Transcriptional Control of Nitric Oxide Reductase Gene (CYP55) in the Fungal Denitrifier Fusarium oxysporum

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Fungal denitrification is a dissimilating metabolic mechanism for nitrate and was first described in Fusarium oxysporum. Here we investigated regulatory systems of expression of CYP55, which encodes cytochrome P450 (P450nor) and is essential for the fungal denitrification. Promoter-reporter analysis of F. oxysporum CYP55 using Escherichia coli β-galactosidase showed that the region between nucleotides −526 and −515 was critical for induction by nitrate. It contained a nucleotide sequence similar to the binding consensus sequence of the pathway-specific transcriptional factor NirA, which induces expression of the nitrate-assimilatory genes. The deletion studies also indicated that the nucleotide sequence between −118 and −107, which was similar to the binding consensus of the yeast Rox1p, which represses the anoxic enzymes under aerobic conditions, was responsible for repression of CYP55 under aerobic conditions. These results indicate that the fungus adapts to the denitrifying conditions by a combination of NirA- and Rox1-like transcription factors.

Key words: denitrification; nitric oxide reductase; cytochrome P450nor; nitrate respiration; Fusarium oxysporum

Denitrification is an anaerobic metabolic mechanism that has been well documented in facultatively anaerobic bacteria by which the bacteria reduce nitrate to nitrous oxide (N2O) or dinitrogen. This reaction is closely linked to the respiratory chain and thus denitrification is thought to function as nitrate respiration.1,2) Bacterial denitrification is an adaptation mechanism for the cell to acquire energy in the absence of oxygen (O2) and is induced under O2-limited conditions.2,3) Meanwhile, fungal denitrifiers were recently found.4–6) Fusarium oxysporum is the first example of denitrifying eukaryotes and can reduce nitrate up to N2O.4) Recent research in our laboratory showed that fungal denitrification functions as nitrate respiration under O2-limited conditions,7 like the bacterial one, and is located in mitochondria.7–9) It has been further found that the denitrifying activity is not restricted to the single species of F. oxysporum but is also distributed among many other filamentous fungi across the phyla including ascomycetous fungi such as Cylindrocarpon tonkinense and Gibberella fujikuroi,5 and the basidiomycete fungus Trichosporon cutaneum.6 These observations were surprising because these denitrifying fungi have been thought to be strictly aerobic organisms and denitrifiers had been found exclusively in bacteria. We previously showed that the fungal denitrifying system is comprised of a set of enzymes, i.e. nitrate reductase, formate dehydrogenase, nitrite reductase,10) and nitric oxide reductase (Nor).11) Among them, Nor of F. oxysporum has been best characterized12,13) and its gene (CYP55) has been cloned.14,15) The fungal Nor belongs to the cytochrome P450 (P450) superfamily on the basis of the primary and tertiary structures,14,16) and thus is designated P450nor. Involvement of P450nor is one of the striking features of the fungal denitrification as all the bacterial Nor known to date contain cytochromes bc in their catalytic centers and are not P450.1,2) The catalytic properties of P450nor are unique among P450 proteins. Conventional P450 catalyze monooxygenation which is supported by other electron-transferring proteins, but P450nor reduces NO into N2O using NADH or NADPH as a direct electron donor and requires no other protein in its reaction.11–13) This is in marked contrast to the bacterial Nor, which receives electrons from the respiratory chain.1,2) F. oxysporum produces two isoforms of P450nor that showed no observable difference in

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Abbreviations: Nor, nitric oxide reductase; P450, cytochrome P450; P450nor, cytochrome P450nor; PCR, polymerase chain reaction; P_CYP55, the gene promoter of cytochrome P450nor
their catalytic properties. They are encoded by the single gene CYP55, in which two putative initiation codons for translation are found. The upstream one is followed by 27 amino acids residues which functions as a targeting signal for transport to mitochondria and are used for translating one of the isoforms (P450norA) that is targeted to mitochondria. Another isoform (P450norB) is translated from the second initiation codon and has the first methionine cleaved off after translation, and the resulting N-terminus alanine is acetylated to form a cytosolic isoform of P450nor.

Molecular genetics of denitrification have been most intensely studied in F. oxysporum. Previous studies on expression of CYP55 suggested that the transcription of CYP55 is induced under denitrifying conditions, that is, the P450nor gene is transcribed in the presence of either nitrate or nitrite and under a limited O2 supply. This is similar to the expression of denitrifying genes of bacteria except that fungal denitrification is induced in the presence of significant amounts of but not in excess O2. In the case of bacterial nitrate respiration systems, transcription factors such as FNR and NarL have been identified, which bind specifically to specific DNA sequences on the gene promoters of the denitrifying enzymes and regulate their expressions. On the contrary, no regulating mechanism of expression of CYP55 has been clarified. Therefore, the promoter analysis of CYP55 from F. oxysporum will be important for elucidating molecular basis of the induction of denitrification of the fungus.

Here we investigated the structure of the gene promoter of CYP55 (P CYP55) and identified for the first time the specific transcription elements regulating gene expression of the eukaryotic denitrifying gene. We also found those elements are similar to the binding sequences of NirA and Rox1p that respond to nitrate and hypoxia in other fungi.

Materials and Methods

Strains, cultures, and media. Fusarium oxysporum MT-811, AET1, AET2 (harboring pLDAL1) and MT811/PLD (harboring pLD10) were previously described. For assaying denitrification, the transformants were cultured at 30°C for 72 h with shaking at 120 rpm on a rotary shaker in 300 ml of GP medium (3% glycerol, 0.2% polypeptone, 10 mM potassium phosphate (pH7.2), 10 μg/ml hygromycin B) in a 500-ml volume Erlenmeyer flask sealed with a cotton plug. Then 300 ml of Dextrose-peptone (DP) medium (2% glucose, 0.2% polypeptone, inorganic salts and 50 μg/ml hygromycin B) containing 0.2% NaN3 in a 500-ml volume Erlenmeyer flask was inoculated with a portion (30 ml) of the first culture and incubated for 72 h under the same conditions as above.

Construction of plasmids. Upstream deletion mutants of P CYP55 were amplified by the polymerase chain reaction (PCR) using the primer, 5'-GTAGGATCCATGTTGATGGAAAT-3' and each of the following primers; 5'-GTCCTTTTGTGACCATGTCG-3' (for pLDAL-A578), 5'-TGTGTTACGCATGTAACCTAAAGACTGC-3' (for pLDAL-A5543), 5'-GGGTACCCAGCATCTCCACGG-3' (for pLDAL-A590), 5'-CAGAATGGTACCTTGATCC-3' (for pLDAL-A510), 5'-CAGAATGGTACCTTGATCC-3' (for pLDAL-A590), 5'-GGGTACCCAGCATCTCCACGG-3' (for pLDAL-A5877), 5'-CAGAATGGTACCTTGATCC-3' (for pLDAL-A5210) and 5'-GGGTACCCAGCATCTCCACGG-3' (for pLDAL-A5877) (Underlines indicate restriction sites for BamHI and KpnI). They were digested with KpnI and BamHI and ligated with pUC19 digested with KpnI and BamHI. The 3.0-kb BamHI fragment encoding lacZ was inserted into the BamHI site of each resulting plasmid DNA. The resulting plasmids were digested with KpnI and SalI, the 3.1- to 3.6-kb fragment containing the promoter-lacZ fusion was purified, and they were inserted into the fungal transformation vector pLD10. Resulting plasmids were designated as pLDAL-A578, pLDAL-A543, pLDAL-A510, pLDAL-A589, pLDAL-A5274, pLDAL-A5245, and pLDAL-A5607, respectively. DNA was manipulated by the standard methods.

The promoters with internal deletions were amplified by PCR by the methods described by Higuchi et al. Primers used were: 5'-GACTGGTTTGCAGATCCGATCTTCCAGG-3' (for pLDAL-IntA), 5'-ATCAATGACACCGCTCAATCTCCAGG-3' (for pLDAL-IntB), and 5'-ATCAATGACACCGCTCAATCTCCAGG-3' (for pLDAL-IntC). These promoter fragments are fused to lacZ and ligated to pLD10 as described above. All the mutations introduced in this study were confirmed by sequencing the inserted nucleotide fragments.

Transformation of F. oxysporum. Protoplasts of F. oxysporum MT-811 prepared as described. Transformation was done by the polyethylene glycol-CaCl2 method using 10 to 30 μg of plasmid DNA as described.

β-Galactosidase assay. Mycelia were suspended in Z buffer (100 mM sodium phosphate (pH 7.2), 1 mM MgSO4, 10 mM KCl, and 50 mM β-mercaptoethanol) and disrupted by sonication as described. The homogenate was centrifuged at 700 × g for 10 min and the resulting supernatant was centrifuged again at 15,000 × g. The resulting supernatant was used for the β-galactosidase assay. β-Galactosidase was as-
Results

Nitrate- and oxygen-dependent regulation of P<sub>CYP55</sub>

In the previous reports we reported the construction of the fusion gene using the 700 bp of the promoter fragment of <i>CYP55</i> (P<sub>CYP55</sub>) and <i>E. coli</i> lacZ, analyzed fungal transformants with that gene, and showed that β-galactosidase activity derived from the P<sub>CYP55</sub>:lacZ fusion is increased in the presence of nitrate and repressed under oxic conditions.15 In that experiment, lacZ was fused to the initiation codon of one of the isoforms (P450norB) which located downstream of the initiation codon of another isoform (P450norA). To minimize DNA regions responsible for the regulated expression of P<sub>CYP55</sub>, we deleted the region between the initiation codons of the isoforms, fused the initiation codon of P450norA in frame to the open reading frame of lacZ (pLDAL1), and examined whether the region is involved in the regulated expression of CYP55. The transformants with pLDAL1 (strains AET1, and AET2) showed significant β-galactosidase activity under denitrifying conditions (Table I). These activities must be derived from the introduced gene, because glucose was added to the media to repress endogenous β-galactosidase, and, moreover, little activity was detected in the control strain MT-811/pLD10 under the culture conditions we used. Southern blot analysis showed no integration event of the plasmid into the chromosomes in these strains (data not shown), which is consistent with the report that pLD10 replicates autonomously in <i>F. oxysporum</i>. Copy-numbers of pLDAL1 in the strains AET1 and AET2 ranged from 2.5 to 2.7 under the four culture conditions used (Table I and data not shown). Copy-numbers of the plasmid in the cells cultured under denitrifying conditions ranged from 2.5 to 2.7 among all the transformants including the deletion constructs shown below (data not shown). The activities per copy number (1000 to 1400 unit mg<sup>-1</sup> copy<sup>-1</sup>) under the denitrifying conditions were similar between AET1 and AET2 (Table I). In both strains, β-galactosidase activity was higher in the presence of nitrate and under the hypoxic conditions (Table I). This is consistent with the expression pattern of CYP5515 and β-galactosidase activity due to lacZ fused to CYP55 at the initiation codon of P450norB.17 These results showed that the promoter region between the two initiation codons was dispensable for regulating expression of the CYP55 gene by nitrate and O2.

Nitrate responsive element in P<sub>CYP55</sub>

To minimize putative regulating elements in P<sub>CYP55</sub>, several deletion mutants of P<sub>CYP55</sub> were constructed and their activities were analyzed using the reporter system above. The fungal transformants containing pLDAL1, pLDAL1-D378, and pLDAL1-D543 were assayed for β-galactosidase activity under the inducing and the repressing conditions. These transformants produced higher levels of β-galactosidase activity under the hypoxic conditions than under the aerobic conditions both in the presence and absence of nitrate in the medium (Fig. 1). β-Galactosidase activity in the presence of nitrate was decreased significantly by eliminating the DNA region between –700 and –543, indicating that the region affects nitrate-dependent expression of P<sub>CYP55</sub>. Further deletion of the promoter region to –510 resulted in a drastic decrease in β-galactosidase activity under the nitrate-inducible conditions. The inducing ratios
were close or less than 1 (0.9 and 0.6 under the aerobic and the hypoxic conditions, respectively). These results indicate that a critical nitrate-responsive element is located between the nucleotides –546 and –510. Comparison of the nucleotide sequence in this region to the nucleotide sequences of the gene promoters of other P450nor isozymes (P450nor1 and P450nor2 of Cylindrocarpon tonkinense) indicated that the nucleotide sequence between –546 to –510 is conserved among these genes (Fig. 2A). Interestingly, the nucleotide sequence of the region contained a similar nucleotide sequence to the consensus sequence of the transcription factor NirA (CTCCGHGG, shown in Fig. 2A) which is characterized in Aspergillus nidulans. NirA and its counterpart in Neurospora crassa (Nit-4) are known to be positive regulators of the nitrate/nitrite-inducible genes involved in the nitrate assimilatory pathway in the fungi.21,28,29 The transformants containing pLDAL1-IntA that has a deletion in the consensus sequence (from –526 to –515) had lower levels of β-galactosidase activity under the inducing conditions (Fig. 1). Another similar sequence for binding NirA (from –508 to –497) is found at 7 bp downstream of the NirA site above although the sequence does not completely match the consensus (Fig. 2). Deletion of this region from P\textsubscript{CYP55} (pLDAL1-IntB) did not cause a significant decrease of β-galactosidase activity (Fig. 1), indicating that this region is not required for induction by nitrate. These results indicate that the NirA-binding sequence located at the region between –526 to –515 is critical for nitrate-dependent induction of P\textsubscript{CYP55} in F. oxysporum.

\textbf{O\textsubscript{2}-responsible element in P\textsubscript{CYP55}}

All the deletion mutants shown in Fig. 1 had higher activity under the hypoxic conditions than under the aerobic conditions. To investigate a putative regulatory element responsible for environmental \textit{O\textsubscript{2}}, we constructed other mutants of P\textsubscript{CYP55} and analyzed their promoter activity (Fig. 3). The \textit{F. oxysporum} transformants with the plasmid pLDAL1-Δ389, pLDAL1-Δ274, and pLDAL1-Δ245 showed higher β-galactosidase activity under the hypoxic conditions than the aerobic conditions. For example, in the absence of nitrate in the medium, the ratio of the activities between the aerobic and the hypoxic cells was nearly 100-fold. Further deletion of P\textsubscript{CYP55} to the nucleotide –107 resulted in a drastic (50-fold) decrease in β-galactosidase activity under the hypoxic conditions (Fig. 3). These results suggest that the O\textsubscript{2}-responsible element should be located in the region between –245 and –107. In the presence of nitrate, the β-galactosidase activity was decreased more severely under the hypoxic conditions by eliminating the region between –389 and –245 than in the absence of nitrate. The plasmid pLDAL-Δ245, for example, resulted in only 4-fold activity under the hypoxic conditions (10 U/mg) over that in the aerobic conditions (2.5 U/mg). This indicates that nitrate in the medium affects the O\textsubscript{2}-dependent regulation of P\textsubscript{CYP55}.

Comparing the nucleotide sequences in the region from –245 to –107 to the gene promoters of other P450nor isozymes, we could find a conserved sequence, as shown in Fig. 2B. This sequence is also found to be similar to the consensus sequence for binding of Rox1p, which has been reported as a repressing factor of transcription of the anoxic genes of \textit{S. cerevisiae} under oxic conditions.22 To investigate the nucleotide sequences corresponding to O\textsubscript{2}, plasmid pLDAL1-IntC, which lacked the conserved 12-bp sequence (from –118 to –107), was constructed. Transformants containing this plasmid produced higher β-galactosidase activity under both the aerobic and the hypoxic conditions, indicating that the nucleotide sequence has an activity for repressing...
transcription under the aerobic conditions. This is consistent with the repressing function of the binding sequence for Rox1p in S. cerevisiae and suggests that the mechanism for repressing hypoxic genes in aerobic growth may be similar between F. oxysporum and S. cerevisiae.

Discussion

Although expression mechanisms of the bacterial denitrifying genes have been studied for decades, there was no report on the transcription mechanism of the fungal denitrifying genes until our study. Here we demonstrated participation of the functional transcription elements the nucleotide sequences of which are similar to the binding consensus sequences of the eukaryotic transcription factors NirA and Rox1p in the expression of CYP55. These results also show that these transcription elements are critical for P_{CYP55} to respond to nitrate and environmental O_2, respectively. This completely corresponds with the previous observations that NirA and Rox1p of other fungi respond to nitrate and O_2 limitation, respectively, and, therefore, suggests that the expression of CYP55 is regulated through these transcription factors.

Although our results showed that expression of CYP55 would be regulated through transcription factors (NirA and Rox1p) both of which belong to the eukaryotic families of transcription factors, they suggest that other transcription elements are likely to be involved in the regulated expression of CYP55. For example, the mutant of the Rox1p binding site (pLDAL-IntC, Fig. 3) partly retains the activity to repress the expression under the aerobic conditions, suggesting the presence of other O_2-responsive element(s) in P_{CYP55}. The deletion mutant of the NirA site (pLDAL-IntA, Fig. 1) is defective in derepression under hypoxic conditions as well as in the nitrate-dependent induction, suggesting this elements affect O_2 responsibility of P_{CYP55}. Otherwise, deletion of the region between −700 and −578 partially lost nitrate-dependent induction activity (Fig. 1) although the NirA site is intact. We previously found in this region a similar nucleotide sequence similar to the binding consensus sequences for the bacterial transcription factors NarL, which regulate expression of the bacterial denitrifying genes. Contribution of this potential element in the expression of P_{CYP55} has not been established from our results. Investigation on further mutation study of P_{CYP55} will established the overall mechanism of the regulated expression of CYP55.

Some bacterial strains both assimilate and dissimilate nitrate. It has been shown that most nitrate-dissimulating bacteria express a set of genes for nitrate respiration under anaerobic conditions in the presence of nitrate. The expression is mediated by a specific transcription factor that belongs to the FNR family. Another transcription factor, NarL, activates the nar operon required for dissimilatory nitrate reduction. In contrast, bacteria use a distinct transcription factor such as NtrC for assimilating nitrate. Furthermore, mutation at the nar locus does not affect the expression of nitrate assimilation, suggesting that bacteria regulate expression of the genes involved in nitrate assimilation and dissimilation by using their respective transcription factors. By contrast, involvement of the cis-element for NirA in expression of CYP55 should indicate that expression of both the nitrate assimilation and denitrification (dissimilation) systems are regulated simultaneously in the fungus, as NirA is concerned with nitrate-dependent induction of the nitrate assimilatory genes in many fungi. This is a unique feature of the fungus. Recently, we showed that the fate of nitrate in the culture of F. oxysporum is dependent on the O_2 supply. When the O_2 supply is enough to support cell growth through O_2 respiration (aerobic conditions), nitrate is reduced to ammonium and assimilated, but it is reduced to N_2O by denitrification under the hypoxic conditions. Our results explain this phenomenon as follows. Under the aerobic conditions the nitrate assimilatory genes are induced in an NirA-dependent manner, but the denitrifying genes are repressed by Rox1p and thus the fungus assimilates nitrate. However, under the hypoxic conditions, Rox1p does not repress expression of the denitrifying genes and NirA-dependent induction of the gene expression occurs. Under these conditions nitrate is reduced by both assimilation and denitrification. This gene regulation mechanism is quite reasonable for the fungus as both nitrate assimilation and denitrification use nitrate not only as a substrate but also as an inducer.

Although there are other instances of anaerobic respiration by eukaryotes, it is a rather rare event as known to date. Fumarate respiration by some parasitic nematodes, and nitrate respiration by ciliates and fungi are such examples. Among them, to our knowledge, this study is the first report on the molecular mechanisms for regulating gene expression under anaerobiosis. The studies on anaerobic metabolisms of the eukaryotic cells have just begun. Future studies on the transcription factors involved in the fungal denitrification will be important for understanding the mechanisms by which the eukaryotic cells adapt into anaerobiosis.

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References

Regulation of a Fungal Denitrifying Gene


