Trx1 from Magnaporthe oryzae (MGG_15004 and MGG_04236), Saccharomyces cerevisiae (SCTR1X, SCTR2X and SCTR3X), Schizosaccharomyces pombe (SPAC7D4.07c and SPBC12D12.07c), Aspergillus nidulans (ANID_00170 and ANID_08571), Botrytis cinerea (BC1G_14403), Sclerotinia sclerotiorum (SS1G_08534), Ustilago maydis (UM06521 and UM01370), Candida albicans (CAWG_02294 and CAWG_04484), and Neurospora crassa (NCU05731, NCU06556 and NCU00598), were analysed by the PhyML3.0 (http://www.atgc-montpellier.fr/phyml/).

Fig. S2. The TRX1 and TRX2 deletion constructs and mutants. A. The TRX1 gene replacement event and Southern blot analysis. Blots of KpnI-digested DNA of Guy11, Δtrx1 mutants (A44G, A52G), and an ectopic transformant (Ect1) were hybridized with a TRX1 fragment amplified with F5 and R6 (Probe 1) or a nedR neomycin resistance gene (Probe 2). B. The TRX2 gene replacement event and Southern blot analysis. Blots of EcoRI-digested DNA of Guy11, Δtrx2 mutant (B9G), Δtrx1 Δtrx2 double mutant (D65), an ectopic transformant (Ect2) of A52G were hybridized with a TRX2 fragment (Probe 3) or a hph fragment amplified with H850 and H852 (Probe 4). The Δtrx2 and Δtrx1 Δtrx2 mutants lacked the wild-type 8.0-kb band hybridized to probe 3 but had the expected 7.7-kb band hybridized to probe 4.

Fig. S3. Expression and localization of Trx2-GFP fusion proteins in appressoria formed on hyphal tips by the Δtrx2/Δtrx2-GFP transformant B9G-C.

Fig. S4. Assays of TRX1 and TRX2 expression by qRT-PCR. A. The abundance of TRX1 and TRX2 transcripts in Guy11 was assayed with RNA isolated from conidia (con), 12 h appressoria (ap), and infected rice leaves 7 dpi (in). Their relative expression levels in conidia were arbitrarily set to 1. Mean and standard deviation were calculated with data from three independent replicates.

Fig. S5. The expression levels of TRX1 and TRX2 in Guy11 treated with or without 6 mM H2O2. Their relative expression level in Guy11 was set to 1. Mean and standard deviation were calculated with data from three independent replicates.

Fig. S6. The expression levels of TRX1 and TRX2 in the wild type and Δtrx1 or Δtrx2 mutant was assayed with RNA isolated from vegetative hyphae. The relative expression level in Guy11 was set to 1. Mean and standard deviation were calculated with data from three independent replicates.

Table S1. PCR primers used in this study.