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The Role of Fungi in Sustainable Agriculture and Energy

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***Abstract*— We have cloned and characterized spr1, a putative serine protease gene, from a nematode-trapping fungus, Monacrosporium megalosporum. The gene was present as a single copy in the genome. The predicted protein sequence of spr1 is homologous to the putative cuticle-degrading serine proteases PII and Azo1 from the nematode-trapping fungus, Arthrobotrys oligospora. In the 5' untranslated region near the initiation codon, consensus sequences to an AreA binding site, a well-known mediator of nitrogen metabolite repression in the fungus Aspergillus nidulans, a CreA binding site, a carbon response regulator in A. nidulans, and a PacC binding site, a transcription factor that responds to ambient pH signals in A. nidulans were found. However, spr1 was not regulated by carbon or nitrogen source, and exogenous protein did not induce expression of spr1. The transcription of the spr1 gene of this fungus was significantly affected by ambient pH. Based on RT-PCR, the product of the spr1 gene was not transcribed at pH 4, whereas under alkaline conditions such as pH 8 and 9, the spr1 gene was transcribed well. These results indicate that the spr1 gene is controlled only by a PacC homologue. Moreover, the expression profile of the spr1 gene corresponded with the pH-dependent physiology of this fungus.**

***Keywords*— Pine wilt disease; Monacrosporum megalosporum; Serine protease**

I. INTRODUCTION

A forest of pine trees such as Pinus thunbergii and P. densiflora is a traditionally important wood resource that is widely distributed in Japan. However, recently the destruction of forests caused by pine wilt disease has been worsening in Japan. One of the causes of wilt disease of pine trees is infection by the pine wilt nematode, Bursaphelenchus xylophilus; thus, it is necessary to exterminate the pine wilt nematode to prevent pine wilt disease and for pine forests conservation.

The nematode-trapping fungi include a large group of fungi that infect nematodes using a special mycelial structure or trap. Interest in studying the biology of infection by these fungi comes from their potential use as biological control agents against plant and animal parasitic nematodes. Utilization of nematode-trapping fungi for agriculture is a powerful tool for biological control of nematodes on the farm. In addition, fungi show host specificity, and their range is limited. Therefore, if a fungus that is specific to pine wilt nematode can be isolated, the fungus can be used as a good biological control agent because it should not affect other nematodes that are important ecologically, It is not clear, however, whether the sole cause of destruction of the for3ests is the pathogenicity of the nematode. It is also not clear whether acid rain affects destruction of pine forests. It is important to investigate the physiology of nematode-trapping fungi to understand the cause of destruction of pine forests and the ecological relationship between acid rain and nematode pathogenicity. Because, nematode-trapping fungi are one of the natural biological control agents of nematodes in the field, and acid rain ay affect the physiology of nematode-trapping fungi, these fungi might be a key to solving the problem of destruction of pine forests.

Fungi respond to their environments by tailoring their output of activities to the ambient pH. Ambient pH affects gene expression and physiology in filamentous fungi, and acid rain may affect the physiology of fungi in the field. Therefore, it is very important for an understanding of ecological effects to investigate the effect of ambient pH on the cellular physiology of filamentous fungi. The nematode-trapping fungi comprise a large group of fungi that can infect nematodes using a special mycelial structure (trap). The interest in studying the infection biology of these fungi comes from their potential use as biological control agents against plant and animal parasitic nematodes[1]. Utilization of nematode-trapping fungi for agriculture is a powerful tool for biological control of nematodes on the farm. In addition, there is host specificity in fungi, and the host range is limited [2]. In this report, we describe the isolation of a nematode-trapping fungus, Monacrosporium megalosporum, which is specific to pine wilt nematode, and its genetic background. Moreover, we describe the effect of ambient pH on cellular physiology, including the growth rate. The present study is part of our attempts to understand the causes of destruction

of pine forests and to develop a biological control system for the pine wilt nematode.

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II. Material and Methods

A. Strains and culture conditions.

Nematode-trapping fungi were isolated from soil and plant residues according to a method previously described [3]. Cultivation of nematodes was carried out as follows: *Botrytis cinerea*, which was used as feed for nematode cultivation, was grown on barley medium (2g barley, 7ml water) in 50ml Erlenmeyer flasks at 25C for 1 week. Then, nematodes were inoculated into the mycelial culture and the flask was incubated at 25C for 1 week. The nematodes were collected by centrifugation after filtration through tissue paper to remove the barley. MYA(2% malt extract, 0.2% yeast extract, 2% agar) medium was used for maintaining the fungus.

B. Nematode-trapping activity.

The host specificity of nematode-trapping activity was examined as follows: the *M. megalosporum* strain was incubated on MYA and incubated at 25C for 2 weeks. One hundred nematodes were inoculated on the medium, and trapped nematodes were observed and counted under a microscope after 1 week.

C. DNA extraction.

The *M. megalosporum* strain was grown in MY medium for later DNA extraction. DNA was extracted from lyophilized tissue of the *M. megalosporum* strains using CTAB (cetyltrimetyl ammonium bromide) procedure essentially as described previously [4]. In general, about 100ng of DNA was used as template in PCR.

D. ITS sequencing

The primers ITS4 and ITS5 were used o amplify the ITS region I, 5.8S rDNA, the ITS region II, and a portion of the 28S rDNA. The same primers were also used for direct sequencing. About primers of serine protease(*spr* 1), all primers were shown in Table 1. PCR was conducted in 100 μℓ reaction volumes. Each reaction tube contained approximately 100ng of DNA template, 10 μℓ 10 X PCR buffer (Sawady, Tokyo, Japan), 200 μM of each dNTP, 0.5pM each of ITS 4 and 5 primers,and 2.5U of Super Taq polymerase (Sawady). The thermal cycling parameters were initial denaturation at 94C for 3 min followed by 30 cycles of denaturation at 94C for 30s and extension at 72C for 45s; a final extension at 72C for 10min was done at the end of amplification. The amplified ITS I were purified with a Microcon 100 filter (Milipore, MA) and used as a DNA template for direct sequencing. DNA sequencing was carried out in an ABI PRISM 310 Genetic Analyzer (Applied Biosystem, Tokyo, Japan) using the chaintermination procedure with a BigDye Terminator Cycle Sequencing Kit(Applied Biosystem) with ITS2,ITS3,ITS4 and ITS5 primers. The sequence data of comlementary strands were compared visually and aligned using CLUSTALW (DDBJ edition) software.

TABLE I
PCR Primers Used in This Study

Primer	Sequence	Remark
SubF2	5′-GGNCAYGGNACNCAYTGYGC-3′	Used for initial amplification of tyrosinase gene (*spr1*)
SubR1	5′-NGGNSWNGCCATNWSNGTNCC-3′	
ActinF	5′-ACATGGAGAAGATCTGGCAC-3′	Used for initial amplification of actin gene (*act1*)
ActinR	5′-CTCGTCGTACTCCTGCTT-3′	
DmegProF	5′-ACTATAACGACAATTTCTCCGG-3′	Used for genomic DNA fragment using probe for Southern hybridization
DmegProR	5′-ATTGATAACCGAACTGAGGACA-3′	
DmegProRTF2	5′-AAGAAAGAGCATTGGCAATTCC-3′	Used for RT-PCR of *spr1*
DmegProRTR	5′-TATTTACGTTCTGGAGGAGCAA-3′	
DmActRTF2	5′-CCAACAGAGAGAAGATGACAC-3′	Used for PCR-RFLP of *act1*
DmActRTR2	5′-ATCAGCAATACCGGGGTACAT-3′	
T7primer	5′-TAATACGACTCACTATAGGG-3′	Used for 3′-RACE
Poly(T)-T7	5′-TAATACGACTCACTATAGGG(polyT)$_{17}$-3′	
HH2BF3	5′-GTNCAYCCNGAYACNGGNAT-3′	Used for initial amplification of histone H2B gene
HH2BR	5′-GCNARYTCNCCNGGNARDAT-3′	

III. Result and Discussion

A. Screening pine wilt nematode-trapping fungi

Two hundred isolates from soil and plant residues were tested for nematode-trapping activity. Among the 200 isolates, 30 isolates formed traps; however, none of these trapped the pine wilt nematodes, four other strains trapped the pine wilt nematode. Based on morphology and ITS sequences, the four strains were identified as *Arthrobotrys cladodes*, *A. oligospora*, *Dactylella lysipaga* and *Monacrosporum megalosporum*. The *M. Megalosporum* strain grew fastest among the four strains. Therefore, further studies used the *M. megalosporum* strain.

B. Effect of temperature on growth of M. megalosporum

The effect of temperature on the growth and nematodetrapping activity of *M. megalosporum* was examined. *M. megalosporum* was inoculated on MYA agar and incubated at from 4C to 37C. Optimum temperature for growth was between 25C and 30C.

C. Host specificity of nematode-trapping activity

In order to investigate host specificity, pine wilt nematode and a non-phytopathogenic nematode, which is morphologically similar to *B. xylophilus*, were inoculated on mycelium of *M. megalosporum* grown on MYA. After 1 week, *M.megalosporum* could capture pine wilt nematodes; however, non-phytopathogenic nematodes were not captured (Fig. 1). These results suggest that the host specificity of this fungus is high, and that this fungus might recognize biochemical differences between *B. Xylophilus* and *B. mucronatus*.

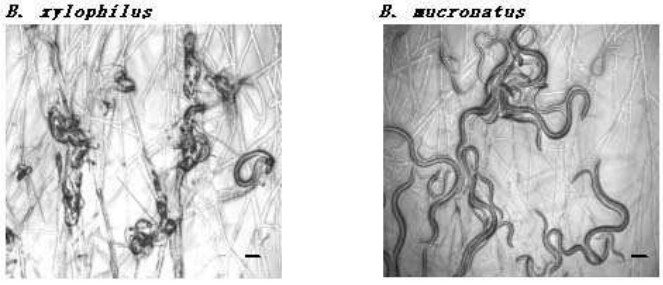

Fig.1. Specificity of nematode trapping activity Scal bar:20 μm

D. Effect of pH on growth.

Growth ability was investigated on MYA plates buffered with some buffers. The growth of this fungus was very fast between pH 7 and pH9. *M. megalosporum* could grow between pH5 and pH9 and very slowly below pH4.

E. Isolation and characterization of a serine protease gene(spr 1)

A plasmid clone, corresponding to the *spr 1* coding region that was on a 4.5-kb *Eco*RI fragment, was isolated from a genomic DNA library of *M. megalosporum.* The nucleotide sequence of *spr* 1 coding region is shown in Fig.2 The locations of exons and introns of *spr* 1 were deduced from interruptions in the portion of the amino acid sequence that was homologous to that of the subtilisin-like serine protease P II from *A. oligospora* [5], as well as from the sites of consensus sequences in conserved 5' and 3' splice sites within introns. In order to predict the regulation of *spr* 1 transcription, consensus promoter sequences in the 5' untranslated region near the initiation codon of *spr* 1 were analyzed. Three consensus sequences of interest, to which transcription factors should bind, were found. The first sequence was a consensus sequence for the *Are*A binding site(5'-GATA-3'), a mediater of nitrogen metabolite repression in the fungus, *Aspergillus nidulans* and other fungi[6]. The second sequence was a consensus sequence for *Cre*A(5'-SYGGRG-3'), a carbon response regulator in *Aspergillus nidulans* and other fungi[7]. The third sequence was a consensus sequence for *Pac*C (5'-GCCARG-3'), a transcription factor that responds to ambient pH signals in *Aspergillus nidulans* and other fungi [8].

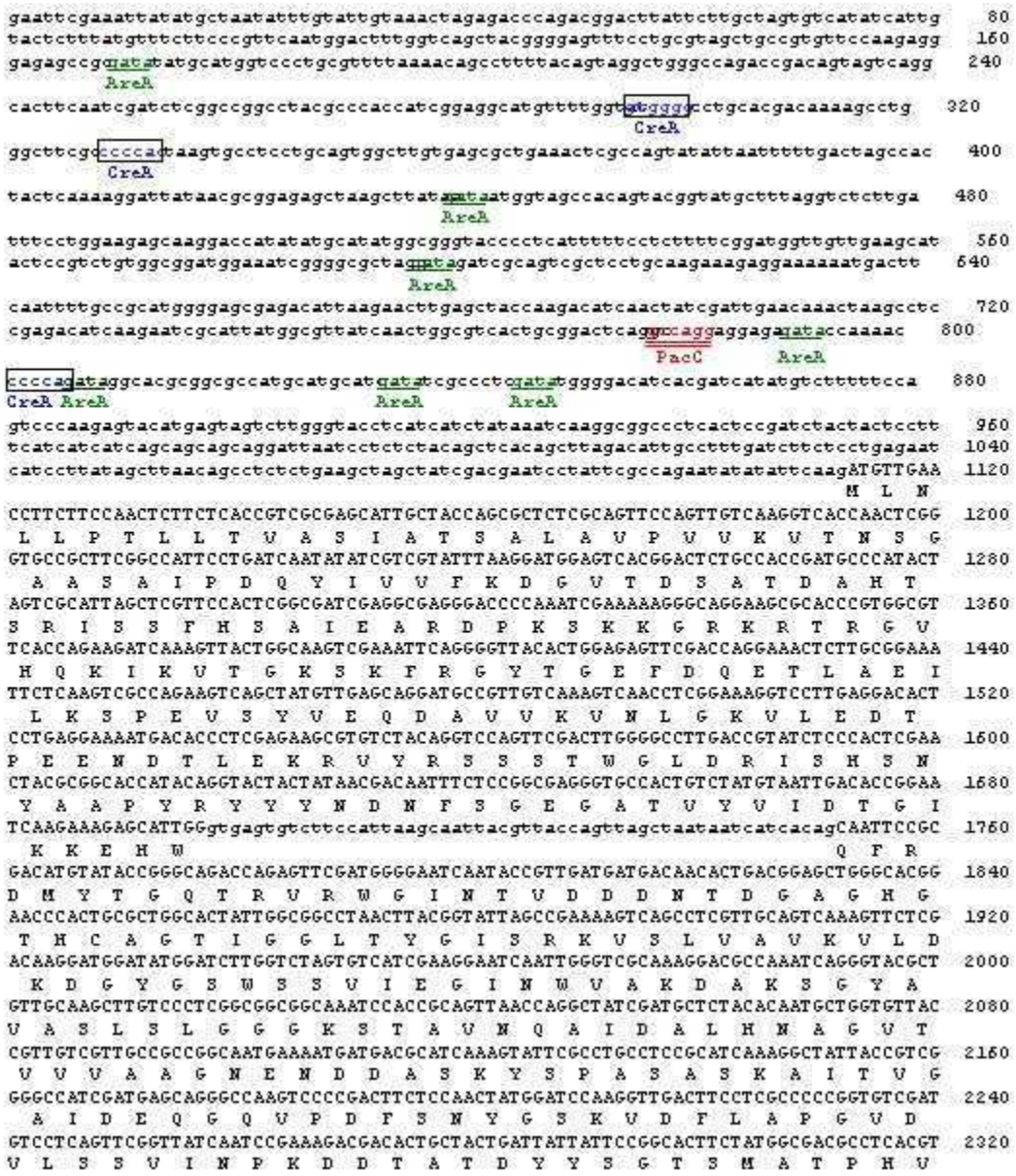

Fig.2. Nucleotide and deduced amino acid sequence of serine protease gene from *M. Megalosporum*

The length of the coding region from ATG to the stop codon was 1407 bp. *M. megalosporum spr* 1 encodes a polypeptide of 450 amino acid; contains one intron; and has 50% amino acid identity and 62% similarity with PII and 49% identity and 61% similarity with Aoz 1 of *A. oligospora*. Thus, Spr 1 protein may be related to the cuticle-degrading serine protease during infection of nematodes.

In order to develop a biological control agent against pine wilt nematode, we isolated nematode-trapping fungi from nature. We succeeded in isolating the *M. Megalosporum* SOM-5 strain, which grew rapidly and was viable at mild temperature. Thus, this fungus could be utilized as a biological control agent in the Japanese climate. The *M. megalosporum* SOM-5 strain that was isolated in this study was specific to pine wilt nematode. It did not capture a morphologically similar non-phytopathogenic nematode. Because there are many useful nematodes in natural ecosystems, a biological control agent should capture only phytopathogenic nematodes. Our results indicate that the *M. megalosorum* SOM-5 strain isolated in this study is a favorable candidate for the biological control of the pine wilt nematode.

IV. Conclusions

Cloned and characterized spr1, a putative serine protease gene, from a nematode-trapping fungus, *Monacrosporium megalosporum* had been done. The gene was present as a single copy in the genome. The predicted protein sequence of spr1 is homologous to the putative cuticle-degrading serine proteases PII and Azo1 from the nematode-trapping fungus, Arthrobotrys oligospora. In the 5' untranslated region near the initiation codon, consensus sequences to an AreA binding site, a well-known mediator of nitrogen metabolite repression in the fungus *Aspergillus nidulans*, a CreA binding site, a carbon response regulator in A.nidulans, and a PacC binding site, a transcription factor that responds to ambient pH signals in *A. nidulans* were found. However, spr1 was not regulated by carbon or nitrogen source, and exogenous protein did not induce expression of spr1. The transcription of the spr1 gene of this fungus was significantly affected by ambient pH. Based on RT-PCR, the product of the spr1 gene was not transcribed at pH 4, whereas under alkaline conditions such as pH 8 and 9, the spr1 gene was transcribed well. These results indicate that the spr1 gene is controlled only by a PacC homologue. Moreover, the expression profile of the spr1 gene corresponded with the pH-dependent physiology of this fungus.

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