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 $\Delta$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 $\text{H}_2\text{O}_2$.

**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

**Introduction**

Thioredoxin (Trx) is a major multifunctional protein containing a conserved redox-active disulfide/dithiol sequence: $–\text{Trp–Cys–Gly–Pro–Cys}$ . 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 sulphydryl 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 nematicidal 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 Aotrx-1 (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 WTA. 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 ΔAotrx1 strains

The WT and ΔAotrx1 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.
Comparison of conidia yield between the WT and ΔAotrx1 strains

The WT and ΔAotrx1 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. 2 Decreased growth rate of strain ΔAotrx1 compared with WT. a–c ΔAotrx1 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 ΔAotrx1 and WT strains on different growth media
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 \( \Delta \text{Aotrx1} \) 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 \( \Delta \text{Aotrx1} \) 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 \( \text{A. oligospora} \) 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 \( \Delta \text{Aotrx1} \) strains

To determine whether Trx plays a role in oxidative and detergent stress tolerance in \( \text{A. oligospora} \), fresh mycelial plugs of WT and \( \Delta \text{Aotrx1} \) were inoculated onto TYGA containing 5, 10, and 15 mM \( \text{H}_2\text{O}_2 \), 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 \( \text{Aotrx1} \) gene from \( \text{A. oligospora} \)

pRS426 containing the \( \text{hph} \) gene as well as partial upstream and downstream sequences of \( \text{Aotrx1} \) was transformed into protoplasts of \( \text{A. oligospora} \) (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 \( \text{Aotrx1} \) genetic locus was successfully replaced by the hygromycin cassette (Fig. S1B). Southern blot analysis using probe P and restriction enzyme \( \text{XhoI} \) detected single bands in both the WT strain and the transformant, and the sizes were consistent with the predicted values (Fig. 1c).

Effect of \( \text{Aotrx1} \) mutation on hyphal growth

To examine whether the disruption of \( \text{Aotrx1} \) influenced the growth of \( \text{A. oligospora} \), we selected different media including PDA, CMY, TYGA, and TG to culture the WT and \( \Delta \text{Aotrx1} \) strains. Compared with the WT strain, \( \Delta \text{Aotrx1} \) 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 \( \Delta \text{Aotrx1} \) strain were < 2 cm on PDA.
Fig. 2a, about 1 cm on CMY (Fig. 2b), and 2 cm on TG medium (Fig. 2c). Moreover, the aerial hyphae of ΔAotrx1 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 nematicidal 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 ΔAotrx1 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 nematicidal activity of strain ΔAotrx1 was also reduced (Fig. 4b).

**Effect of SDS and H_2O_2 stress on the growth of the WT and ΔAotrx1 mutant**

The growth rate and colony morphology of the WT and the ΔAotrx1 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
medium containing 0.01–0.03% SDS and could not grow on medium containing 5–15 mM H₂O₂ (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, Aotrx1, in the nematophagous fungus A. oligospora. Compared with the WT strain, conidia production in the Aotrx1 deletion strain decreased by 70%, and the germination rate also decreased, suggesting that Aotrx1 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 ΔAotrx1 mutant was unable to produce traps and kill nematodes in the presence of urea. This result suggests that Aotrx1 may participate in some process(es) related to urea transport and metabolism. The fact that the ΔAotrx1 mutant is incapable of forming traps in the presence of C. elegans also suggests that Aotrx1 is...
likely involved in cell wall biogenesis (Yang et al. 2011). Moreover, it showed inhibitory effects on the mycelial growth of the \( \Delta \)Ottrx1 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 nematicidal activity of A. oligospora. In addition, Trx has been reported to reduce \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \) in many organisms (Armér and Holmgren 2000). In our experiments, tolerance of the Aotrx1 mutant to \( \text{H}_2\text{O}_2 \) 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 \( \text{trxA} \) 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 \( \Delta \)Ottrx1 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 multifunctional 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 nematicidal 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.

**Informed consent** N/A

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