

イネいもち病菌 *Magnaporthe grisea* シアン耐性呼吸欠損株におけるalternative oxidase遺伝子の解析

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Analysis of Alternative Oxidase Gene in Mutants of *Magnaporthe grisea* Deficient in Alternative, Cyanide-resistant, Respiration

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Abstract

The fungicide SSF-126 [(*E*)-2-methoxyimino-*N*-methyl-2-(2-phenoxyphenyl)acetamide] induces cyanide-resistant, alternative respiration in the fungus *Magnaporthe grisea*. The activity of alternative respiration correlates with the expression level of alternative oxidase (AOX). Seven SSF-126-sensitive mutants (named MS) of *M. grisea* were completely deficient in alternative respiratory activity. Here, a genomic copy for the alternative oxidase gene (*AOX*) was isolated and analyzed. A 2661-bp *AOX* genomic DNA fragment containing a 613-bp of the promoter region of *AOX* was isolated from *M. grisea* wild type strain by using an inverse PCR method. The *AOX* was organized as three exons that are separated by short introns. Several *cis*-acting elements were detected in the 5'-upstream region of *AOX*. Northern blot analysis of *AOX* in MS mutants revealed that all the mutants expressed *AOX* mRNA in response to SSF-126 treatment, but the levels of mRNA in MS2, 5, 8, 9 and 11 were reduced several fold relative to the wild-type. All seven mutants had altered sequences in the *AOX* gene. These results demonstrate that sensitivity to SSF-126 and activity of alternative respiration in *M. grisea* are regulated by the *AOX* gene.

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Key words : alternative oxidase, cyanide-resistant respiration, fungicide SSF-126, *Magnaporthe grisea*.

INTRODUCTION

The fungicide SSF-126 [(*E*)-2-methoxyimino-*N*-methyl-2-(2-phenoxyphenyl)acetamide], developed for rice blast control, strongly inhibits mycelial respiration in the causal fungus *Magnaporthe grisea* by blocking electron flux in mitochondria through the cytochrome *bcl* complex^{14,21}. One hour after treatment with SSF-126 under normal growth conditions, respiration of cells has recovered. Growth then continues with a delayed generation time¹⁴. In field trials, however, SSF-126 markedly suppresses the development of rice blast¹¹. Because conidial germination of *M. grisea* was inhibited by SSF-126 in the concomitant presence rice-leaf homogenate⁷, SSF-126 can controls rice blast in combination with plant components.

Fungal mitochondria contain a cyanide-resistant, salicylhydroxamic acid (SHAM)-sensitive, alternative oxidase (AOX) in addition to cytochrome oxidase. This alternative respiratory pathway branches from the cytochrome pathway at the ubiquinone pool and directly reduces O₂ to H₂O. Electrons flowing through the pathway between ubiquinone and oxygen are not linked to oxidative phosphorylation; all of the free energy re-

leased is lost as heat^{15,22}. In the thermogenic plant *Arum maculatum*, this heat is utilized to volatilize foul-smelling compounds to attract insect pollinators¹². In non-thermogenic plants and fungi, however, the primary role of AOX has not been elucidated well.

In *M. grisea*, no AOX activity has been detected under normal growth conditions, although AOX activity can be induced by respiratory inhibitors such as SSF-126 and antimycin A¹⁴. The induced AOX activity coincided with the appearance of the 40-kDa AOX protein in *M. grisea* mitochondria^{13,24}. To characterize the SSF-126-resistant respiratory system in *M. grisea*, we previously isolated twelve mutants (named MS) deficient in the activity of AOX by screening for increased SSF-126 susceptibility after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) mutagenesis²⁴. Seven mutants which were completely deficient in AOX activity were classified into two groups. Four mutants produced the inactive AOX protein, whereas the other three did not express detectable amounts of the protein. In addition, we have cloned the *AOX* cDNA from *M. grisea* wild-type and analyzed the expression of *AOX* mRNA²³. The 1.4-kb mRNA accumulated in response to SSF-126 treatment. Expression of the mRNA was also induced by hydrogen peroxide or cycloheximide in the absence of SSF-126,

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suggesting that the *AOX* gene in the fungus is stress-induced.

To study the induction and catalysis of AOX, we isolated the *AOX* genomic DNA fragment containing the promoter region of the gene with an inverse PCR method. Sequence and expression analyses of the *AOX* gene were carried out in the mutants which are completely deficient in AOX activity.

MATERIALS AND METHODS

Fungus and culture conditions *Magnaporthe grisea* Herbert (rice pathogen, race 003) wild-type and MS mutants were cultured in CM liquid medium as described previously²⁴.

Isolation of a genomic clone and gene analysis of *AOX* *M. grisea* genomic DNA was isolated from mycelia using a modified CTAB method¹⁰. The 5'-upstream and 3'-downstream region of *M. grisea AOX* gene were cloned by using the inverse polymerase chain reaction (PCR) method¹⁷. Using *AOX* cDNA sequence information, five restriction enzymes, *Hae*III, *Hap*II,

*Sau*3AI, *Sph*I and *Xho*I, were selected and four primers were designed to obtain the 5'-upstream region. The primer sequences were as follows: 5'-ACA CAC CTT GCC AAA GTG GT-3' (*AOX*-F1), 5'-AGG CTT CTT CTG TGT ACT CG-3' (*AOX*-F2), 5'-GCT TCG TGT TGA CCT GAT GA-3' (*AOX*-R1) and 5'-AGA CGG TAC GTT CAG TGT TC-3' (*AOX*-R2). *M. grisea* genomic DNA was digested with each restriction enzyme, and digested DNA was self-ligated with T4-ligase (Takara, Kyoto, Japan) at 16°C for 12 hr. The first PCR was carried out with Taq DNA polymerase (Takara) using *AOX*-F1 primer, *AOX*-R1 primer and self-ligated genomic DNA as a template. The conditions for the PCR were as follows: denaturation for 1 min at 94°C, annealing for 2 min at 54°C, and extension for 3 min at 72°C. The reactions were carried out for 30 cycles. In the second, the PCR was carried out with *AOX*-F2 and *AOX*-R2 primers by using the first PCR products (3 µl) as a template. To obtain the 3'-downstream of *AOX* gene, the inverse PCR method was also used. Four restriction enzymes, *Hae*III, *Sau*3AI, *Sph*I and *Taq*I, were used. The sequences of primer were as follows:

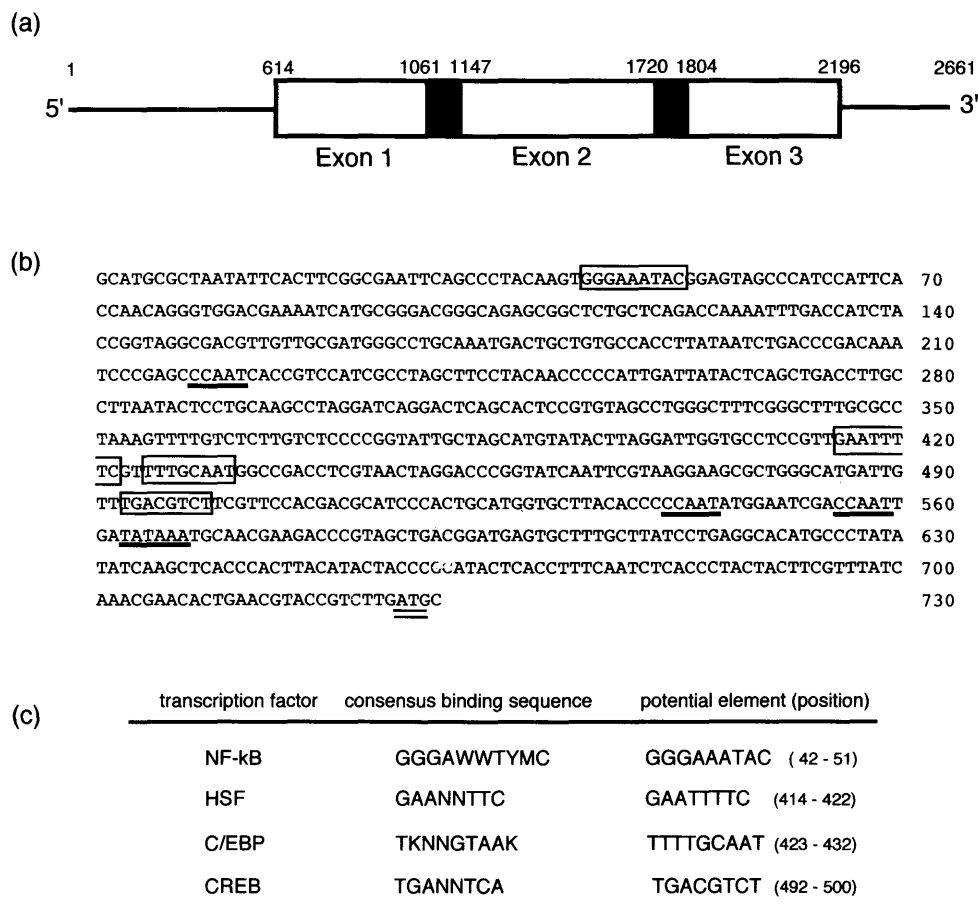


Fig. 1. Characterization of *M. grisea AOX* gene. (a) Schematic diagram of *AOX* gene. Exons are shown as open boxes, introns as filled boxes, and untranscribed regions as lines. (b) Nucleotide sequence of 5'-upstream region of *AOX* gene. Translation initiation site (ATG) is double-underlined. The putative TATA box and potential CAAT boxes are underlined. Four potential *cis*-acting transcriptional elements are shown in boxes. (c) The families of potential *trans*-acting factor on the *AOX* gene promoter. Four families of potential transcription factors and the consensus binding sequences are indicated. Abbreviations: W, A+T; Y, T+C; M, A+C; K, T+G; N, A+T+G+C.

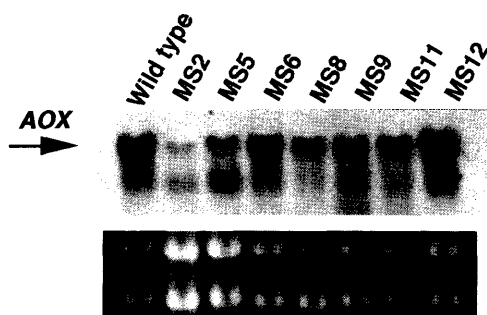


Fig. 2. Expression analysis of AOX mRNA in MS mutants. Upper panel: Probing with the digoxigenin-labeled AOX cDNA of MS mutants and wild-type strain total RNA. Total RNA was extracted from mycelia grown under SSF-126 supplemented medium. Lower panel: Ethidium bromide-stained agarose gel (1.0%).

5'-CGC AAG ACT GAC ACA AAG AG-3' (AOX-3F1), 5'-TTG GCA CAA CAG ATG ACA TC-3' (AOX-3F2), 5'-TTC CTC AAA CTG AAA CCT GC-3' (AOX-3R1), and 5'-CCT CCT TTC CTA TAA CAT CC-3' (AOX-3R2). The amplified fragments were subcloned into plasmid pCR2.1 (Invitrogen, Leek, Netherlands) and the DNA sequences were determined with a Thermo Sequenase dye terminator cycle sequencing kit (Amersham, OH, USA) using an automated DNA sequencer (373XL, Perkin-Elmer Applied Biosystems, CA, USA). Additional specific primers were designed to amplify the full length AOX gene containing 5'- and 3'-non-coding regions: 5'-GCA TGC GCT AAT ATT CAC TTC-3' (Paax-1) and 5'-TCG AGG CTG TGC TAC TCG CG-3' (Taax-1). PCR amplification was carried out using the primers and *M. grisea* genomic DNA, yielding a single band of 2.6-kb. The product was separated on an agarose gel (0.8%) to remove uncoupled primers and nucleotides. The corresponding band was isolated by a QIAEX II gel extraction kit (QIAGEN, Hilden, Germany) and purified fragment was directly sequenced using Thermo Sequenase dye terminator cycle sequencing kit (Amersham) according to instructions. The nucleotide sequence was analyzed by the DNASIS and Sequencher programs (Hitachi Software, Kanagawa, Japan).

Northern blot analysis Total RNA was extracted from mycelia of *M. grisea* wild-type and MS mutants, which were treated with SSF-126 (50 μ M) for 1 hr, by the acidic guanidium thiocyanate method⁹⁾. Total RNA (10 μ g) was separated on a 1% (w/v) agarose gel containing 1% (v/v) formaldehyde, and blotted on a nylon membrane (Hybond N+, Amersham). The hybridization solution contained 0.1% (w/v) *N*-laurylsarcosine, 7% (w/v) SDS, 2% (w/v) Blocking reagent (Boehringer Mannheim, Mannheim, Germany), 0.75 M NaCl, 75 mM sodium citrate, 50 mM sodium phosphate buffer (pH 7.0), yeast tRNA (200 μ g/ml), and denatured salmon sperm DNA (100 μ g/ml). The blots were probed with cDNA labeled with digoxigenin (Boehringer Mannheim). Hy-

Table 1. Mutations in MS alleles

Strain	Mutation
MS2 and 5	G to A transition at codon 184 (changes Trp to Stop)
MS6 and 12	C to T transition at codon 196 (changes Arg to Cys)
MS8 and 9	C to T transition at codon 158 (changes Ala to Val)
MS11	multiple changes that alter the sequence GAGGCAC to TAGTCAT at position 614 to 620

Abbreviations: Trp, tryptophan; Arg, arginine; Cys, cysteine; Ala, alanine; Val, valine.

bridization was allowed to proceed overnight at 60°C. Membranes were washed twice in 2×SSC/0.1% SDS at room temperature and twice in 0.1×SSC/0.1% SDS at 68°C. Signals were detected with the chemiluminescent substrate CSPD (Tropix, MA, USA).

RESULTS

Cloning and sequence analysis of AOX genomic DNA

To obtain the 5'-upstream region of *M. grisea* AOX gene, the inverse PCR method was used. In the second PCR, a 900-bp fragment was obtained from the first PCR product using *Sph*I-digested genomic DNA as a template. The fragment contained the 287-bp sequence of the 5' region of *M. grisea* cDNA and a 613-bp sequence of the 5'-upstream region. A 555-bp fragment of 3'-downstream region of AOX was obtained by inverse PCR reaction using *Taq*I-digested DNA as a template. Then, full-length PCR yielded a 2661-bp fragment (Fig. 1). Comparison of the sequences of cDNA and the genomic DNA indicated that the *M. grisea* AOX gene contained three exons. Two introns had 85-bp and 83-bp. The 5'-end of the longest cDNA obtained was at position 619 (Fig. 1). The putative TATA box with the sequence TATAAA was located at bases 562 to 568. Three putative CAAT boxes were at bases 219 to 223 (CCAAT), 540 to 544 (CCAAT) and 555 to 559 (CCAAT). Several *cis*-acting transcriptional elements were identified in the promoter region of AOX: nuclear factor kappa B⁴⁾ (NF- κ B, at bases 43 to 52), heat shock factor⁹⁾ (HSF, at 414 to 423), CAAT enhancer binding protein¹⁾ (C/EBP, at 421 to 433) and cyclic AMP response element binding protein⁵⁾ (CREB, at 493 to 500).

AOX gene expression in MS mutants

We have isolated seven *M. grisea* mutants deficient in alternative respiratory activity by screening with a replica method for increased SSF-126 sensitivity after MNNG mutagenesis²⁴⁾. These mutants were classified into two groups: four mutants, MS6, 8, 9 and 12, produced inactive AOX protein, and the other three mutants, MS2, 5 and 11, did not produce detectable amount of AOX. To characterize the mutants, we analyzed

AOX gene expression in MS mutants by Northern blot analysis using *AOX* cDNA as a probe²³). None of the mutants expressed *AOX* mRNA in the absence of SSF-126 (data not shown). In the presence of SSF-126, MS6 and 12 expressed 1.4-kb *AOX* mRNA as seen in wild type strain, but the other mutants showed reduced expression (Fig. 2). A faint signal with 1.2-kb was also detected in all strains, suggesting that a gene containing a similar sequence of *AOX* may be expressed in the fungus. This signal, however, was not detectable under the same conditions of Northern analysis in other experiments²³). No further characterization of the transcript was done.

Sequence analysis of *AOX* in MS mutants

To identify the mutation in MS mutants, PCR products from the 2.6-kb region of the *AOX* gene from MS mutants were prepared and both strands were sequenced (Table 1). MS6, 8, 9 and 12 contained single missense mutations in the coding sequences. Interestingly, MS6 and 12 had the same missense mutation, resulting in a change of arginine to cysteine at codon 196. MS8 and 9 also contained the same single mutations at codon 158, resulting in a change of alanine to valine. MS2 and MS5 had a single nonsense mutation that changed tryptophan to a stop codon at 184. MS11 had multiple changes at the 5' end of the 5'-non-coding region that altered GAGG-CAC to TAGTCAT.

Phenotypic characterization of MS mutants

Conidia formation, germination, appressorium formation and pathogenicity to *Oryza sativa* (cv. Aichiasahi) in MS 9 were examined to determine whether *AOX* activity is related to phenotypic expression. No significant differences in the phenotypes were detected between *M. grisea* wild-type and MS9 (data not shown).

DISCUSSION

By using the inverse PCR, we obtained a 2661-bp genomic DNA fragment from *M. grisea* wild type strain. Comparison with the cDNA sequence indicated that the *AOX* gene of *M. grisea* was organized as three exons that are separated by two short introns (Fig. 1). This structure was similar to that of *Neurospora crassa AOX* gene⁸), whereas *Sauromatum guttatum aox1* gene was organized as four exons¹⁹).

The mutant MS9, which is completely deficient in *AOX* activity, showed normal growth and infectivity, suggesting that *AOX* activity in *M. grisea* is not required for growth and infection. In some plants, the physiological role of *AOX* has been investigated. Respiration through the alternative pathway is involved in the heat generation to volatilize foul-smelling attractants for insects engaged in the pollination process in *Araceae*¹²), as well as in the defense response against tobacco mosaic virus (TMV) infection in tobacco²). In other plants and fungi, however, the role of *AOX* has not been elucidated well. Under the presence of SSF-126, the mutants lacking the *AOX* activity did not continue to

grow, whereas the wild type strain did²⁴). Our previous results have shown that inhibition of the cytochrome pathway by SSF-126 resulted in the generation of superoxide anion¹³) and further that hydrogen peroxide could induce the expression of *AOX* gene in the absence of SSF-126²³). Therefore, *AOX* in the fungus may play a protective role against oxidative stress in the mitochondria. We have also suggested that the *AOX* gene is a member of a very early gene, because the *M. grisea AOX* gene and very early mammalian genes showed similar responses to cycloheximide. Sequence analysis of the promoter region of the *AOX* gene revealed the presence of several sequences similar to consensus binding sites for transcriptional factors which are involved in stress-responsive induction.

We demonstrate here that both sensitivity to SSF-126 and activity of cyanide-resistant respiration in the fungus are regulated by *AOX* gene. The mutant MS5, which did not produce detectable amounts of *AOX* protein, expressed *AOX* mRNA in response to SSF-126 treatment (Fig. 2). This mutant had a nonsense mutation at codon 184 as did the MS2 mutant. The MS11 mutant did not contain a mutation in the coding sequence but had multiple mutations in the 5' non-coding region. These mutations may prevent the translation of *AOX* gene. Four mutants, MS6, 8, 9 and 12, contained a single missense mutation in the *AOX* coding sequence. These mutations occurred in positions which are conserved in all other *AOX*s sequenced to date^{6,8,18,20}), and were presumed to inactivate the protein. One of these (codon 158) found in MS8 and 9, is located in the putative transmembrane region¹⁶), whereas the other mutation (codon 196) was located in the intermembrane space (between inner and outer membranes) in mitochondria. Five mutants, MS2, 5, 8, 9 and 11, also showed reduced expression of *AOX* mRNA as compared to the other mutants and wild-type strain. These results imply a possibility that additional mutation occurs in a gene encoding a component that regulates induction of *AOX* activity.

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和 文 摘 要

雪岡日出男・稲垣秀一郎・田中玲爾・加藤研治・三木信夫・水谷 章・益子道生：イネいもち病菌 *Magnaporthe grisea* シアン耐性呼吸欠損株における alternative oxidase 遺伝子の解析

イネいもち病殺菌剤 SSF-126 はイネいもち病菌 *Magnaporthe grisea* ミトコンドリア電子伝達系の複合体 III (cytochrome bc1 complex) に作用し、本菌のチトクローム系の呼吸を阻害する。しかし、いもち菌は alternative oxidase (AOX) を誘導し、SSF-126 非感受性のいわゆるシアン耐性呼吸により酸素消費を回復させる。われわれは、本菌におけるシアン耐性呼吸機構解明のためシアン耐性呼吸欠損株 (MS1-12) を分離し、解析をおこなってきた。これまでに欠損株 7 株のうち AOX 蛋白質が検出されない株が 3 株 (MS2, 5, 11) あり、残り 4 株 (MS6, 8, 9, 12) は不活性 AOX 蛋白質を生産している株であることを示した。また野生株から AOX cDNA をクローニングし、SSF-126 非存在下で AOX が過酸化水素やシクロヘキシミドにより発現誘導されることを明らかにした。今回、野生株から 5' 上流非翻訳領域 726 bp を含む AOX ゲノム DNA 2661 bp を inverse PCR 法によりクローニングした。AOX 遺伝子は 3 つのエクソンからなっており、その 5' 上流非翻訳領域には数種の転写因子結合配列が確認された。シアン耐性呼吸欠損株 7 株における AOX 遺伝子発現解析の結果、すべての株において AOX mRNA の発現が確認されたが、そのうち MS2 株は発現が極端に低かった。塩基配列解析の結果、7 株すべての AOX 遺伝子塩基配列内に塩基置換が確認された。以上の結果は、AOX 遺伝子が本菌のシアン耐性呼吸活性発現を担っていることを示している。