Identification of a Putative Vacuolar Serine Protease Gene in the Rice Blast Fungus, *Magnaporthe grisea*

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We identified and cloned a gene designated *SPM1*, encoding a serine protease from the rice blast fungus *Magnaporthe grisea*. *SPM1* is a single-copy gene, encoding a subtilisin-like serine protease with 536 amino acids. Analyses of the deduced amino acid sequence of *SPM1* suggested that *SPM1* would be localized in a vacuole, an important organelle in pathogenicity.

Key words: *Magnaporthe grisea*; rice blast disease; gene cloning; vacuolar subtilisin-like serine protease; vacuole

A heterothallic ascomycete, *Magnaporthe grisea*, the causal agent of rice blast disease, is one of the best-characterized plant pathogenic fungi.¹ The disease cycle of *M. grisea* includes complex morphological developments, such as conidiogenesis and appressorium formation, which require *de novo* protein synthesis. These developments occur in nutritionally limited conditions, like those in disease lesions and on the rice leaves. Nitrogen starvation, which may reflect the environment in a plant tissue, has been suggested as one of the inductive cues for the expression of the genes needed for disease symptom development in *M. grisea*.²

Fungal vacuolar proteases are reported to be important for morphogenesis and adaptation to ambient nutritional conditions. For example, they are involved in spore morphogenesis and adaptation to nutritional stress in *Saccharomyces cerevisiae*.³ In *Aspergillus fumigatus* also, a homolog of *S. cerevisiae* vacuolar proteinase B,⁴ ALP2, is involved in conidiogenesis.⁵ These findings suggest the importance of vacuolar proteases in *M. grisea* as novel candidates for pathogenicity-related proteins, which will be a target for disease control.

From this point of view, we tried to identify vacuolar protease genes in *M. grisea*. At first, we identified a cDNA clone designated C50 (DDBJ/EMBL/GenBank accession No. AB066277) encoding a part of a serine protease through the sequencing analyses of a *M. grisea* specific cDNA library during the early stage of appressorium formation.⁶ The deduced amino acid sequence of one C50 ORF had high similarity to the C-terminal part of other fungal serine proteases, especially to that of *Podospora anserina* (DDBJ/EMBL/GenBank accession No. AJ251921).

In *M. grisea*, one thousand kinds of expressed sequence tag (EST) sequences of the appressorium formation stage had already been submitted to a DNA database (Choi, W., and Dean, R. A., unpublished results). We tried to identify ESTs encoding a part of the objective serine protease in their library by translated-BLAST search against the nucleotide database,⁷ using the *P. anserina* serine protease as a query sequence. Seven ESTs that had exhibited high similarity to the *P. anserina* serine protease were identified. These seven ESTs and C50 were aligned based on their position in the serine protease of *P. anserina* (Fig. 1). The alignment could cover all of the *P. anserina* serine protease.

A sense primer, 68959-S1 (5’-CACAGTCATTGCCCCACGATAT-3’) was constructed from an EST clone mgae0004dE11f (Accession No. AI068959) and an antisense primer, C50-AS2 (5’-TCTTGCATCGTGTATCCTCCCA-3’) was constructed from the C50 to amplify the full-length ORF of the gene. Reverse transcription-polymerase chain reaction (RT-PCR) was done with an One-step RT-PCR kit (QIAGEN) using these primers and 5 ng of total RNA from growing mycelia of *M. grisea* strain Hoku1. An approximately 1.9-kbp DNA fragment was successfully amplified, and cloned into the plasmid vector pUC19. Three independent clones, designated pMC50-1 to 3, were put through sequencing analysis to correct any sequence variation derived
from the misreading of RT-PCR. Among the three clones, sequences were different at eight nucleotide positions and these differences were corrected by a majority rule to make a consensus sequence (Fig. 2). There was a 1,611-bp ORF encoding a protein with 536 amino acids in an insert DNA fragment of 1,843 bp. The deduced amino acid sequence of the ORF had high similarity to the full length of other fungal serine proteases. The gene for this cDNA was designated SPM1 (Serine Protease of *Magnaporthe* 1).

Since the target of this study is vacuolar proteases, characteristics of the deduced amino acid sequence of *SPM1* were rapidly investigated by various computer analyses. At first, a motif search for amino acid patterns was done using a Scan PROSITE tool in the ExPASy (Expert Protein Analysis System) proteomics server. Among the serine protease families, *SPM1* had significant identity to the subtilisin family. The subtilisin family was a catalytic triad (aspartic acid, histidine, and serine) which provides catalytic activity by a charge relay system. *SPM1* had these three catalytic triad residues (Asp 192, His 224, Ser 390) and the sequences around these three residues were nearly identical to the consensus sequences of the subtilisin family except for Thr 195 (Fig. 2). These results suggested that the *SPM1* protein would belong to a subtilisin-like serine protease.

Analysis based on the signal sequence cleavage prediction program SignalP V1.1 indicated that a signal sequence existed in the N-terminal of *SPM1* and a possible cleavage site was between fifteenth and sixteenth alanine residues (Fig. 2). This result suggested that the *SPM1* protein would be translocated into the endoplasmic reticulum.

The result of similarity analysis of the deduced amino acid sequence of *SPM1* indicated that the *SPM1* protein had high similarity to other fungal serine proteases. *SPM1* protein shared a 65% sequence identity with the vacuolar serine protease of *Penicillium oxalicum* and a 50% sequence identity with the vacuolar proteinase B of *Saccharomyces cerevisiae*. There was a tendency to have a high similarity to putative cellular or vacuolar serine proteases (50 to 72%) rather than extracellular ones (maximum 40%). The number of amino acids encoded by *SPM1* gene was also more similar to putative cellular or vacuolar serine proteases (500 amino acids in rough average) than extracellular ones (400 amino acids in rough average). Furthermore, an extracellular serine protease MP1 of *Magnaporthe poae*, a species related to *M. grisea*, did not have high identity to *SPM1* (36%).

In addition, prediction of subcellular localization of *SPM1* by the PSORT program suggested that the most certain localization of *SPM1* was in the vacuole (certainty: 0.822) and the second most probable one was extracellular (certainty: 0.714). These results suggest that *SPM1* protein would be a vacuolar subtilisin-like serine protease, which could be expressed in the early stage of appressorium formation. Thus, we further proceeded for cloning of genomic DNA of the *SPM1* gene.

A genomic DNA library of *M. grisea* strain Hoku1 was constructed with Lambda FIX II (Stratagene). After two rounds of plaque hybridization with cDNA clone, we finally found a *HindIII-DraI* fragment about 3 kbp in size, which contains the *SPM1* gene. The fragment was then subcloned into *HindIII* and *HinCII* digested pUC19 and sequenced as described above.

Complete nucleotide and deduced amino acid sequences of *SPM1* are given in Fig. 2 (DDBJ/EMBL/GenBank accession No. AB070268). The length of the insert DNA fragment was 2,837 bp. The
Fig. 2. Complete Nucleotide and Deduced Amino Acid Sequences of SPM1.

An intron is indicated in lower case. Putative signal peptide is highlighted. Active sites of subtilase family are boxed and their consensus sequences are underlined. Primer sequences used for RT-PCR are double underlined. A motif search for amino acid patterns was done using the Scan PROSITE tool in the ExPASy (Expert Protein Analysis System) proteomics server from the Swiss Institute of Bioinformatics for the SWISS-PROT and TrEMBL database, which is available online at http://www.expasy.org/tools/scnpsit2.html.
SPM1 gene was 1,709 bp in length and consisted of two exons and a 98-bp intron (Fig. 2).

It is essential for a gene disruption experiment that a target gene exists as a single copy in the genome. To investigate the copy number of SPM1 in the M. grisea haploid genome, Southern hybridization analysis was done with an Alkphos direct labeling and detection system (Amersham Pharmacia Biotech) using the insert fragment of pMC50-1 as a probe. Southern hybridization of genomic DNA following digestion with four restriction enzymes (ApaI, EcoRI, HindIII, and XbaI) which don’t have cutting sites in the SPM1 sequence, resulted in the appearance of single band in each single digestion, suggested that the SPM1 gene exists as a single copy in the M. grisea haploid genome (data not shown).

Recently, the vacuole has been revealed as a central element of the lytic system in maturing appressoria of M. grisea and has an important role in pathogenicity.10 SPM1 might be an element of such a vacuolar lytic system, because it is derived from a clone which was originally found in a specific cDNA library of appressorium formation. Further analyses of SPM1 including expression, reporter gene, target gene disruption, and phenotypic analyses of such null mutants will elucidate the role of the vacuolar protease in development of pathogenicity of M. grisea.

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References


