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Involvement of FpTRP26, a thioredoxin-related protein, in oxalic acid-resistance of the brown-rot fungus *Fomitopsis palustris*

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Abstract **Brown-rot fungus *Fomitopsis palustris* grows vigorously at high concentrations of oxalic acid (OA), which is fungal metabolite during wood decay. We isolated a cDNA FpTRP26 from *F. palustris* by functional screening of yeast transformants with cDNAs grown on plates containing OA. FpTRP26 conferred a specific resistance to OA on the transformant. OA-content in transformants grown with 2 mM OA decreased by 65% compared to that of the control. The amount of FpTRP26 transcript in *F. palustris* amplified with increasing OA-accumulation, and was maintained at high levels even in the stationary phase. Its transcription in *F. palustris* was inducible in response to exogenously added OA. These results suggest that FpTRP26 is involved in the OA-resistance in *F. palustris*.**

Keywords: Oxalic acid; Wood-rotting fungi; Oxalic acid resistance; Thioredoxin-related protein; *Fomitopsis palustris*

1. Introduction

Oxalic acid (OA), common in plants, fungi, and animals, plays a wide variety of roles in ecosystems [1]. As far as physiological effects of OA on organisms, OA potentially inhibits growth of organisms. For example, OA produced by symbiotic ectomycorrhizal fungi is proposed to be an antifungal compound contributed to disease-resistance of the host woody plants, which is based on in vitro results showing that 0.2 mM of OA caused 43% suppression of the sporulation of *Fusarium oxysporum* [2], and 20 mM of OA inhibited the growth of *Phythium vexans* by 38% [3] compared to controls without OA. Furthermore, OA is widely used to control the mite *Varroa destructor* in honeybees in Europe and Canada [4]. OA has a number of known biochemical properties: (1) induction of reactive oxygen in cells to disrupt the cell membrane [5]; (2) it is one of the strongest carboxylic acids to decrease the pH value; (3) strong chelating agent to precipitate many divalent metal ions; and (4) electron source [6]. However, it is still unclear how these features contribute exactly to growth inhibitory effects of OA on organisms.

On the other hand, brown-rot fungi, which cause severe damage to wooden structures, grow even in the presence of large amounts of OA produced by fungus [7]. For example, in liquid culture, the brown-rot basidiomycete *Fomitopsis palustris* ferments OA to acquire energy for growth [6,8], and grow vigorously concomitantly with an accumulation of 30–78 mM OA resulting in a pH decrease from 5 to 2.5–2.0 [6]. Because the concentration is significantly greater than those showing growth inhibition mentioned previously [2,3], under such conditions several organisms probably experience some level of growth inhibition.

Therefore, *F. palustris* must have a strong OA-resistance system to prevent any toxicity effects by intra- and extra-cellular OA. Another wood-rot white-rot fungus is known to have an OA-resistance system [9–11], whereas the system has not been studied in *F. palustris*.

Therefore, we were motivated to isolate *F. palustris* cDNA involved in OA-resistance. In order to isolate the cDNA, we screened a yeast transformant with cDNAs surviving on an OA-containing plate. We isolated a cDNA encoding thioredoxin-related protein 26 kDa, and thus named it *FpTRP26*. FpTRP26 conferred resistance specifically to OA on the yeast transformant, suggesting that this polypeptide contributes to the OA-resistance in *F. palustris*.

2. Materials and methods

2.1. Culture conditions of F. palustris

Two plugs of mycelia (5 mm in diameter) of *F. palustris* TYP6137 (Berkely et Curtis Murill) were grown as stationary culture in a 200-ml Erlenmeyer flask with 40 ml liquid medium as previously reported [12].

2.2. Cloning of F. palustris cDNA conferring oxalic acid-resistance

A *F. palustris* cDNA library was constructed by TAKARA BIO INC. with yeast expression vector pDR196 [13] using mRNA extracted from 5 day-old *F. palustris* mycelia. The cDNA library thus prepared was introduced into a *Saccharomyces cerevisiae* AD (1–8) strain (Δ*yor1*, Δ*snq2*, Δ*pdr5*, Δ*pdr10*, Δ*pdr11*, Δ*ycf1*, Δ*pdr3*, Δ*pdr*) [14] by the lithium acetate method. Transformants selected on SD plates lacking uracil, SD(-Ura), were replicated on a 12 mM OA-containing SD(-Ura) plate and grown for 5 days at 30 °C (first screening). Clones obtained were transferred into liquid SD(-Ura) and grown overnight. Cell densities were adjusted to OD_{600} 0.5, and cells (10 μl each) were spotted on the same OA-containing SD(-Ura) (second screening). Because the cDNA fragment recovered from OA-resistance yeast

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Abbreviations: OA, oxalic acid; ROS, reactive oxygen species; a.a., amino acid; GLOX, glyoxylate; NBT, nitrobluetetrazolium

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showing a strong phenotype lacked the 5′ region, the 5′ end of the cDNA was determined by PCR with primers of sense (5′-GAAAGAAAAAAAATATACCCCAGC-3′) and antisense (5′-TTGAGGTCGCTAATAGTGGGGAAG-3′), which anneal at PMA-promoter in the pDR196 vector [13], and an internal sequence of the cDNA, respectively, using pDR196 cDNA library as template. Subsequently, the coding region of the cDNA was amplified with primers of sense (5′-GGAATTCATGTCTCATCACCACGGCC-3′) and antisense (5′-CTCGAGGCGTTGCATGTTGGG-3′), which were gene specific primers containing *Eco*RI and the *Xho*I restriction site, respectively. The coding region thus obtained was named *FpTRP26*.

2.3. Characterization of FpTRP26 for oxalic acid resistance

The plasmid containing *FpTRP26* was re-introduced into *S. cerevisiae* AD (1–8) strain to characterize their OA-resistance. An empty vector without insert was used as control. Cells were grown until the logarithmic growth phase, adjusted to OD_{600} 1.0, and subsequently diluted to 1/2, or 1/4 with SD(-Ura) liquid medium. Next, 10 μl of each diluted culture was spotted on SD(-Ura) solid medium with (0–12 mM) OA. The cultures were incubated at 30 °C for 4 days. The OA-resistance was determined by the growth of yeast transformant. Similarly, the transformants were cultivated separately on SD(-Ura) plates containing different HCl concentrations (pH 1.5, 1.6, 1.7), and H_2O_2 concentrations (1.0, 1.25, 1.5 mM).

2.4. Quantification of oxalic acid

OA in culture medium of *F. palustris* and in yeast cells was quantified by a commercial kit (Roche, Germany) [10] and GC–MS analysis [15], respectively. Yeast (OD_{600}0.1) was cultured at 30 °C in 50 ml of SD(-Ura) liquid medium containing 2 mM OA until OD_{600} 1.0–2.0. They were harvested at 1000 × *g* for 10 min and washed twice with cold distilled water. After cells were lyophilized for 5 h, their dry weight was determined. To the dried cells, 250 μl of 1 N HCl and 600 μl of ethylacetate was added, and cells were homogenized using glass beads (Toshinriko, No. 04) for 4 min. The OA extracted with ethylacetate was quantified as previously described [15] with a slight modification. GC–MS (EI) was performed on a Shimadzu GC–MS QP-5050A, column: CBP1-M25-025, 25 m × 0.22 mm (i.d.), column temperature: 80–240 °C (8 °C/min), carrier gas: He, carrier gas flow rate: 0.8 ml/min.

2.5. Detection of superoxide by nitrobluetetrazolium assay

The amount of superoxide in yeast cells was determined by the nitrobluetetrazolium (NBT) method [16]. Cells were cultured in 50 ml of SD(-Ura) medium containing 2 mM OA from an OD_{600} of 0.1 at 30 °C. Cells were cultured until OD_{600} of 1.0–2.0. They were then harvested as described in Section 2.4. Cultured cells were homogenized in 700 μl of 50 mM Tris–HCl buffer (pH 7.5) using glass beads for 4 min then centrifuged at 12000 × *g* for 10 min at 4 °C to remove cell debris. The supernatant (400 μl) was mixed with 100 μl of 6 mM NBT solution, and was incubated for 1 h at room temperature. The amount of superoxide of the samples was compared based on the absorbance at 560 nm. The amount of protein was determined by the Bradford method [17] with bovine serum albumin as a standard.

2.6. Real-time quantitative PCR analysis of gene transcription

Total RNA was isolated from *F. palustris* mycelium using a RNasy Plant Mini Kit (Qiagen) and applied (0.2 μg) to first-strand cDNA synthesis performed using Superscript II reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed using a 7300 Real Time System (Applied Biosystems). The SYBR Green (Applied Biosystems) was used to detect amplicons. The quantifications of the amplicons were done based on standard curves prepared for each of target cDNAs. The gene-specific primers: 5′-GACAATGTCATAGCCCTCAATGC-3′ and 5′-GCTGATCCCAGGGTTTGATG-3′, were used to generate a 69 bp-amplicon for *FpTRP26* transcripts. The amount of transcripts was normalized by comparison with those of a 75 bp-amplicon derived from 28S rRNA (GenBank Accession No. AY515333) with primers (5′-TGACACGGACTACCAGTGCTTT-3′ for sense; 5′-CACCCATTTTGAGCTGCATTC-3′ for antisense), or total RNA.

Furthermore, for comparison, a 64 bp-amplicon for *FPICL1* transcripts (GenBank Accession No. AB079254) was generated with primers (5′-TCTGGGCGTCGCTCACA-3′ for sense, 5′-GTCAAGGGCGCCGTATGT-3′ for antisense). Separately, the amount of *FpTRP26* transcript in *F. palustris* was compared between the addition of H_2O (control) and 100 mM OA after 12-h incubation at the culture phase on day 3.

2.7. Statistical analysis

Analyses were carried out by Student's *t*-test. *P* values < 0.05 are regarded significant.

3. Results and discussion

3.1. Cloning of F. palustris cDNA conferring oxalic acid-resistance on yeast transformant

In the first functional screening, approximately 50 yeast clones showing OA-resistance were obtained from 1.0×10^6 transformants with a primary *F. palustris* cDNA library (1.0×10^6 cfu). We sequenced eight different cDNAs obtained by the second screening. However, a BLASTp (http://www.ncbi.nlm.nih.gov/BLAST/) analysis showed that there was no cDNA showing any similarity with proteins involved in OA-degrading enzymes, such as oxalate decarboxylase and oxalate oxidase. Neither was there any similarity with OA-transporting proteins such as the oxalate:formate exchange protein of *Oxalobacter formigenes* [18] or the multifunctional anion exchanger SLC26 found in humans [19]. Accordingly, we proceeded to clarify the function of the protein encoded by cDNA obtained from a transformant showing a strong phenotype on the OA-containing plate. The coding region of cDNA (654 bp) obtained by PCR coded a deduced 25,940 Da protein. A BLASTp search revealed that the obtained cDNA showed a similarity with thioredoxin-related proteins 26 kDa (TRP26). Analysis with the conserved domain architecture retrieval tool (http://www.ncbi.nlm.nih.gov/Structure/sdd/sdd.shtml) showed that TRP26 members contain a Domain of Unknown Function 1000 (DUF 1000), but not a thioredoxin (Trx) family domain containing a Trx active site, whereas another thioredoxin-related proteins 32 kDa (TRP32) contains both. Our deduced protein contained DUF 1000, but no Trx family domain. Therefore, we named the cDNA obtained *FpTRP26* (*F. palustris*, thioredoxin-related protein, 26 kDa, GenBank Accession No. AB275458). Due to the presence of a Trx domain in TRP32, TRP32 has been proposed to be a cytoplasmic regulator of the redox state in higher eukaryotes [20], and involved in the cellular response against glucose deprivation [21]. However, no possible role of TRP26 members has been reported. Thus, we have characterized the transformant with *FpTRP26* (*FpTRP26*-transformant) regarding its resistance to OA.

3.2. Characterization of FpTRP26 regarding oxalic acid-resistance of the yeast transformant

On plates containing 8.5–10 mM OA, growth was observed for the *FpTRP26*-transformant, whereas no growth was observed for the control group (Fig. 1A and B). At OA-concentration below 8.5 mM both control and *FpTRP26*-transformants grew with no difference. In order to eliminate the possibility that FpTRP26 was resistant to low pH (2.5–2.2) but not specifically to OA, we investigated growth of the *FpTRP26*-transformant on a HCl-containing plate (Fig. 1C). No difference in growth between the control and *FpTRP26*-transformant was observed at pH 1.5–1.7, and pH 5. Next, we investigated if FpTRP26 conferred its resistance to