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Cloning, sequencing and expression analysis of the *NAR* promoter activated during hyphal stage of *Magnaporthe grisea*[*]

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Abstract: The promoter of *NAR* gene in *Magnaporthe grisea* was isolated and sequenced. The promoter sequences contained the "TATA" box, the "CAAT" box, and binding sites for fungal regulatory proteins. Programs that predict promoter sequences indicated that promoter sequence lies between locations 430 and 857 of the *NAR* promoter fragment. *GFP* expression under the *NAR* promoter and *NAR* transcript analysis revealed that this promoter is activated primarily at the mycelial stage in the rice blast fungus and could be used to express native or extrinsic genes in the mycelia of the rice blast fungus.

Key words: *Magnaporthe grisea*, Promoter, *NAR*, Clone, Hypha
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INTRODUCTION

The fungus *Magnaporthe grisea* is the cause of the rice blast disease found the world over wherever rice is grown and is increasingly used as a model system to study host-pathogen interaction (Dean *et al*., 2005). Introducing such extrinsic genes as *GFP*, or the gene for fusion protein, into the blast fungus under the control of a native promoter is an effective method to study this fungus. The powerful active promoter of *M. grisea* can also be used for gene expression in other pathogenic fungi or fungi of industrial significance to study the function of a gene or to produce the protein. Although many promoters of this fungus have been obtained during studies on the function of pathogenic genes (Bruno *et al*., 2004; Foster *et al*., 2003; Fudal *et al*., 2007; Xue *et al*., 2002), these promoters were generally expressed weakly in the fungus. Also, a powerful promoter actively expressed during the hyphal stage of this fungus has never been reported. In this paper, we report a typical hypha promoter, which is actively expressed in the hyphae of *M. grisea*, and its cloning, sequencing, and expression analyses.

MATERIALS AND METHODS

Isolates of the fungal pathogen and growth conditions

M. grisea strain Guy-11 was cultured on complete medium (Talbot *et al*., 1993) at 25 °C in a cycle comprising 14 h of light alternating with 10 h of darkness. Conidia were harvested and resuspended (1×10^5 spores/ml), and a 20-μl drop was placed on each plastic coverslip for studying conidial germination and appressorium formation, following the procedure described before (Lu *et al*., 2005).

Cloning and sequencing of the *NAR* promoter

The DNA fragment containing promoter sequences of the *NAR* gene (putative protein MG10274.5 in the *M. grisea* genome database) was amplified from Guy-11 genomic DNA by polymerase

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chain reaction (PCR) using primers 5′-CAGGATC CGGGAAGCGATTGCGTT-3′ and 5′-GGTGCC ATGGTGTCGGTTGTGGTG-3′ and cloned into a T-vector, namely pUCm-T (Sangon, Shanghai, China). The recombinant cDNA clones were directly sequenced on an ABI 377 DNA sequencer (Applied Biosystems, USA) with primers M13R and M13F. DNA isolation, PCR, ligation reaction, restriction digest, and gel electrophoresis were carried out following standard procedures (Sambrook *et al*., 1989).

The structure of the *NAR* promoter was predicted using online promoter analysis programs, such as Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html, version 2.2), TSSP (http://www.softberry.com/berry.phtml, prediction of plant promoters), and TFSCAN (http://bioweb.pasteur.fr/seqanal/interfaces/tfscan.html).

Construction of green fluorescent protein (GFP) expression vector and fungal transformation

The promoter DNA fragment of the *NAR* gene was cut out from the T-vector containing the *NAR* promoter using restriction endonuclease *Bam*HI and *Nco*I and inserted into *Bam*HI-*Nco*I sites of pEGFP (Clontech, USA) to produce an interim vector, namely pEGFP-NAR. Then a hygromycin-resistant gene (*HPH*) was cut out using *Sal*I from pCB1003 vector (Carroll *et al*., 1994) and inserted at the *Sal*I sites of pEGFP-NAR to generate pNAR. In the pNAR, the *GFP* expression construct was under the control of the native *NAR* promoter.

The resulting vector pNAR was transformed into protoplasts of Guy-11 and transformants were selected on the complete medium with 20% sucrose and 200 μg/ml hygromycin B (Roche Diagnostics GmbH, Germany). The hygromycin-resistant transformants were further confirmed by passing them through one more round of selection and then individually confirmed by PCR and DNA gel blot analysis. The transformation procedure including protoplast generation was the same as that described before (Liu *et al*., 2007).

Analyses of *GFP* expression

The expression of *GFP* in cells during the development stage of *M. grisea* was examined and photographed using an Olympus-BX51 epifluorescence microscope with a cooled CCD camera DP50 (Olympus, Japan). And the expression of *GFP* in the hyphae grown in CM liquid medium (complete medium) and CM-N liquid medium (complete medium sans the nitrogen source) (Talbot *et al*., 1993) was also examined.

RESULTS AND DISCUSSION

Cloning of *NAR* promoter

After searching the phytopathogenic fungi and oomycete EST database in COGEME (http://cogeme.ex.ac.uk) (Soanes and Talbot, 2005), an expressed sequence tag (EST) (unigene ID: DNMag0422) was found, primarily expressed in the hyphae as revealed by EST transcript analysis (Table 1). The locus (putative protein MG10274.5) of this EST in the genomic DNA of *M. grisea* strain 70-15 was identified through a homology search of the *M. grisea* database (http://www.broad.mit.edu/annotation/fungi/magnaporthe) (Dean *et al*., 2005) by BLASTN (Altschul *et al*., 1997). The putative protein MG10274.5, a norsolorinic acid reductase (Nar) in *M. grisea*, is a homolog of the Nar in *Aspergillus flavus*, which is involved in the aflatoxin biosynthetic pathway (Hua *et al*., 1999).

Table 1 Analysis of transcript abundance of an EST (unigene ID: DNMag0422) from four cDNA libraries (data from http://cogeme.ex.ac.uk)

Library[1]	Mag02	Mag06	Mag07	Mag08
No. of ESTs[2]	0	178	4	1
%ESTs[3]	0	5.649	0.118	0.023

[1]Composition of the cDNA libraries was as follows (numbers in parentheses are the total number of ESTs sequenced from each library): Mag02, 70-15 appressorium (2553); Mag06, Guy-11 mycelium grown in complete medium (3193); Mag07, Guy-11 conidia (3405); Mag08, Guy-11 mycelium grown in nitrogen starvation medium (4310). [2]Number of ESTs representing this unigene sequenced from each cDNA library. [3]%ESTs representing this unigene shown as percentage of the total number of ESTs sequenced from each library

Primers for cloning the promoter fragment of *NAR* gene were designed based on the DNA sequence of *NAR* gene in *M. grisea* strain 70-15. A 1157-bp PCR product amplified from the genomic DNA of *M. grisea* strain Guy-11 was cloned to the T-vector and sequenced. The sequence data were analyzed using VecScreen program (NCBI) for vector masking. The promoter sequence of *NAR* gene was submitted to GenBank (GenBank_Accn: EF486491).

Analyses of the *NAR* promoter

Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html) indicated that the basic promoter sequence starts at position 807 and ends at position 857 of the *NAR* promoter DNA sequences (score=0.95, underlined in Fig.1) and that the transcription start lies at location 847 (Fig.1). TSSP (http://www.softberry.com/berry.phtml) indicated that the promoter position lies at 849 (Fig.1**)** and "TATA" box at position 813 (Fig.1) and found 19 binding sites of the transcription factor of *NAR* promoter sequences ("+" strand), all located between locations 592 and 802. TFSCAN for fungi (http://bioweb.pasteur.fr/seqanal/interfaces/tfscan.html) predicted that the *NAR* promoter fragment contains 7 known binding sites for regulatory proteins of *Saccharomyces cerevisiae* (Table 2) and that 20% of the sites are distributed between positions 59 and 283, 60% between positions 430 and 840, and 20% between positions 915 and 1145.

On searching the EST database in GenBank by BLASTN (Altschul *et al*., 1997) using putative *NAR* coding sequence (CDS), many ESTs were found, with sequences starting at position 874 of the *NAR* promoter DNA fragment (e.g. GenBank_Accn: BM862247). This implied that the transcription start lies at a location before 874 (Fig.1). Therefore, the promoter sequence of *NAR* promoter fragment probably lies between locations 430 and 857 and the transcription starts at location 847 or 849.

Table 2 Putative regulatory protein binding sites of *Saccharomyces cerevisiae* in *NAR* promoter DNA fragment predicted by TFSCAN program

Binding factors	DBF-A	MCBF	GCN4	GCN4	GAL4	GAL4	GAL4	GAL80	RAF	ADR1
Sequences	TTATC	ACGCGT	AAGTCA	CAGTCA	GGGG	GATAA	GAGGA	GAGGA	CCGA	TCTCC
Positions	551-555	701-706	1087-1092	59-64	837-840	728-732	279-283	279-283	1142-1145	926-930
(Start-end)			996-1001		836-839	456-460			915-918	689-693
			68-73		624-627				726-729	554-558
					464-467				576-579	430-434
					463-466				136-139	
					453-456					

1 GGGAAGCGATTGCGTTTGTCGGAAAGATATCTGATTGTTCCAAATACGACGAGACCCGCA
61 GTCACCCAAGTCATACTGGATTTGTCAGCTTGTGATACATTGCAAGCCGCCGCCATACAG
121 TACTGTAAGCCAGCGCCGATTTCAGCATCTGGTGTCCGTCCACTCCAGGCCATTTTCATT
181 TCGGTTCGTCGCTAAGACCCCTGTCGGCTTAGGTCCCTGTCTGCTGCAGCGGTCGAAAAG
241 CTGCGAAAGTTGTCATGTTGCTGCTGGAGTCTTTGGCAGAGGAGCTGCAGTCCTAGGCGT
301 TACAGGTGCCTACCTTACCTAGGGACAGTAACAAGACAAGAAAAAACAAGGCATAGTCAC
361 GGAACCCAATCCAAGTGAATCAAAGTCCTTCGGAATCTCGCATGCTCGGGTGGTAAAACG
421 GACTTTTTTTCTCCTGCACAACGCACGAGTATGGGGATAAGTGGGGGTTTAGCTGGGTAA
481 AGCCAGCAACAAGCTTGCCGCCGTTACATGTTGACGTGTTTGTCACCACTTTTTATGTAC
541 CTGCGTTCGTTTATCTCCGTCGCCCGTTCTTGAGTCCGAACAAACCTCGGCGACTTTTTG
601 CCTGCCGCAAAATCCCGTAACTCGGGGCCTTTTTTTTTGTGCTCGGTAAACGCCATTGCG
661 CCATTCAAATCATTCCGTTTCCGGCTAATCTCCGCATCACACGCGTGCAAACCCTGGCGC
721 CAGATCCGATAAATATTTGTAATACGACGTTGGTAAGCGCAGGTGGGTTAGAATATGGTA
781 GAAATCCACTATCAACAACCCCAGTTGTCGATTCGCTATAAGTAGTCCAGCATCTGGGGG
841 CTATTCTCTGTTTTAGTTTTGTTGCAAGTTCGCCTCAAATAAACCTTTTTAGCACAGGTG
901 CAAATATCCACGATCCGAAAGTTATTCTCCGGCTGGTTTGTTGCAACTGCGAATTGAATA
961 ACCTTTTCATAAGTCTGCTTATATCCGGCATCTGAAAGTCAATCTCAGATTGATTTGTTC
1021 CGCATCGAATAAACTGTGCAGAAATTCGCAACTCAAGCTTGCCTCAGATCCCTTCTCTTG
1081 CGACGAAAGTCACCCTCCCGGCAAACACTGCACACCAGAACCCTTACCAACCACACCACA
1141 ACCGACACCATGGCACC

Fig.1 The sequence of *NAR* promoter. The *NAR* gene initiation codon "ATG" is marked by a double underline. The first four basic groups "CTCA", appearing in the ESTs found in GenBank using BLASTN, are enclosed within a frame. The basic promoter sequence predicted by Neural Network Promoter Prediction is marked by a single underline (807~857). The "TATA" box or "CAAT" box is marked with a wavy underline. The transcription start "T" (847, Neural Network Promoter Prediction) or "T" (849, TSSP) is shaded light grey

Analyses of *GFP* expression

The pNAR, in which the *GFP* gene is under the control of the *NAR* promoter, was introduced into protoplasts of *M. grisea* strain Guy-11. Twelve hygromycin-resistant transformants were selected and confirmed by PCR and DNA gel blot analysis. The expression of *GFP* in cells, including hyphal cells (on CM plate), ungerminated spores, germinating spores, and appressoria, was examined under an epifluorescence microscope. The green fluorescence emitted by GFP protein was bright in the hyphae, weaker in ungerminated and germinating spores (2 h post-incubation, hpi), and the weakest in incipient appressoria (4 hpi) and fully formed appressoria (24 hpi) (Fig.2), thereby implying that the *NAR* promoter is activated mainly at the mycelial stage in the rice blast fungus. These results were similar to those of *NAR* transcript analysis (Table 1). No transcripts of *NAR* gene, which was faintly expressed in appressoria, were found in the appressorial cDNA library, perhaps because the transcript was lost during the building of the library. Furthermore, when inoculated in CM-N medium (CM medium sans the nitrogen source), the green fluorescence emitted by GFP protein in hyphae was brighter than those inoculated in complete medium. This cue implied that the *NAR* promoter was induced by starvation, and was consistent with the predicted results that this promoter was regulated by Gcn4 protein, which is a transcription factor in response to starvation (Tournu *et al*., 2005).

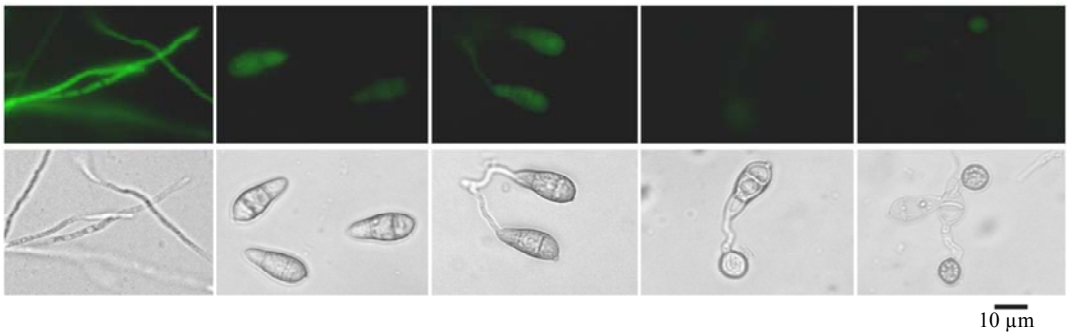

Fig.2 Differential expression pattern of *NAR* promoter. *GFP* under the control of the *NAR* promoter was expressed strongly in hyphae (a), weakly in ungerminated (b) and germinating conidia (c) (2 hpi), and faintly in incipient appressoria (d) (4 hpi) and fully formed appressoria (e) (24 hpi) of *M. grisea*. The mycelia, conidia, and appressoria were observed by fluorescence microscopy (top) and by light microscopy (bottom) in each panel

CONCLUSION

The *NAR* promoter containing the "TATA" box, the "CAAT" box, and fungal regulatory protein binding sites was isolated from *M. grisea*. Analyses of *GFP* expression under the *NAR* promoter showed that the promoter was primarily activated in hyphae. The successful expression of Mgatg1-GFP fusion protein and Mnh6-GFP fusion protein under *NAR* promoter in *M. grisea* (Liu *et al*., 2007) also showed that *NAR* promoter isolated in this study could be used to express native or extrinsic genes in the mycelia of the rice blast fungus.

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