Gene Silencing by RNA Interference in the White Rot Fungus *Phanerochaete chrysosporium*[▽]

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The effectiveness of RNA interference (RNAi) is demonstrated in the lignin-degrading fungus *Phanerochaete chrysosporium*. The manganese-containing superoxide dismutase gene (*MnSOD1*) was used as the target for RNAi. The plasmid constructed for gene silencing contained a transcriptional unit for hairpin RNA expression. Significantly lower *MnSOD* expression at both the mRNA and protein activity levels was detected in RNAi transformants. Furthermore, even though *P. chrysosporium* possesses three copies of the *MnSOD* gene, this RNAi construct was sufficient to decrease the enzymatic activity by as much as 70% relative to control levels. Implementation of the RNAi technique in *P. chrysosporium* provides an alternative genetic tool for studies of gene function, particularly of essential genes or gene families.

The white rot fungus *Phanerochaete chrysosporium* can degrade and metabolize lignin, as well as a broad range of recalcitrant organopollutants, more rapidly and more extensively than any other microbial group (18, 34). Its lignin-degrading system consists of two families of hydrogen peroxide (H₂O₂)-requiring extracellular heme peroxidases designated lignin peroxidase (LIP) and manganese-dependent peroxidase (8, 25). LIP production in liquid cultures of *P. chrysosporium* occurs either when they are flushed with pure O₂ or when the medium is deficient in manganese ion (Mn²⁺) (3, 13, 26, 38). A high O₂ concentration or Mn²⁺ deficiency stimulates increased production of reactive oxygen species (ROS), subjecting the fungus to remarkable oxidative stress, as confirmed by high levels of ROS, oxidative damage, and antioxidant enzyme activity. Thus, ROS are key factors as inducers of *lip* expression (5, 6). In oxygenated cultures of *P. chrysosporium*, the major response of the antioxidant system involves increased expression and activity of manganese-containing superoxide dismutase (MnSOD). In contrast, in Mn²⁺-deficient cultures, MnSOD protein is produced but its enzymatic activity is rather low (5, 6). Formation of MnSOD[−] mutants will enable us to examine the role or influence of Mn²⁺ ions and MnSOD in the production of the relevant ROS necessary for LIP induction.

Superoxide dismutases (SODs) are the first and most important line of enzymatic defense systems against ROS. They catalyze the dismutation of the highly reactive superoxide radical anions (O₂^{•−}) to O₂ and H₂O₂ in all oxygen-metabolizing organisms (17, 46). SODs are metalloproteins containing iron, manganese, copper plus zinc, or nickel as prosthetic groups (2). MnSOD has been found in the cytosolic fractions of prokaryotes and in the mitochondrial matrix of eukaryotes. In eukaryotic cells, MnSOD is synthesized in the cytosol and

imported posttranslationally into the mitochondrial matrix (2, 23). The *P. chrysosporium* genome contains at least three MnSOD genes, *MnSOD1* (GenBank accession no. AF388395), which is located in scaffold 23 (nucleotides 84573 to 85297), and the two additional putative MnSOD genes *MnSOD2*, which is located in scaffold 8 (nucleotides 478804 to 479683), and *MnSOD3*, which is located in scaffold 9 (nucleotides 1861632 to 1862495); on the other hand, no CuZnSOD activity or homologous sequence has been detected in this organism (7). *MnSOD* expression is essential for survival under aerobic conditions and for the development of cellular resistance to oxygen radical-mediated toxicity (24). Mutations generating a defective SOD cause hypersensitivity to oxidative damage in bacteria, yeast, and *Drosophila melanogaster* (42). Inactivation of the *sodA* and *sodB* genes in *Escherichia coli* increased the mutation frequency when the bacteria were grown under aerobic conditions (15). Elimination of the *MnSOD* gene in *Saccharomyces cerevisiae* increased its sensitivity to oxygen (20, 42). Heterozygous *MnSOD* knockout mice exhibit a 50% reduction in MnSOD activity, while mice with homozygous null mutations in *MnSOD* die within 1 to 18 days (43). Thus, it is not clear how *P. chrysosporium* manages to survive with reduced MnSOD activity in Mn²⁺-deficient cultures.

RNA interference (RNAi), initially reported in *Caenorhabditis elegans* (16), is a posttranscriptional gene-silencing phenomenon in which double-stranded RNA (dsRNA) triggers the degradation of related mRNA in a sequence-specific manner (31). This technique is induced by the introduction or production of dsRNA molecules homologous to the gene being targeted for silencing in the cell of interest. This dsRNA is processed into 21- to 25-nucleotide fragments which associate with a nuclease complex (RNA-induced silencing complex) and are used as a guide for homology-dependent degradation of the target mRNA (11, 14). RNAi has been used as a method to study gene function and for specific inhibition of gene expression in a range of organisms, including several basidiomycetes and ascomycetes such as *Aspergillus fumigatus* (30), *As-*

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TABLE 1. Primers used in this study

Primer	Description	Sequence (5'-3') ^a
AM42	18S rRNA gene antisense	CAACTACGAGCTTTTAACTGC
AM43	18S rRNA gene sense	CAAATTACCAATCCCGACAC
AM60	PstI- <i>MnSOD1</i> promoter forward	AACTGCAGCATCGGGTCAGCCATTGG
AM62	XbaI-linker reverse	GCTCTAGAGTCGGCCTTGACGTTGAG
AM63	XbaI- <i>MnSOD1</i> exon1 reverse	GCTCTAGAGAGCCAGCCCCAGCC
AM64	BamHI- <i>MnSOD1</i> exon1 forward	CGGGATCCATGTCCGGCCAGCACAC
AM65	BamHI- <i>MnSOD1</i> terminator forward	CGGGATCCCTCTGAGTCCCCGCGTG
AM59	EcoRI- <i>MnSOD1</i> terminator reverse	GGAATTTCGACTCACGACCGCC
AM69	<i>bar</i> gene forward	GCCGTGCCACCGAGG
AM70	<i>bar</i> gene reverse	AGCTGCCAGAAACCC
AM73	<i>MnSOD1</i> exon 2 forward	GGCTACAACCCGAGC
AM75	<i>MnSOD1</i> exon 3 reverse	TCAGAGCTTGGCGCC
AM89	<i>MnSOD2</i> reverse	GACCAATCACACTTAAGATGA
AM90	<i>MnSOD2</i> forward	CAGCTAGCAGGGCATTGAGG
AM91	<i>MnSOD3</i> reverse	ATTTAGTCCTTTTAAGCAGATT
AM92	<i>MnSOD3</i> forward	GAGCTCTTTCGCGCGCGC

^a Restriction sites are in bold.

pergillus oryzae (45), *Coprinus cinereus* (32, 44), *Schizophyllum commune* (12), *Cryptococcus neoformans* (27), and *Neurospora crassa* (10). This procedure may be particularly useful for the simultaneous suppression of closely related genes, as well as the partial suppression of essential genes (31, 36). We report here the silencing of the *MnSOD* gene in *P. chrysosporium* by RNAi.

MATERIALS AND METHODS

Strains and culture conditions. The widely used dikaryotic fungus *P. chrysosporium* Burds BKM-F-1767 (ATCC 24725) was used for this study. The fungus was maintained at 4°C on 2% (wt/vol) malt extract agar slants and inoculated by the method of Tien and Kirk (41). The growth medium was prepared as previously described (37, 38) with initial concentrations of glucose, diammonium tartrate, and $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ of 56, 2.4, and 0.225 mM, respectively. The fungus was grown in submerged liquid culture (90 ml) at 175 rpm and 37°C in 250-ml flasks sealed with rubber stoppers, and the headspace was flushed twice a day with O_2 for 2 min at a flow rate of 1 liter/min (oxygenated cultures). The oxygen gas used was of medical-grade purity.

DNA manipulation. All DNA manipulations were performed by standard methods as described by Sambrook et al. (39). Genomic DNA was extracted from *P. chrysosporium* by a method for the rapid isolation of genomic DNA from filamentous fungi (35) and used for PCR amplification and Southern blot analysis. The PCR mixtures (50 μl) contained 0.5 U of *Taq* DNA polymerase (Sigma-Aldrich, Rehovot, Israel), each deoxynucleoside triphosphate at 200 μM , and 20 pmol of each primer. The PCR program was 5 min at 94°C and 40 cycles of 30 s at 94°C, 30 s at the optimal annealing temperature (52 to 60°C), and 30 s at 72°C, followed by 5 min at 72°C. For Southern blot analysis, genomic DNA (1 μg) from each transformant was digested with a selected restriction enzyme and the resulting fragments were separated on 0.8% agarose gel and subsequently transferred to a nylon membrane (Amersham Biosciences, United Kingdom). The promoter and first exon sequence of the *MnSOD1* gene were used as the template to synthesize random primed [α - ^{32}P]dCTP-labeled probes. Southern hybridization and autoradiography were performed according to the Amersham Biosciences protocol.

Construction of pMSC. The construct for RNAi was designed with inverted 398-bp repeats of the first exon of the *MnSOD1* gene from *P. chrysosporium* (GenBank accession no. AF388395) separated by a 188-bp linker segment composed of the first intron, followed by the second exon of *MnSOD1*. A 1,181-bp fragment of *MnSOD1* containing the promoter, the first and second exons, and the first intron was PCR amplified from *P. chrysosporium* genomic DNA with primers AM60 and AM62 to add a PstI and a XbaI restriction site, respectively (Table 1). For reverse orientation cloning, the first exon of *MnSOD1* was PCR amplified from genomic DNA with primers AM63 and AM64 to add an XbaI and a BamHI restriction site, respectively (Table 1). A 642-bp fragment corresponding to the *MnSOD1* gene's 3' untranslated region was amplified by PCR from genomic DNA with primers AM65 and AM59 to add a BamHI and an EcoRI

restriction site, respectively (Table 1). The PCR fragments were digested and ligated into the PstI and EcoRI sites of plasmid pBar3.8, containing the selectable marker gene *bar* for resistance to the herbicide phosphinothricin (PPT; Sigma-Aldrich, Rehovot, Israel) (28). A new plasmid, designated pMSC, was obtained (Fig. 1).

Fungal transformation. A transformation procedure was performed as described by Chakraborty and Kapoor (9), with slight modifications. Ten-day-old conidia were collected with ice-cold 1 M sorbitol and filtered through sterile 200- μm -pore-size nylon mesh (Amiad Filtration Systems, Amiad, Israel). The conidia were washed three times and suspended in ice-cold 1 M sorbitol to a final concentration of 3×10^9 conidia per ml. Conidia (9×10^7) were mixed with 1 μg of linearized pMSC DNA and subjected to a prechilled electroporation cuvette. The electroporator was set up to 1.5 kV, a capacitance of 50 μF , a resistance of 200 Ω , and an 8- to 9-ms pulse length. After electroporation, 360 μl of ice-cold 1 M sorbitol was added to the cuvette. The transformation mixture was incubated at 25°C for 3 h. Transformants were selected by plating on potato dextrose agar (PDA) plates containing the herbicide PPT for selection (28).

RNA extraction and Northern blot analysis. Total cell RNA was purified with Tri Reagent (Sigma-Aldrich, Rehovot, Israel) according to the manufacturer's instructions. The isolated RNA was separated by agarose-formaldehyde gel electrophoresis, blotted onto a Hybond-N⁺ nylon membrane (Amersham Biosciences, United Kingdom), and hybridized with a ^{32}P -labeled probe according to Sambrook et al. (39). To make this probe, the *bar* gene fragment was PCR amplified with oligonucleotide primers AM69 and AM70 (Table 1) and plasmid pBar3.8 as the template.

Real-time PCR. Total cDNA was generated by reverse transcription with the Reverse-IT Max 1st Strand Synthesis kit (ABgene, Epsom, United Kingdom). The amount of *MnSOD* transcript in relation to 18S rRNA gene transcript was

FIG. 1. Construction of pMSC for induction of *MnSOD* RNAi. Schematic diagram of the *MnSOD*-silencing cassette. A 398-bp fragment, corresponding to the first exon of the *MnSOD* gene, was cloned in the forward and reverse orientations separated by a 188-bp linker segment and under the control of the *MnSOD* promoter and terminator. The linker was composed of the first intron and second exon of *MnSOD*. The cassette was ligated to the pBar3.8 plasmid harboring the selectable marker gene *bar* for resistance to PPT. CDS, coding sequence.

TABLE 2. Orthologs of RNAi in *P. chrysosporium*

Accession no.	Protein	E value	Ortholog in <i>P. chrysosporium</i> (nucleotide position)
AAF31695	<i>Neurospora crassa</i> QDE3	0	Scaffold 15 (353724–354932)
XP_747330	<i>Aspergillus fumigatus</i> QDE2, putative	0	Scaffold 10 (630398–633207)
BAC15768	<i>Mus musculus</i> Piwi/Argonaute family protein meIF2C3	0	Scaffold 5 (959815–962759)
XP_001276635	<i>Aspergillus clavatus</i> QDE2, putative	0	Scaffold 10 (636512–645176)
NP_171612	<i>Arabidopsis thaliana</i> DCL1	8e-45	Scaffold 1 (2858263–2862332)
CAK32533	<i>Mucor circinelloides</i> putative Dicer-like protein	1e-39	Scaffold 8 (260504–264943)

determined by real-time PCR, which is based on the high-affinity double-stranded DNA-binding dye Sybr green (Absolute QPCR Sybr green ROX mix; ABgene, Epsom, United Kingdom) and was performed in triplicate in a spectrofluorometric thermal cycler (Rotor-Gene 3000; Corbett Research, Sydney, Australia) with the primers AM42 and AM43 for the 18S rRNA gene, AM63 and AM64 for *MnSOD1*, AM89 and AM90 for *MnSOD2*, and AM91 and AM92 for *MnSOD3* (Table 1). The real-time PCR program included a 15-min polymerase activation step at 95°C, followed by up to 45 cycles of 15 s at 95°C, 20 s at the optimal annealing temperature (60°C for the 18S rRNA gene, *MnSOD1*, and *MnSOD3* and 45°C for *MnSOD2*), and 25 s at 72°C. Assay specificity was confirmed by subjecting the PCR products to Sybr green I melting curves. The efficiency of real-time amplification was determined by running a standard curve with serial dilutions of cDNA and defined as $E = 10^{-1/m} - 1$, where m is the slope of the reaction. The optimal melting points of *MnSOD1*, *MnSOD2*, *MnSOD3*, and the 18S rRNA gene were 93, 86.5, 88.2, and 84°C, respectively.

Protein extraction and determination of MnSOD activity. Samples were homogenized in the cold for 2 min in 50 mM phosphate buffer, pH 7, with an Ultra-Turrax T25 homogenizer (IKA, Staufen, Germany). The homogenate was centrifuged at $20,000 \times g$ for 20 min at 4°C. Phenylmethylsulfonyl fluoride (1 mM; Sigma-Aldrich, Rehovot, Israel) was added to each sample during homogenization. The protein samples were then analyzed by nondenaturing polyacrylamide gel electrophoresis (PAGE) according to Sambrook et al. (39). The assay of MnSOD activity was based on its ability to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide anions, produced photochemically by riboflavin. Activity staining for MnSOD on a nondenaturing polyacrylamide gel was performed according to the method proposed by Beauchamp and Fridovich (4). The gels were incubated in the dark for 30 min in 80 ml of a reaction mixture containing 0.1 M potassium phosphate buffer (pH 7.8), 1 mM EDTA, 33 μ M riboflavin (Sigma-Aldrich, Rehovot, Israel), 245 μ M NBT (Sigma-Aldrich, Rehovot, Israel), 17 mM *N,N,N',N'*-tetramethylethylenediamine (TEMED). The gel was then incubated in 0.1 M potassium phosphate buffer (pH 7.8) with 1 mM EDTA and exposed to light for 30 min (38). MnSOD activity was detected by the appearance of transparent bands, representing the inhibition of NBT reduction by superoxide anions, on a blue background (reduced NBT). The densities of the areas of activity were measured and compared by using TINA program software (Raytest Isotopenmessgeräte GmbH). Soda from *E. coli* (Sigma-Aldrich, Rehovot, Israel) was used as a control.

RESULTS

Orthologs of RNAi in *P. chrysosporium*. Orthologous predicted proteins for different genes known to be involved in RNAi in different organisms were found by searching the *P. chrysosporium* genome database (<http://www.jgi.doe.gov/>) with BLASTp. Homologues of QDE2 and Argonaute 1, components of the RNA-induced silencing complex (1), a homologue of QDE3, similar to RecQ DNA helicase and believed to be involved in the activation step of gene silencing (1), and a homologue of DCL1, a Dicer-like protein, were all identified in the *P. chrysosporium* genome database with high identity (Table 2). The presence of orthologs to these proteins suggested that RNAi should be functional in *P. chrysosporium*.

RNAi of the *MnSOD* gene. The genome of *P. chrysosporium* contains at least three *MnSOD* genes. Multiple sequence alignment of the *MnSOD1* gene and the two additional putative *MnSOD* genes from *P. chrysosporium* showed 66 to 76% amino

acid identity, with the greatest sequence conservation within the first exon. This homology enables the formation of 21- to 25-nucleotide fragments, along the first exon of the three *MnSOD* genes, needed for gene silencing. With the objective of simultaneously suppressing the three genes, we focused on the first exon of *MnSOD1* for construction of the hairpin RNA cassette (Fig. 1). The fragments used for the construction of the *MnSOD*-silencing cassette were PCR amplified from *P. chrysosporium* genomic DNA. The PCR fragments were digested and ligated into the PstI and EcoRI sites of the pBar3.8 plasmid (28), producing the new plasmid pMSC. This plasmid contained the selectable marker gene *bar* for resistance to the herbicide PPT. The plasmids pMSC and pBar3.8 (as a control) were transformed into *P. chrysosporium* conidia for the formation of MSC and BAR transformants, respectively. Six transformants were prepared by electroporation, and four of them were chosen for further analysis. These transformant colonies were grown in PDA medium containing 600 μ g/ml PPT at 37°C and selected for the ability to produce mycelium on the selective medium. Radial growth of representative transformants is shown in Fig. 2. MSC1, MSC2, MSC5, MSC6, and BAR5 colonies were resistant to PPT. In contrast, the nontransformed control colony (P.c) did not grow in the presence of PPT. After several rounds of growth on PDA plates containing PPT, transformants MSC1 to MSC6 were checked by PCR for

FIG. 2. Radial growth of *P. chrysosporium* transformants on PPT-containing medium. Transformant colonies were grown on PDA medium containing 600 μ g/ml PPT at 37°C for 7 days. MSC1, MSC2, MSC5, and MSC6 were transformed with pMSC containing the *MnSOD*-silencing cassette. BAR5 was transformed with plasmid pBar3.8. P.c, nontransformed *P. chrysosporium*.

FIG. 3. Presence of the *bar* gene and silencing cassette (MSC) in transformants. PCR with specific primers of *bar* (540 bp), the silencing cassette (1,040 bp), and a fragment (279 bp) harboring exon 2, intron 2, and exon 3 of the *MnSOD1* performed with *P. chrysosporium* (P.c) and MSC1 transformant genomic DNA.

the presence of the *bar* gene with primers AM69 and AM70 (Table 1), the presence of the silencing cassette with primers AM59 and AM63 (Table 1), and the presence of exon 2, intron 2, and exon 3, corresponding to endogenous *MnSOD1*, with primers AM73 and AM75 (Table 1) as a control. The presence of the *bar* gene (540-bp fragment), the silencing cassette (1,040-bp fragment), and endogenous *MnSOD1* (279-bp fragment) was confirmed in all of the transformants. The PCR results obtained for MSC1 in comparison to the nontransformed control colony (P.c) are presented in Fig. 3. In transformants MSC2 to MSC6, faint bands were obtained, probably

FIG. 4. (A) Southern blot analysis of DNAs from P.c, MSC1, and MSC2 transformants. The blot was hybridized with the 32 P-labeled *MnSOD* sequence. Lanes 1 to 3, undigested genomic DNAs (gDNA) from P.c, MSC1, and MSC2, respectively; lanes 4 to 6, the same genomic DNAs as in lanes 1 to 3 digested with PstI; lanes 7 to 9, the same genomic DNAs as in lanes 1 to 3 digested with EcoRI. The endogenous *MnSOD1* gene is marked by arrow. (B) Schematic restriction map of the surrounding sequences of the wild-type *MnSOD1* gene by PstI and EcoRI.

FIG. 5. MnSOD activity in *P. chrysosporium* transformants. (A) Protein samples from *P. chrysosporium* transformants were analyzed by activity staining in nondenaturing PAGE. MnSOD activity is indicated by the appearance of transparent bands, representing inhibition of NBT reduction by superoxide anions, on a blue background (reduced NBT). (B) Protein level was measured by staining the gel with Coomassie blue. (C) Areas of activity were measured by the TINA program, and the relative activities of MnSOD in the different transformants were compared. The BAR5 activity level was set arbitrarily to 1. Data represent the average $\pm$ the standard deviation of three culture replicates.

because of a low ratio of transformed nuclei (data not shown). The presence of *bar* expression in the BAR5, MSC1, and MSC2 transformants was also verified by Northern blot analysis (data not shown). Southern blot analysis was performed on DNA extracted from P.c and the transformants MSC1 and MSC2 with the 32 P-labeled promoter and the first exon sequence of the *MnSOD1* gene as the probe. The absence of rapidly migrating bands with undigested transformant DNA (lanes 2 and 3, Fig. 4A) indicates that the transformed plasmids were integrated into the chromosomes rather than carried in an autonomously replicating form. Bands of 7.5 and 4.4 kb were obtained by Southern blotting of P.c, MSC1, and MSC2 genomic DNA digested with EcoRI and PstI, respectively (Fig. 4A). These results were in accordance with the restriction sites flanking the endogenous *MnSOD1* gene (Fig. 4B). The additional bands obtained in MSC1 (lanes 5 and 8, Fig. 4A) and MSC2 (lanes 6 and 9, Fig. 4A) indicated the integration of one and two copies of the silencing cassette, respectively. The signal retrieved from the additional bands is rather weak relative to the endogenous bands, possibly due to the low percentage of transformed nuclei in this heterokaryotic fungus. The presence of endogenous *MnSOD1* exon 2, intron 2,

FIG. 6. MnSOD expression in *P. chrysosporium* transformants. The expression of *MnSOD1*, *MnSOD2*, and *MnSOD3* transcripts relative to 18S rRNA gene transcript was measured by real-time PCR. Data represent the average $\pm$ the standard deviation of three culture replicates.

and exon 3 in both P.c and the transformant MSC1 (Fig. 3) and the Southern blotting results indicate that no gene replacement or disruption occurred at the *MnSOD1* locus.

MnSOD gene expression and activity in *P. chrysosporium* transformants. To verify that *MnSOD* had been silenced in the MSC transformants, the expression of the *MnSOD* gene at both the mRNA and protein levels was measured in transformants grown under conditions suitable for the induction of *MnSOD* expression, i.e., under pure oxygen flushed into flasks for 2 min twice a day for 5 days. Protein extracted from the same biomass of each transformant was analyzed by nondenaturing PAGE for MnSOD activity (Fig. 5A and B). The densities of the areas of MnSOD activity were measured by TINA program software, and the relative activities of MnSOD in the different transformants were compared. MnSOD activity in the MSC transformants was decreased to various degrees in comparison to that in the control (BAR5) transformant. Activity decreases of 70, 31, 16, and 6% were obtained in transformants MSC1, MSC2, MSC5, and MSC6, respectively (Fig. 5C). The radial growth of the MSC1 transformant, which showed the lowest MnSOD activity, was slow, with only a little mycelium development, relative to the other MSC and BAR transformants (Fig. 2).

For measurement of the three *MnSOD* transcripts, real-time reverse transcription-PCR was performed with gene-specific primers (Fig. 6). Examination of melting curves indicated highly specific amplification of the respective cDNAs (data not shown). The reaction efficiencies were 80, 89, and $\sim$ 100%, for *MnSOD1* (398 bp), *MnSOD2* (638 bp), and *MnSOD3* (636 bp), respectively. *MnSOD1* gene expression was decreased by 51, 76, 67, and 17% in MSC1, MSC2, MSC5, and MSC6 transformants, respectively, in comparison to the expression in the BAR5 transformant. A significant decrease in *MnSOD2* and *MnSOD3* gene expression was also observed in MSC2, MSC5, and MSC6 transformants. *MnSOD2* gene expression was decreased by 78, 96, and 75% in MSC2, MSC5, and MSC6 transformants, respectively, in comparison to the expression in the BAR5 transformant. *MnSOD3* gene expression was decreased

by 93, 85, and 15% in the MSC2, MSC5, and MSC6 transformants, respectively, in comparison to the expression in the BAR5 transformant. In contrast, a fourfold increase in *MnSOD2* and *MnSOD3* gene expression was observed in the MSC1 transformant, in comparison to the expression in the BAR5 transformant (Fig. 6).

DISCUSSION

We selected *MnSOD* as a target to investigate the gene-silencing effect by using RNAi in *P. chrysosporium*. Unlike knockout techniques, RNAi does not completely block gene expression and therefore is less likely to be lethal when the targeted gene is essential (30). Furthermore, RNA silencing can be used for the simultaneous suppression of an entire gene family, consequently avoiding gene compensation (30, 31, 36). Simultaneous interference with homologous family members by using dsRNA has been demonstrated in trypanosomes and *Drosophila* (40, 47). In fact, in most protozoan parasites and fungi, this approach is significantly more efficient than constructing multiple knockout strains (11). The approach is particularly appealing for *P. chrysosporium*, where gene families are common, where gene replacement or disruption is extremely difficult, and where we have identified components of probable orthologs of the genes supporting an RNAi system (Table 2). It has recently been demonstrated that vectors generating hairpin RNAs are highly effective in inducing silencing (19, 21, 22, 29, 31). With this in mind, we constructed the pMSC plasmid containing the inverted-repeat sequences of our target gene for silencing.

The *MnSOD*-silencing cassette was integrated into the *P. chrysosporium* genomic DNA, as shown by Southern blot analysis. The weak signals of certain bands in the Southern blot analysis of MSC1 and MSC2 suggest that the transformants are heterokaryotic. Integration of the *MnSOD*-silencing cassette caused a reduction in *MnSOD* expression at both the mRNA and protein levels in the transformants. A wide range of decreased MnSOD activity (6 to 70%) and

MnSOD1 mRNA level (17 to 76%) was observed among transformants. Such variation among RNAi mutants has been observed in other microorganisms exposed to RNAi, including *C. neoformans* (27) and *Trypanosoma brucei* (33). The variation may be due to differences in the genome context of ectopic integration events (30). The slow growth of the MSC transformants suggests that the reduced activity of MnSOD achieved by RNAi hinders growth. These results support an important role for MnSOD in *P. chrysosporium* viability. RNAi was also proven to be a useful method for downregulation of gene expression and for reduction of essential enzyme activity in *P. chrysosporium*.

It was previously reported that *MnSOD* expression can change as a function of culture age (6, 7), and it will be interesting to examine the degree of silencing as a function of age in future studies.

Oxidative stress and a ROS-rich environment are key factors in LIP expression in oxygenated or Mn^{2+} -deficient cultures of *P. chrysosporium* (5, 6). Since the principal difference between oxygenated and Mn^{2+} -deficient cultures is the activation or inactivation of MnSOD, respectively, it would be most interesting to clarify the function of MnSOD and Mn^{2+} ions in the LIP expression pathway. The conditional $MnSOD^-$ mutants prepared here by the RNAi technique will enable us to ascertain the role or influence of Mn^{2+} ions and MnSOD, as well as other antioxidant enzymes, in the production of the relevant ROS necessary for LIP induction.

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