RNA-Mediated Gene Silencing of Superoxide Dismutase (*bcsod1*) in *Botrytis cinerea*

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ABSTRACT

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Gene silencing is a powerful tool utilized for identification of gene function and analysis in plants, animals, and fungi. Here, we report the silencing of superoxide dismutase (*bcsod1*) in *Botrytis cinerea* through sense and antisense-mediated silencing mechanisms. Because superoxide dismutase (SOD) is a virulence factor, transformants were tested for phenotypic silencing in vitro and reduction in pathogenicity in planta.

Plate-based assays with and without paraquat were performed to screen initial silencing efficiency, and a subset of transformants was used for in planta studies of virulence. Transformants exhibiting strongly decreased transcripts levels were recovered with both constructs but none of those exhibited a reduction in virulence in planta. Our investigations may help optimize a high-throughput gene silencing system useful for identifying potential gene targets for future fungal control.

Additional keywords: posttranscriptional gene silencing, RNAi.

Sequence-specific RNA silencing is an invaluable tool that allows for the functional analysis and screening of genes in order to identify potential pathogenicity factors. Silencing was first observed in *Petunia* and termed “cosuppression” (24). It is also referred to as RNA interference (RNAi) in animals, posttranscriptional gene silencing (PTGS) in plants, and quelling in fungi (13,18,35). Collectively, these processes are referred to as PTGS. Because this phenomenon has been observed in a wide range of organisms, its mechanism and biological purpose is thought to be highly conserved across kingdoms. Fundamentally, gene silencing is mediated by RNA molecules and is intricately linked with transcription of the target gene. It results from a sequence-specific degradation of messenger RNAs (mRNAs) induced by the formation of aberrant double-stranded RNA (dsRNA). The dsRNA is processed into 21- to 25-nucleotide fragments called short interfering RNAs (siRNA) by a Dicer enzyme (3,11,21). Individual siRNAs are then integrated into a RNA-induced silencing complex (RISC), an endonuclease that targets mRNA complementary to the incorporated siRNA sequence (12,38).

Although gene silencing is normally involved in genome defense, researchers are able to manipulate gene expression of endogenous- and trans-genes through the generation of dsRNA molecules complementary to the target gene, making it an invaluable tool for gene discovery and function (23). It is believed that the benefits observed from gene silencing may be demonstrated for the plant pathogen *Botrytis cinerea*, a necrotrophic fungus capable of infecting over 200 plant species worldwide (37). Primarily, the fungus infects dicotyledonous crops such as fruit berries, vegetables, cut flowers, potted plants, and grapevine. Unlike many plant pathogens, *B. cinerea* is prominent all year round and causes infection under a broad range of environmental

conditions (15). Because *B. cinerea* is resilient against natural plant defensive measures and various fungicides (6,17,19), new target genes affecting pathogenicity must be uncovered before additional forms of control can be developed.

To develop a gene silencing system in *B. cinerea*, we selected the endogenous gene superoxide dismutase (*bcsod1*), an H₂O₂-generating enzyme (25). Superoxide dismutases (SODs) belong to a family of enzymes that neutralize the effect of active oxygen species (AOS). Specifically, SOD catalyzes the conversion of O₂⁻ into H₂O₂ (8). The expression of *bcsod1*, a single-copy gene, has been characterized in vitro and in planta, and knockout mutants in this gene show reduction in virulence (25). As a result, silencing efficiencies can be assessed or compared in vitro and in vivo to better understand SOD production levels. Because this is the first report of gene silencing in *B. cinerea*, RNAi technology may be used in screening unknown genes from the recently sequenced *B. cinerea* genome.

MATERIALS AND METHODS

Fungal strains and growth conditions. Experimental analyses and transformation were performed on *B. cinerea* haploid strain B05.10 (4). *B. cinerea* conidia were stored as glycerol stocks at -80°C. Three SOD deletion mutants— $\Delta bcsod1$, -2, and -3 (kindly provided by P. Tudzynski, University of Munster, Germany)—were used to establish the maximum reduction in growth in vitro and in planta (25). The fungus was grown on malt agar containing 5% malt extract and 1.8% agar and incubated at 20°C under near-UV light to induce sporulation. Conidia were harvested 7 to 14 days postinoculation in 10 ml of water containing 0.05% (vol/vol) Tween 80 and collected by centrifugation at 3,000 × g for 5 min. For DNA extraction, 5% (wt/vol) malt extract broth was inoculated with 5 × 10⁸ spores and incubated at 20°C and 180 rpm for 24 h. Fungal mass was harvested by centrifugation and lyophilized.

Transformation of *B. cinerea*. Transformants were produced using methodology described by Hamada (10) with modifications described by van Kan et al. (33).

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Standard nucleic acid techniques. Fungal genomic DNA was isolated as described (30). Polymerase chain reaction (PCR) analysis was performed as described by Saiki et al. (27) using Reddy Mix (Bioline) and amplification products were separated by gel electrophoresis. Primers used during PCR analysis are listed in Table 1. Purified products (Wizard Gel Extraction Kit; Promega Corp., UK) were cloned using the pCR 2.1 TOPO Cloning Kit (Invitrogen, UK). Ligation strategies were designed using Clone Manager Suite (Science & Educational Software). Sequence analysis and alignment were aided by BLAST analysis at the National Center for Biotechnology Information (1). Northern analysis of total RNA was completed according to Sambrook et al. (28) with slight modifications. Total RNA was electrophoresed through a denaturing gel (1.2% agarose, 2.2 M formaldehyde, 1× MOPS gel). Autoradiographs were quantified by densitometry analysis (Gene Tools; Syngene, UK) within a linear signal range and normalized to ribosomal 28S RNA levels.

SOD plate-based assay. SOD activity was tested using malt extract plates supplemented with 15 mM paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride hydrate; ABCR GmbH & Co., Germany). Plates were incubated at 20°C for 3 days before radial measurements were taken. Silencing frequencies (percent) were calculated by comparing the colony diameters in the presence and absence of paraquat for both the transformant and the wild-type recipient strain.

Pathogenicity assays. In planta studies were conducted on 14-day-old dwarf French bean plants (cv. Tendergreen). Conidia of the sporulating wild type and transformants of *B. cinerea* were harvested and resuspended in water at a working concentration of 1×10^6 conidia ml⁻¹. Punctured plant leaves were then inoculated with a single droplet containing 5×10^3 conidia from the wild type and a transformant of *B. cinerea*. Leaves were incubated over a period of 5 days with diurnal cycles of 16 h of light and 8 h of darkness and 100% humidity before being photographed. Areas of infection were then determined with ImageJ software.

Construction of sense and antisense silencing vectors. The principal aim of this study was to induce gene silencing in *B. cinerea* so that it may be optimized for gene function investigations. To do so, we utilized an effective vector system, pLOB1 (R. M. Patel, unpublished data). The pLOB1 vector system consists of the pLOB1-MCS expression vector (with a 120-bp multiple cloning site) and pLOB1-HYG hygromycin resistance cassette (Fig. 1A). Both vectors are controlled by the *Aspergillus nidulans* *oliC* promoter and what is reported to be the *B. cinerea* *tubA* terminator, each isolated from the pLOB1 hygromycin resistance cassette (33). Sense and antisense *bcsod1* silencing constructs were amplified from *B. cinerea* cDNA (GenBank accession no. AJ555872), with primers SSOD F and SSOD R, and ASOD F and ASOD R (Table 1), respectively. Amplified fragments (581 bp in length) represent bases 12 to 465 of the coding region and 128 bp of the 3' untranslated region. The sense construct was designed with a start-stop (ATG/GGG/TAA) sequence to prevent transcriptional readthrough and gene overexpression. Both PCR fragments were flanked by *NcoI* and *BamHI* restriction sites and ligated into the *NcoI*- and *BamHI*-digested pLOB1-MCS vector,

between the promoter and terminator regions. The resulting silencing vectors, pLOB1-SSOD and pLOB1-ASOD (Fig. 1B and C), were subsequently transformed into *B. cinerea* to test for silencing capabilities in *B. cinerea*.

RESULTS

Selection and screening of SOD-silenced transformants.

Silencing of the *bcsod1* gene was achieved by co-transforming either the sense or antisense silencing constructs along with pLOB1, encoding the hygromycin resistance gene. After several rounds of subculturing, transformants were screened on paraquat plate-based assays to determine silencing efficiencies by phenotypic screening (Fig. 2). Colony radii were measured 3 days post-inoculation. The *bcsod1*-knockout mutants ($\Delta bcsod1$ -1, $\Delta bcsod1$ -2, and $\Delta bcsod1$ -3) exhibited minimal growth (radial growth of 3 mm) on paraquat but demonstrated wild-type growth (radial growth of 21 mm) rates in the absence of paraquat, whereas the wild type demonstrated a 29% reduction of growth with paraquat. A ratio of 36% of all recovered transformants (27% of sense and 47% of antisense) demonstrated intermediate (33 to 65%) or high levels (66 to 85%) of growth reduction when compared with the wild type (Table 2). In all, 11 transformants (6 sense: S44, S150, S154, S156, S160, and S165; and 5 antisense: A76, A100, A101, A162, and A164) showing varying degrees of growth reduction, indicative of silencing, were chosen for further experimentation. Select transformants exhibited a significant (*F* was significant at *P* = 0.0002) reduction in radial growth compared with the wild type on plates supplemented with paraquat. Overall, the *bcsod1* knockout mutant showed an 85% reduction in colony diameter on plates supplemented with paraquat (compared with growth without treatment), whereas the wild type demonstrated a 29% reduction in growth. Selected transformants exhibited a range of growth reduction between 32 and 71% (Fig. 3).

Analysis of construct integration. PCR was performed on chosen transformants (S44, S150, S154, S156, S160, S165, A76, A100, A101, A162, and A164) and the wild-type strain to examine integration of the silencing construct into the *B. cinerea* genome. Primers SOD DNA F and SOD DNA R (Table 1) amplified the *bcsod1* gene (with introns), confirming the presence of an intact endogenous *bcsod1* gene in all transformants and wild-type samples. The presence of an intact silencing and hygromycin construct in all tested transformants was confirmed using primers OLIC2 and TUB2, which anneal to the *oliC* promoter and terminator region.

Determination of mRNA expression levels. The *bcsod1* transcript levels were determined by northern analysis using a DNA probe amplified from *bcsod1* cDNA with primers SOD RNA F and SOD RNA R (Table 1). Densitometry analysis was used in normalizing for differences in loading based on rRNA levels (Fig. 4A and B). High levels of silencing are defined as SOD transcript levels 1 to 30% compared with the wild type, while medium and low levels of silencing are defined as SOD transcript levels of 31 to 60% and 61 to 90%, respectively. According to the autoradiograph result and subsequent densitometric comparison with

TABLE 1. Primer list and sequences utilized for vector construction, screening, and northern analysis of *Botrytis cinerea* isolates

Primer name	Primer sequence 5'–3'
ASOD F	GGGGATCCGCGAGTTGCTACCGTCCGCGGTG
ASOD R	GGCCATGGAGTAGAACGATTGCTTTTCTGTAAAAAC
OLIC2	ATTAAGCGGGAGACGTATTT
SOD DNA F	ATTCTTCTCATATGTTGCTTTCCTCTT
SOD DNA R	TCATGTCAATTTGTATGCTACATTTA
SOD RNA F	ATGGTTAAAGCAGTTGCTACCGTCC
SOD RNA R	TTATGCAGCAATACCGATAACACC
SSOD F	GGCCATGGGGTAAGCAGTTGCTACCGTCCGCGGTG
SSOD R	GGGGATCCAGTAGAACGATTGCTTTTCTGTAAAAAC
TUB2	GGGACTTGAGACCGATGAAAC

the wild type, transformants S154, S156, S160, and A162 exhibited high levels of silencing, while samples S150 and A164 demonstrated medium levels of silencing. Samples S165 and A101 displayed low levels of silencing, while S44, A76, and

A100 showed levels equal to or greater than the wild type. Of all tested transformants, S160 demonstrated the greatest reduction in *bcsod1* transcript levels (79.2%). As expected, the knockout mutant ($\Delta bcsod1$ -1) did not generate detectable levels of *bcsod1*.

Fig. 1. pLOB1-multiple cloning site (MCS) and silencing plasmids. Regulatory sequences are the *Aspergillus nidulans oliC* promoter and what is reported to be the *Botrytis cinerea tubA* terminator (27). **A**, pLOB1-MCS contains a 120-bp MCS. **B**, pLOB1-SSOD contains the sense superoxide dismutase (SOD) silencing construct with start-stop sequence (;ATG/GGG/TAA, checked box), while **C**, pLOB1-ASOD contains SOD in the antisense orientation. Amplified fragments (581 bp in length) represent bases 12 to 465 of the coding region and 128 bp of the 3' untranslated region. Transcript and regulatory sequence sizes are found above each segment, while construct sizes are denoted at the end of each plasmid.

Fig. 2. Representation of the paraquat plate assay used to screen of *Botrytis cinerea* isolates for *bcsod1* silencing (3 days postinoculation). Samples in the upper row correspond with those in the lower row; the top row shows plates without paraquat whereas plates in the bottom row contain paraquat (15 mM). Wild-type and knockout samples were grown according to Rolke et al. (25). Transformants S160 and S150 demonstrate the silenced phenotype.

Pathogenicity of transformants. Previous studies reported that *bcsod1* mutants $\Delta bcsod1$ -1, $\Delta bcsod1$ -2, and $\Delta bcsod1$ -3 displayed a reduction in virulence (25). Therefore, silenced transformants can be similarly tested for a reduction in pathogenicity, which may determine the residual SOD concentration required for normal (wild-type) virulence. Transformants selected demonstrated high (S154, S156, S160, and A162) or intermediate (S150 and A164) levels of *bcsod1* downregulation and, therefore, were expected to also exhibit a reduction in virulence. Primary leaves of dwarf French bean plants were inoculated with spores of the wild type, knockout, and six silenced transformants (Fig. 5). The area of the lesion was recorded after 72 and 96 h postinoculation (hpi). In total, each sample (wild type, knockout, and transformants) was tested four times (four replicates in one experiment). Initial observations found that lesion development was similar in all strains, including the wild type and knockout, regardless of activity observed during plate-based analysis; all samples exhibited an average radius of growth of 3 to 4 mm at 72 hpi, 8 to 9 mm at 96 hpi, and complete maceration after 5 days. The equivalent growth rates between the wild-type and knockout samples were unexpected; therefore, an additional 15 French bean leaves were inoculated with the wild-type and knockout strains to confirm effects of pathogenicity in planta. Overall, infection rates (Fig. 5) of the knockout mutant were rather variable, as previously described by Rolke et al. (25) However, statistical analysis indicates that the area of necrosis for the wild type and knockout were equivalent (*t* test, *F* value not significant at *P* = 0.914).

DISCUSSION

Gene silencing has become a powerful tool for reverse genetics; by refining methodologies, silencing can serve to investigate and determine gene function quickly and efficiently. In contrast to gene knockout by homologous recombination, gene silencing is a dominant trait, which makes it feasible to perform gene function analysis in organisms with a diploid genome or in haploid fungi a multinucleate thallus, such as *B. cinerea*. In plant-pathogenic fungi, successful gene silencing was first reported in *Magnaporthe grisea* (16) and *Venturia inaequalis* (7). The *B. cinerea* SOD gene *bcsod1* was targeted to determine the potential and efficiency of silencing in *B. cinerea*. Paraquat plate-based assays served to determine initial SOD silencing frequencies. As expected, the wild type grew on plates with paraquat more slowly than on plates without paraquat. The $\Delta bcsod1$ gene knockout mutants exhibited minimal growth with paraquat, whereas transformants containing silencing constructs exhibited varying degrees of growth reduction with paraquat supplementation (ranging from similar to the wild type to 71% reduction in colony radius), signifying a range of SOD silencing levels. Knockout mutants and silenced transformants exhibited growth similar to the wild type without paraquat supplementation. Overall, plate-based analysis indicated that silencing was feasible using sense and antisense silencing constructs. Antisense-based constructs yielded a higher percentage of transformants with silenced phenotypes compared with sense-based constructs. The increase in observed antisense silencing frequencies may be attributed to the fact that complementary gene sequences promote the formation of RNA–RNA complexes between antisense and sense endogenous RNA mo-

lecules (2,36). Previous studies have found that, although sense and antisense silencing constructs are both capable of inducing gene silencing, they are only moderately efficient. For instance, it has been observed that only 5 to 20% of transformants showed a decrease in mRNA production in *Arabidopsis thaliana* (29,32). Because *bcsod1* transformants demonstrated a significantly higher percentage of transformants displaying silencing during plate-based assays, comparisons cannot be made with mRNA silencing frequencies observed in *A. thaliana*. However, when *Neurospora crassa* was transformed with a plasmid containing the entire *al-1* gene, a simple plate-based visual inspection revealed that $\approx 30\%$ of recovered transformants exhibited silencing phenotypes (9,26). These frequencies are similar to those observed during sense-mediated silencing in *Botrytis* spp. ($\approx 27\%$). However, it should be noted that all recovered transformants were subjected to in vitro plate-based analysis without the confirmation of silencing cassette integration. As a result, SOD silencing frequencies may be greater than those observed in *N. crassa*. Overall, plate-based and northern analyses confirm *bcsod1* downregulation, the first report of gene silencing in *B. cinerea*.

Fig. 3. Paraquat plate assay showing radial mycelial growth of six chosen transformants compared with the wild type (WT) and knockout (KO) strain, $\Delta bcsod1$ -1 of *Botrytis cinerea*. **A**, Average raw data showing radial growth (mm) of the wild type and transformants with and without paraquat; **B**, Percent decrease in radial growth (sample growth with paraquat treatment compared with growth without treatment) with respect to the data in **A**.

TABLE 2. Frequency of RNA-mediated silencing of *bcsod1* in *Botrytis cinerea*

Transformant	Total (no.)	Silencing levels (no.)			Transformants with silencing (%)
		Not detectable	Intermediate	High	
Sense + antisense	96	61	19	16	36
Sense	51	37	7	7	27
Antisense	45	24	12	9	47

Fig. 4. Northern blot analysis confirming superoxide dismutase (SOD) transcript expression in *Botrytis cinerea*. **A**, Total RNA was hybridized with a *bcsod1* probe. **B**, Total RNA was electrophoresed through two denaturing gels (italicized and bolded samples). **C**, SOD expression percentages (determined by densitometry analysis) indicate total detectable transcript compared with that of the wild type (WT1 or WT2) and are used when determining silencing levels. Italicized samples were compared with WT1 while bolded samples were compared with WT2. **D**, Silencing levels with respect to the WT are also indicated by the letters NS (not silenced), L (low, 61 to 90% *bcsod1* transcript levels), M (medium, 31 to 60% *bcsod1* transcript levels), and H (high, 1 to 30% *bcsod1* transcript levels). All samples exhibit silencing compared with the WT as determined by paraquat sensitivity.

Fig. 5. Example of in planta pathogenicity assay for determining virulence of *bcsod1*-silenced transformants of *Botrytis cinerea* compared with the wild type (WT). Sample S156 demonstrates a lesion size equivalent to the wild type (BO5.10). The *bcsod1* knockout mutant (KO; $\Delta bcsod1$ -2) displays similar levels in pathogenicity. Punctured plant leaves were inoculated with a single droplet containing conidia from the wild type and transformant (or knockout). Leaves were incubated over a period of 3 days before being photographed. Areas of infection were determined with ImageJ software.

Effects of SOD silencing were also assessed in planta. Previous investigations have found that $\Delta bcsod1$ knockout mutants demonstrated a reduction of virulence on French bean leaves (25). As a result, silenced transformants were hypothesized to vary in virulence levels, which would help to determine residual SOD levels required for normal (wild-type) virulence. Nonetheless, all tested $\Delta bcsod1$ knockout mutants and the silenced transformants were able to invade plant tissue and cause lesions of sizes similar to the wild type under laboratory conditions. The $\Delta bcsod1$ knockout mutant did result in highly variable lesion sizes as described by Rolke et al. (25). Even so, statistical analysis indicates that the wild type and knockout mutant do not differ in virulence capabilities. The variations in observed virulence may be caused by differences in French bean cultivar as well as growth conditions (light duration, fertilizers, and so on). For example, in a previous study, broad bean leaflets exhibited an increase in *B. cinerea* resistance when treated with yellow and red lighting (14). Other studies have shown that plant cultivar and growth condition may increase or decrease fungal virulence. For instance, Matheron and colleagues found that powdery mildew infection was dependent upon lettuce cultivar and growth conditions (22). Nutrient availability (nitrogen, sulfur, potassium, and so on) is also able to

affect fungal virulence. As a result, variations in fertilizer may directly affect plant resistance capabilities (34). The variations between plate-based analysis and in planta studies of select transformants may result from the high concentrations of free radicals found in the plate-based analyses compared with those of ensuing host-pathogen interactions or the complexities of the AOS pathway. High concentrations of free radicals found in the plate-based assays may not be overcome by the fungus, resulting in a diminished growth rate, while the host tissue defensive response may not employ a high oxidative stress response, allowing unhampered fungal growth rates. Recent studies have quantified free radical concentration on infected host tissue (20) but pose ambiguous results; it is unknown what proportion of free radicals is produced by the host versus the pathogen or the ratio of stable to unstable free radicals. The detoxification of intracellular H_2O_2 (generated by the fungus) by *B. cinerea* is also essential to fungal detoxification during infection. Enzymes involved in the detoxification process are unclear and need to be characterized (5,31). Hence, further investigation of total free radical production and neutralization during *Botrytis* spp. infection is needed before conclusions can be made. Therefore, our investigations have shown that, although silencing is possible in *B. cinerea*, initial plate-based screening may not be sufficient for identifying potential pathogenic factors. Instead, a combination of in vitro and in planta analyses should be used to determine true fungal virulence.

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