Thioredoxin1 regulates conidia formation, hyphal growth, and trap formation in the nematode-trapping fungus *Arthrobotrys oligospora*

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Abstract

Purpose *Arthrobotrys oligospora*, a model nematophagous fungus that produces specific adhesive networks to capture nematodes, has been proposed as a potentially effective biological agent to control harmful plant-parasitic nematodes. Although thioredoxin has been characterized as playing important roles in many cellular processes in other species, its function in nematophagous fungi has not been studied. Here, the function of a thioredoxin homolog, Aotrx1, was investigated in *A. oligospora*.

Methods The encoding gene of Aotrx1 in the nematophagous fungus *A. oligospora* was knocked out by homologous recombination; strain growth was assessed.

Results The Δ Aotrx1 strain of *A. oligospora* showed a significant decrease in growth rate on different media (PDA, CMY, and TG), a 70% decrease of conidia production, and a lower germination rate compared with the wild type. The mutant strain was unable to form traps to capture nematodes and was more sensitive to SDS and H₂O₂.

Conclusion Thioredoxin is involved in conidia development, trap formation, normal mycelial growth, and resistance to environmental stresses in the nematode-trapping fungus *A. oligospora*.

Keywords *Arthrobotrys oligospora* · Thioredoxin1 · Conidiation · Nematicidal activity · Phenotypic characteristic

Ji-ai Wang and Xiaowei Huang contributed equally to this work.

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Introduction

Thioredoxin (Trx) is a major multifunctional protein containing a conserved redox-active disulfide/dithiol sequence: –Trp–Cys–Gly–Pro–Cys. This ubiquitous disulfide reductase serves as an electron donor for diverse enzymes (Arnér and Holmgren 2000). It can break disulfide bridges of target proteins and, rarely, promote the formation of disulfide bridges (Sahrawy et al. 1996). Consequently, its expression can directly cause the reduction of disulfide groups in proteins (Belozerskaia and Gessler 2007). Together with thioredoxin reductase and NADPH, Trxs form the “thioredoxin system” that can balance the levels of sulfhydryl groups and disulfides to maintain redox homeostasis (Arnér and Holmgren 2000). The thioredoxin system is highly conserved and is involved in a variety of important physiological processes throughout prokaryotic and eukaryotic cells (Thon et al. 2007). In 1964, Trx was first discovered in *Escherichia coli*, acting as an electron donor for ribonucleotide reductase (Laurent et al. 1964). In the pathogenic microorganism *Cryptococcus neoformans*, Trx

was found to contribute to virulence (Missall and Lodge 2005). In mammalian cells, the lack of cytosolic Trx causes embryonic death. In addition, Trx is known to play important roles in defense against oxidative stress (Arnér and Holmgren 2000); participates in programmed cell death (Ravi et al. 2005), inflammatory responses (Nakamura et al. 2005), lifecycles of viruses and phages (Holmgren 1989), and breast cancer development (Bhatia et al. 2016); and acts as an S-desulphydrase (Collet and Messens 2010).

Previous studies of Trx in the model fungus *Aspergillus nidulans* demonstrated that deletion of the encoding gene *trxA* caused decreased vegetative growth, increased catalase activity, and failure to form reproductive structures such as conidiophores or cleistothecia (Thon et al. 2007). Interestingly, in *Beauveria bassiana*, the yield of conidia was found to decrease by 42% in a *trx2* deletion strain but increase by 21% in a *trx4* deletion strain (Zhang et al. 2015).

In this study, we were interested in the potential roles of Trx in *Arthrobotrys oligospora*, a widely distributed nematode-trapping fungus which produces adhesive networks to capture nematodes. The gene encoding thioredoxin 1 (*Aotrx1*) was knocked out of *A. oligospora* by homologous recombination. The knockout strain showed significant changes in many morphological and physiological characteristics, such as conidia yield, hyphal growth, trap formation, and nematocidal activity, suggesting important roles of Trx 1 in *A. oligospora*.

Materials and methods

Strain and growth conditions

The wild-type (WT) strain of *A. oligospora* (ATCC24927) was purchased from the American Type Culture Collection

and maintained on potato dextrose agar (PDA). This fungus was originally isolated from soil in Sweden and provided to ATCC by Nordbring-Hertz. The nematode *Caenorhabditis elegans* was maintained in oatmeal water agar medium.

Gene knockout of *Aotrx1* from *A. oligospora*

The *A. oligospora* gene *Aotrx1* (GenBank XM_011125943.1) was knocked out by using a gene replacement method described previously (Colot et al. 2006). The hygromycin cassette (*hph*) was obtained from plasmid pSCN44 using primers *hph*-F and *hph*-R (Staben et al. 1989). Partial upstream and downstream *Aotrx1* sequences were amplified using primers *Aotrx1*-5F/5R and *Aotrx1*-3F/3R (Table S1). These three fragments were inserted into the yeast shuttle vector pRS426 (a gift from Prof. KA Borkovich, University of California) (Christianson et al. 1992). Then, the PCR fragment containing the gene replacement cassette was introduced into protoplasts of the WT *A. oligospora* strain and hygromycin-resistant transformants were selected (Margolin et al. 1997). PCR and Southern blotting were used to confirm the *Aotrx1* deletion.

Comparison of growth rates between the WT and $\Delta Aotrx1$ strains

The WT and $\Delta Aotrx1$ strains were incubated on PDA (200 g/L potato, 20 g/L dextrose, 20 g/L agar), TYGA (10 g/L tryptone, 5 g/L yeast extract, 10 g/L glucose, 5 g/L molasses, 20 g/L agar), CMY (20 g/L maizena, 5 g/L yeast extract, 20 g/L agar), and TG (10 g/L tryptone, 10 g/L glucose, 20 g/L agar) media, respectively. Colony morphology and growth rate were observed for 7 days.

Fig. 1 Knockout and verification of *Aotrx1* deletion in *Arthrobotrys oligospora*. **A** Illustration of the method for replacement of *Aotrx1*. Primers 5F/5R and 3F/3R were used to amplify 5'- and 3'- flanking fragments of *Aotrx1*, and YF/YR were used for identification of mutants. P indicates the probe and *Xho*I was the endonuclease used for Southern

blot analysis. **a** The wild type (WT); **b** the mutant. **B** Verification of *Aotrx1* deletion by PCR. The 2078-bp fragment proved the success of the *Aotrx1* knockout (WT 1391 bp). **C** Southern blot confirming the knockout in $\Delta Aotrx1$

Fig. 2 Decreased growth rate of strain $\Delta Aotrx1$ compared with WT. **a–c** $\Delta Aotrx1$ did not grow well on PDA, CMY, and TG media. **d** There was little impact on growth of the $Aotrx1$ deletion strain on TYGA medium. **e**

Morphological features of aerial hyphae of $\Delta Aotrx1$ and WT strains on different growth media

Comparison of conidia yield between the WT and $\Delta Aotrx1$ strains

The WT and $\Delta Aotrx1$ strains were respectively incubated on CMY medium at 28 °C for 15 days, and then, mycelia with the

same biomass were collected and filtered through six layers of lens tissue to collect the conidia. The conidia in suspension were counted using a hemocytometer (Xie et al. 2012). The germination rate of conidia and the mycelial morphology were characterized at 24 and 48 h.

Fig. 3 Conidiation and morphological characteristics of spore germination. **a** Conidiation of the mutant showed an obvious decrease compared with the WT strain ($P < 0.001$). **b** Germination of spores at 24 and 48 h, which showed the lower ability of strain $\Delta Aotrx1$

Induction of trap formation

To analyze trap formation ability, both urea and nematodes were employed to induce traps. Firstly, conidial suspensions of the WT and $\Delta Aotrx1$ strains were spread over a water agar plate with urea (500 mg/L) and then induced at 28 °C for 16 days. In addition, approximately 10^4 conidia of the WT and $\Delta Aotrx1$ strains were inoculated on CMA medium and incubated at 28 °C. After 4–5 days of growth, about 200 adult nematodes were added to the *A. oligospora* mycelia. The traps were counted at specified time intervals using a light microscope (Olympus, Tokyo, Japan).

Effect of different stress conditions on the growth of the WT and $\Delta Aotrx1$ strains

To determine whether Trx plays a role in oxidative and detergent stress tolerance in *A. oligospora*, fresh mycelial plugs of WT and $\Delta Aotrx1$ were inoculated onto TYGA containing 5, 10, and 15 mM H_2O_2 , and 0.01, 0.02, and 0.03% SDS, respectively. The diameters of colonies were measured on the seventh day.

Statistical analysis

Three biological replicates were performed for each experiment, and all data were analyzed using GraphPad Prism version 5 (GraphPad Software, USA). P values < 0.05 were considered significant. Error bars indicate standard deviations (SD).

Results

Knockout and verification of the deletion of the *Aotrx1* gene from *A. oligospora*

pRS426 containing the *hph* gene as well as partial upstream and downstream sequences of *Aotrx1* was transformed into protoplasts of *A. oligospora* (Fig. 1a). After growing on selective medium, nine hygromycin-resistant clones were obtained (Fig. S1A). Compared with a 1391-bp fragment from the WT strain, a 2078-bp fragment was amplified from one transformant using primers YF/YR (Fig. 1b and Table S1). Sequencing analysis of the PCR product confirmed that the *Aotrx1* genetic locus was successfully replaced by the hygromycin cassette (Fig. S1B). Southern blot analysis using probe P and restriction enzyme *XhoI* detected single bands in both the WT strain and the transformant, and the sizes were consistent with the predicted values (Fig. 1c).

Effect of *Aotrx1* mutation on hyphal growth

To examine whether the disruption of *Aotrx1* influenced the growth of *A. oligospora*, we selected different media including PDA, CMY, TYGA, and TG to culture the WT and $\Delta Aotrx1$ strains. Compared with the WT strain, $\Delta Aotrx1$ showed a significant decrease in growth rate on PDA, CMY, and TG media (Fig. 2a–c), but a relatively small difference was observed on TYGA medium (Fig. 2d). After 7 days of incubation, the colony diameters of the WT strain were up to 6 cm, while those of the $\Delta Aotrx1$ strain were < 2 cm on PDA

Fig. 4 Trap formation and nematocidal activity. **a** Trap formation from spores of WT *A. oligospora* and the $\Delta Aotrx1$ mutant at 65 h and 16 days after urea induction. No traps were observed for the *Aotrx1* mutant strain, while the WT formed traps by 65 h. **b** Spores of the $\Delta Aotrx1$ strain did not form traps when induced by *C. elegans* for 20 h. Nematodes were still alive around $\Delta Aotrx1$ hyphae, while those around the WT strain of *A. oligospora* were killed and digested. The arrows indicate the three-dimensional traps

(Fig. 2a), about 1 cm on CMY (Fig. 2b), and 2 cm on TG medium (Fig. 2c). Moreover, the aerial hyphae of $\Delta Aotrx1$ were sparser, and the colonies were less cottony and thicker than those of the WT strain (Fig. 2e).

Effect of *Aotrx1* mutation on conidiation, trap formation, and nematocidal activity

Compared with conidiation of the WT strain (3.36×10^5 /biomass), the production of conidia in *A. oligospora* was severely decreased by disruption of the *Aotrx1* gene (1.01×10^5 /biomass) (Fig. 3a). Moreover, the *Aotrx1* deletion strain also showed a lower germination rate than the WT strain (Fig. 3b).

To investigate the ability of *Aotrx1* to form nematode traps, both urea and the nematode *C. elegans* were used to induce three-dimensional adhesive networks in the WT and mutant

strains with the same hyphal density. After induction with urea for 65 h, the WT formed traps. However, no trap was observed in the $\Delta Aotrx1$ strain (Fig. 4a). Similarly, the WT strain formed many adhesive networks to capture nematodes within 20 h, but the nematodes were still alive in the *Aotrx1* mutant test, which means that the nematocidal activity of strain $\Delta Aotrx1$ was also reduced (Fig. 4b).

Effect of SDS and H_2O_2 stress on the growth of the WT and $\Delta Aotrx1$ mutant

The growth rate and colony morphology of the WT and the $\Delta Aotrx1$ mutant were compared on TYGA medium with different stress factors (Fig. 5a and b). The mutant exhibited significantly slower growth rates than the WT strain on

Fig. 5 Stress tolerance. **a** Mycelial growth of WT and the $\Delta Aotr1$ knockout strain showed no significant difference on TYGA medium. **b** Colony morphology of $\Delta Aotr1$ and WT strains on TYGA medium

containing sodium dodecyl sulfate (SDS) or H_2O_2 . **c** Statistics on colony diameter of $\Delta Aotr1$ and WT strains on TYGA medium containing different concentrations of SDS or H_2O_2

medium containing 0.01–0.03% SDS and could not grow on medium containing 5–15 mM H_2O_2 (Fig. 5b and c).

Discussion

Trx is extensively distributed from archaea to human and is involved in multiple cellular roles (Arnér and Holmgren 2000). In this study, we characterized the function of a homolog of the Trx encoding gene, *Aotr1*, in the nematophagous fungus *A. oligospora*. Compared with the WT strain, conidia production in the *Aotr1* deletion strain decreased by 70%, and the germination rate also decreased, suggesting that *Aotr1* may be essential for the normal morphological development of conidia and mycelium. It has been reported that the *trxA* deletion strain of *Aspergillus nidulans* is unable to form

conidiophores (Thon et al. 2007), while in *B. bassiana*, the yield of conidia decreased by 42% in a *trx2* deletion strain, but increased by 21% in a *trx4* deletion strain (Zhang et al. 2015). Our results and those of others suggest that Trx plays different roles in different fungal species.

Previous studies have shown that bacteria can release urea to trigger nematode-trapping fungi to form traps to capture nematodes. The deletion of genes related to urea transportation and metabolism abolished urea-inducible trap formation in *A. oligospora* (Wang et al. 2014). Surprisingly, the $\Delta Aotr1$ mutant was unable to produce traps and kill nematodes in the presence of urea. This result suggests that *Aotr1* may participate in some process(es) related to urea transport and metabolism. The fact that the $\Delta Aotr1$ mutant is incapable of forming traps in the presence of *C. elegans* also suggests that *Aotr1* is

likely involved in cell wall biogenesis (Yang et al. 2011). Moreover, it showed inhibitory effects on the mycelial growth of the $\Delta Aotrx1$ strain in a cell wall-perturbing agent (SDS), suggesting that Aotrx1 likely participates in cell wall synthesis by regulating the expression of other genes essential for nematocidal activity of *A. oligospora*. In addition, Trx has been reported to reduce H_2O_2 to H_2O in many organisms (Arnér and Holmgren 2000). In our experiments, tolerance of the *Aotrx1* mutant to H_2O_2 was almost abolished compared with the WT implying the vital role of the thioredoxin system in the cellular response to oxidative stress.

Aside from the processes described above, our results are also consistent with Trx regulating other processes in *A. oligospora*, as found previously in other organisms. In the *Aotrx1* deletion strain, aerial hyphae were sparser, the mycelial growth rate decreased significantly, and the colonies were less cottony and thicker on PDA, CMY, and TG media, which is similar to the phenotype of the *trx1* mutant of *C. neoformans* (Missall and Lodge 2005) and the *trxA* deletion strain of *A. nidulans* (Thon et al. 2007). However, our results were inconsistent with those in the model yeast *Saccharomyces cerevisiae*, which suggested that the thioredoxin system is not essential in normal growth conditions (Grant 2001). Here, a relatively small phenotypic effect of $\Delta Aotrx1$ was observed on TYGA, a nutrient-rich medium. Therefore, Trx may provide some important intermediates for normal fungal growth in the case of nutrient limitation.

Based on our experimental results, Trx is a multi-functional regulator of various physiological processes during asexual reproduction of *A. oligospora*. The regulated phenotypic traits include conidiation, mycelial morphology, spore germination, and trap formation. Trx participates in normal mycelial growth, resistance to environmental stresses, and nematocidal activity. At present, it is not known how Trx regulates these processes. In addition, the functions of other Trxs in this fungus are not clear. Regardless, our results clearly demonstrate the importance of *Aotrx1* in the nematode-trapping fungus *A. oligospora*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals N/A

Informed consent N/A

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