NOTE

Cloning and Characterization of a Thioredoxin Gene, CpTrx1, from the Chestnut Blight Fungus Cryphonectria parasitica

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A differential display for the expression profiles of wild-type Cryphonectria parasitica and its virally-infected isogenic hypovirulent strain revealed several transcripts of interest, which evidenced significant matches with fungal genes of known function. Among which, we have further analyzed an amplified PCR product with significant sequence similarity to the known fungal stress-responsive thioredoxin gene from Neurospora crassa. The product of the cloned thioredoxin gene, CpTrx1, consists of 117 amino acids, with a predicted molecular mass of 13.0 kDa and a pI of 5.4. Sequence comparisons demonstrated that the deduced protein sequence of the CpTrx1 gene evidenced a high degree of homology to all known thioredoxins, with the highest degree of homology with trx1, a thioredoxin gene from Saccharomyces cerevisiae, and evidenced a preservation of the conserved hall mark-residues (Trp-Cys-Gly-Pro-Cys) at the active site of thioredoxin. The E. coli-generated CpTRX1 manifested thioredoxin activity, according to the insulin reduction assay, which indicates that the cloned gene does indeed encode for the C. parasitica thioredoxin.

Keywords: Cryphonectria parasitica, hypovirulence, thioredoxin

Cryphonectria parasitica (Murrill) Barr, the agent causative of chestnut blight, was responsible for the destruction of the chestnut forests of North America in the early 20th century (Van Alfen, 1982). Furthermore, disease severity has been becoming greater in some areas of Korea, in which chestnut blight has traditionally presented only minor problems. The results of recent studies have demonstrated that more than 30% of necrotic lesions in chestnut trees in Korea evidenced C. parasitica infection, and the putative mycovirus-containing strains were detectable among the Korean population of C. parasitica (Ju et al., 1999). In addition, several disease severity variations were observed among the currently available chestnut varieties, which had been considered to have been resistant (Lee et al., 1999).

Strains harboring double-stranded (ds) RNA viruses exhibit characteristic symptoms of lowered virulence,

a phenomenon which is referred to as hypovirulence, (Van Alfen et al., 1975; Anagnostakis, 1982; Nuss, 1992) as well as a variety of hypovirulence-associated phenotypic changes, including reduced sporulation, pigmentation, laccase production, and oxalate accumulation (Havir and Anagnostakis, 1983; Elliston, 1985; Rigling et al., 1989). The molecular bases for these diverse but specific changes result in alterations in host transcriptional profiles in response to hypoviral infection (Kazmierczak et al., 1996; Allen et al., 2003; Allen and Nuss, 2004). Although the specific relationships between the development of each symptom and a limited set(s) of fungal genes expressed aberrantly in the hypovirulent strain remain to beelucidated, it appears that a broad but specific set(s) of fungal genes may be modulated via hypoviral infection. Accordingly, several studies have demonstrated that hypoviral perturbation of the fungal signal transduction pathway may function as a means for the coordination of the control of specific set(s) of fungal genes during the development of viral symptoms (Chen et al., 1996; Gao and Nuss, 1996; Kasahara

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and Nuss, 1997; Kim et al., 2002; Park et al., 2004).

In previous studies regarding transcriptional profiles using RNA differential displays (Kang et al., 1999), it was suggested that, among the genes modulated by the presence of the hypovirus, more genes are waiting to be characterized. In addition, recent microarray studies have expanded the number of identified hypoviral-specific gene sets, from less than 20 to nearly 300 (Allen et al., 2003). These sets represent a broad spectrum of biological functions, including stress responses, carbon metabolism, and transcriptional regulation. Among these, the stress responsive pathway was of specific interest. This is because neither a large number of additional nor classical host genes, including heat shock protein 70 (HSP70) and glutathione S-transferase (GST), have been selected as other putative targets for viral regulation, suggesting that it may not be a general, but rather a specific function (Allen et al., 2003; Allen and Nuss, 2004).

Thioredoxin (TRX) is a small, ubiquitous, multifunctional protein, with a conserved active site sequence (Trp-Cys-Gly-Pro-Cys) that catalyzes a variety of redox reactions via reversible redox-active disulfide/dithiol. When thioredoxin is in a reduced state, Trx-$(SH)_2$, the two active-site cysteins, form a dithiol group able to catalyze the reduction of disulfides in a number of proteins. Oxidized thioredoxin (Trx-S_2) can be reduced by NADPH via the catalytic action of the flavoenzyme, thioredoxin reductase (Trr). Thus, Trx, Trr and NADPH form a system that functions as a general disulfide reductase system. Thioredoxin was identified initially in E. coli as a hydrogen donor for ribonucleotide reductase, an enzyme essential to DNA synthesis (Laurent et al., 1964). Since then, genes encoding for TRX have been detected in a variety of sources (Cho et al., 2001). Eukaryotic TRX has been implicated in a wide range of biological functions, including protein folding and regulation, dehydroascorbate reduction, the repair of oxidatively damaged proteins, and sulphur metabolism (Trotter and Grant, 2002). Considering the multiple functions of TRX in living cells, the regulation of TRX genes may perform an important role in its biological functions. Moreover, the fact that host TRX is an essential accessory protein for viral polymerase makes it of interest in determinations of whether the hypovirus modulates TRX gene expression.

In this study, we describe the cloning and characterization of a gene encoding for a thioredoxin from the virus-free wild type C. parasitica strain, EP155/2 (ATCC 38755). The methods used in the preparation of the primary inoculum for liquid cultures, as well as the relevant culture conditions, have been previously described (Puhalla and Anagnostakis, 1971; Kim et al., 1995).

RNA differential display, using an ordered differential display using RT-PCR (ODD-PCR) resulted in the cloning of a 350 bp PCR amplicon which evidenced a high degree of homology with all known thioredoxin genes (Kang et al., 1999). The cloned amplicon was radioactively labeled and utilized as a probe for the screening of a genomic λ library of C. parasitica, in order to generate a full-length genomic clone (Kim et al., 2002). Among the total of 10,000 screened plaques, two evidenced profound hybridizing results, and a 2.0 kb ApaI-BamHI digested λ DNA fragment harboring a full-length thioredoxin was selected and sub-cloned into pBSSKII for further analysis. Based on the results of genomic sequence analysis, the nearly full-length cDNA clone was generated via RT-PCR using the primer pairs CpTrx-mF1 and CpTrx-mR1 at nucleotide positions (nt) -81 to -64 and nt 652 to 668 (relative to the start codon), respectively. The resultant 526 bp amplicon was then cloned into pGEM-T vector. A sequence comparison with the corresponding genomic sequence showed that the CpTrx1 gene consisted of three exons, with two intervening sequences of 94 bp and 129 bp. A sequence analysis of the cDNA clone revealed one long open reading frame (ORF) of 354 bp, which started at the first methionine codon (ATG, 800 nt position) and ended at a stop codon (TAA, 1,376 nt position). The deduced CpTrx1 protein product (CpTRX1) was comprised of 117 amino acids, with an estimated molecular mass of 13.0 kDa and a pI of 5.4 (the GenBank accession number for CpTrx1 is DQ346658). A primer extension experiment demonstrated that the transcription start site of CpTrx1 was located 85 bp upstream of the putative start codon. We discovered two consecutive CAAT boxes at −20 nt in the promoter region of CpTrx1. However, no canonical TATA box was detected. The sequence surrounding the first ATG was in good agreement with Kozak's consensus sequence, in that the nt −3 position was the A in CAAGATG. The 5'-end, 3'-end, and internal consensus sequence of the introns were compared to the consensus intron splice signals of S. cerevisiae, N. crassa, and C. parasitica (Robert et al., 1988; Choi et al., 1992; Kim et al., 2002). The 5'-ends and 3'-ends of both intron structures, with the exception of the 3' end of the 5' slice site of intron 2, matched closely with the known consensus sequences, 5'-GTRRGT-3' and 5'-YAG-3' of C. parasitica, respectively (Choi et al., 1992; Zhang et al., 1994). The internal consensus sequence of 5'-NCTRAC-3' was observed at 32 bp and 22 bp upstream from the 3'-ends of introns 1 and 2, respectively (Gurr et al., 1987; Robert et al., 1988; Choi et al., 1992). In addition, the poly (A) site was located between 92 and 95 bp downstream of the stop codon TAA, and the putative poly (A) signal

structures are shown in Fig. 2A. The sequence comparison of CpTRX1 to other protein thioredoxins revealed a significant number of residues that were identical to those of all other described TRXs, particularly the conserved hallmark sequences of the active site (Trp-Cys-Gly-Pro-Cys) (Eklund *et al.*, 1991). Furthermore, the overall CpTRX1 sequence similarity evidenced a 55% identity, which could be increased further increased to 74% with the positive residues. In addition, our computer analysis revealed a close sequence alignment of *C. parasitica* TRX with other thioredoxins from *M. grisea*, *N. crassa*, *P. anserina*, *S. cerevisiae* (trx1), and *S. pombe* (trx1).

Phylogenetic comparison of fungal Trxs was conducted via UPGMA (unweighted pair group method using arithmetic means) using the Genetyx program (Software Development, Japan). The numbers above the horizontal lines indicate the frequency with which a given branch appears over 1,000 bootstrap replications. The amino acid sequence of the TRXs from *Magnaporthe grisea* (Mgri), *Neurospora crassa* (Ncra), *Podospora anserina* (Pans), *Saccharomyces cerevisiae* (Scer) and *Schizosaccharomyces pombe* (Spom), were analyzed using the GROWTREE algorithm in the GCG software package to generate the phylogram (Fig. 2B). The phylogram showed that the TRXs could be clustered into two subfamilies, one for filamentous fungi and the other for yeast-like fungi. CpTRX1 is a member of the subfamily that includes other filamentous fungal TRXs.

The *CpTrx*1 gene was cloned into the *E. coli* expression vector and expressed in *E. coli*, in order to determine whether or not the cloned *CpTrx*1 gene evidenced functional TRX activity. The full-length *CpTrx*1 protein product, CpTRX1, was expressed in *E. coli* as a hexahistidine fusion protein, and was purified via nickel affinity chromatography, in accordance with the manufacturer's instructions (Novagen, USA). The cDNA encoding for the full-length CpTRX1 was PCR amplified using the following primers: 5'-CCATATGACTGTCCACAACGTC-3' (forward-*del*) and 5'-CTCGAGCTCGGCCTTCTCCTC-3' (reverse-*hol*). The primers were modified to incorporate the restriction sites (underlined) for *Nde*I and *Xho*I, respectively. Full-length *CpTrx*1 (361 bp) was inserted into the *Nde*I/*Xho*I site in the pET28b expression vector. The resulting recombinant plasmids were then transformed into *E. coli* strain BL21. The induction and confirmation of recombinant CpTRX1 using anti-exahistidine antibody were conducted in accordance with the manufacturer's instructions (Novagen, USA). The induced cells were harvested via centrifugation (10,000 × g, 15 min, at 4°C) at the exponential phase, washed once in 50 mM Tris-HCl (pH 7.5), and subjected to sonication (3 × 1 min). The soluble protein
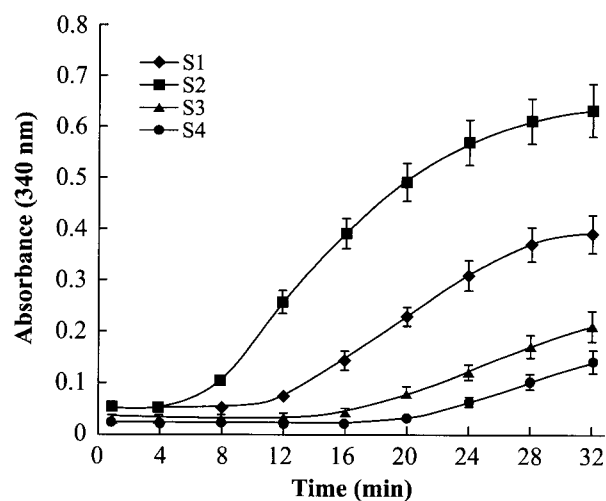

Fig. 3. Analysis of *E. coli*-expressed CpTRX1 activity via insulin reduction assay. S1, S2, S3, and S4 represent the TRX activity of preparations from the soluble proteins of the induced CpTRX1 expressing *E. coli*, uninduced, mock-transformed *E. coli*, and BSA, respectively.

was obtained after centrifugation, and was employed in order to measure the catalytic activity of the *E. coli*-expressed CpTRX1. A spectrophotometric insulin reduction assay was applied to determine the TRX activity of the recombinant CpTRX1, in accordance with the instructions of Luthman and Holmgren (1982). This method is predicated on increases in turbidity due to the reduction of insulin levels in the presence of *E. coli* thioredoxin reductase and NADPH. In brief, 500 μl of the assay mixture (50 mM potassium phosphate; pH 7.0, 1 mM EDTA, 80 μM insulin and 0.2 mM NADPH) was utilized in the presence of 1 μg of yeast thioredoxin reductase (LabFrontier, Korea), coupled with 100 μg of protein preparation at 25°C. As is shown in Fig. 3, the soluble proteins from the induced recombinant *E. coli* showed an increase in enzymatic activity, whereas the mock-transformed cells manifested low levels of optical changes, similar to those seen with BSA as a negative control. This indicates that the cloned *CpTrx*1 gene encodes for a protein with thioredoxin activity. However, the soluble proteins from the uninduced recombinant *E. coli* manifested an intermediate level of TRX activity, thereby suggesting the basal level expression of the cloned *CpTrx*1 gene.

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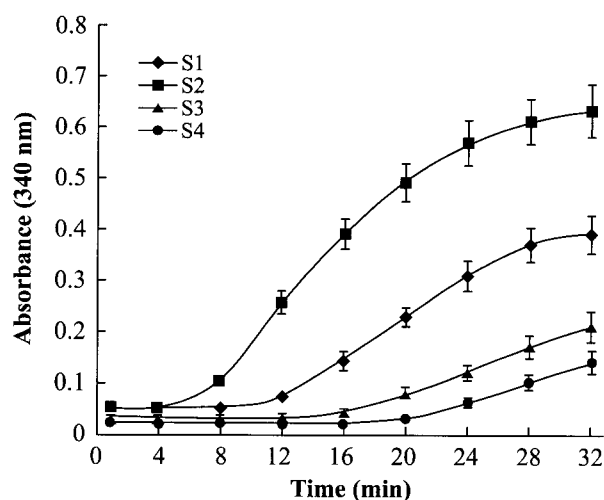

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