The *Rhizopus oryzae* secreted aspartic proteinase gene family: an analysis of gene expression

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***Rhizopus oryzae* was shown to possess a secreted aspartic proteinase gene family (*sap*) of at least four members (*sap1*–*sap4*). Within the family there was 77–87% identity at the nucleotide level and 76–92% identity at the amino acid level. Transcription of three members of this gene family (*sap1*–*sap3*) required an acidic medium (pH < 4.5) and either nitrogen or sulphur derepression. Regulation was co-ordinate and hierarchical, with pH occupying the higher position in the hierarchy. Exogenous protein increased transcript levels, probably via the provision of metabolic intermediates rather than by direct induction of gene expression. *sap4* was not expressed under these conditions. SAP1–SAP4 are predicted to have almost identical substrate-binding sites and therefore substrate specificity. It is proposed that *sap1*–*sap3* exist to provide amplified expression of the secreted aspartic proteinase because protein, an important secondary nitrogen source for this fungus, requires extensive degradation to make its nitrogen available to the cell.**

Keywords: *Rhizopus oryzae*, filamentous fungi, sulphur metabolism, nitrogen metabolism, secreted aspartic proteinase

INTRODUCTION

Rhizopus oryzae, a filamentous fungus which is ubiquitous in soil (Webster, 1980), is also medically and economically important. It is the most frequent aetiological agent of zygomycosis (Frye & Reinhardt, 1993; Espinel-Ingroff *et al.*, 1987; Bouchara *et al.*, 1996), and it is used in a solid-substrate fermentation to produce tempe from soya beans (Nout & Rombouts, 1990) and in industrial fermentations for the production of glucoamylase (Radford *et al.*, 1996) and fumaric acid (Friedberg *et al.*, 1995; Longacre *et al.*, 1997). *R. oryzae* secretes aspartic proteinases which are critical to the production of good quality tempe (Wang & Hesseltine, 1965; Lim *et al.*, 1987) and, by analogy with other pathogenic fungi, might contribute to the virulence of the organism (Fallon *et al.*, 1997).

Proteolytic enzymes are secreted by a variety of fungi. Where they have been studied, these enzymes are often found to be the product of a single-copy gene; for example, the aspartic proteinases secreted by *Glomerella cingulata* (Clark *et al.*, 1997), *Aspergillus oryzae* (Berka *et al.*, 1993) and *Aspergillus awamori* (Berka *et al.*, 1990), and the serine proteinase secreted by *Aspergillus niger* (Jarai *et al.*, 1994). However, the proteinases secreted by *Rhizopus* spp., *Candida* spp. (best studied in *C. albicans*) (Monod *et al.*, 1994) and *Tritirachium album* (Samai *et al.*, 1989, 1990) are the products of multigene families.

The *C. albicans* secreted aspartic proteinase (SAP) gene family comprises at least seven members which show 32–91% identity in the amino acid sequence of the mature proteins. This family comprises three distinct groups: SAP1–SAP3 (70–74% identity within the group), SAP4–SAP6 (75–91% identity within the group) and SAP7 (possibly a pseudogene). The *T. album* secreted serine proteinase gene family comprises at least two members (those encoding proteinase T and proteinase R) which show 54% identity in the amino acid sequences of the proteins. The gene encoding proteinase K, which is secreted by a different strain of *T. album* (Gunkel & Gassen, 1989), belongs to this family.

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Abbreviation: SAP, secreted aspartic proteinase.

The GenBank accession numbers for the sequences reported in this paper are: AF026128 (*sap1*), AF026129 (*sap2*), AF026130 (*sap3*), AF026131 (*sap4*) and AF026127 (*rpys18*).

Rhizopus SAPs (the rhizopuspepsins) have been studied, not only in *R. oryzae*, but also in *Rhizopus chinensis* and *Rhizopus niveus*. Five isoenzymes are known to be secreted by *R. chinensis* (Ohtsuru *et al.*, 1982) and five genes encoding SAPs have been identified in *R. niveus* (Horiuchi *et al.*, 1988; Sakaguchi *et al.*, 1992), which show 72–90% identity in their predicted amino acid sequences. In addition, the cDNA for one of the *R. chinensis* enzymes has been sequenced (Delaney *et al.*, 1987). The amino acid sequence of two of the *R. chinensis* enzymes has been determined (Takahashi, 1988), showing that more than 97% of the residues are identical, and the three-dimensional structure of one of them has been solved (Suguna *et al.*, 1987).

Proteinases typically have an extended substrate-binding site (Barrett, 1994). Structural studies have allowed the amino acid residues which define the extended substrate-binding site to be defined for both the *R. chinensis* and *C. albicans* SAPs and for proteinase K. Proteinase K has broad substrate specificity (Betz *et al.*, 1988, 1993) and because its substrate-recognition site is defined by two strands of β -sheet which are able to move relative to each other, permitting the entry of any substrate into the active site, other members of the *Tritirachium* serine proteinase gene family can be expected to show similarly broad substrate specificity. Of the 27 subsite-defining residues in the *C. albicans* SAP, there are only 11 differences between Sap1–Sap6, of which four might alter the enzyme's conformation (Cutfield *et al.*, 1995). Furthermore, the experimental evidence suggests that there are no major differences in specificity amongst Sap1–Sap3 (Smolenski *et al.*, 1997) in spite of the presence of three residues which might cause differences in the conformation of the substrate-binding sites of these three isoenzymes. Of the 26 subsite-defining residues in the *R. chinensis* SAP (Suguna *et al.*, 1987, 1992), there is only one difference between the two isoenzymes (a Val/Ile substitution at position 15). Although no three-dimensional structures are available for the *R. niveus* proteinases, their substrate-binding sites may be identified by analogy with those of the *R. chinensis* enzyme. There are only four possibly significant differences out of a total of six differences between the residues defining the extended substrate-binding site in this SAP family. These observations suggest that there are unlikely to be major differences in specificity between the different isoenzymes of either the *R. chinensis* or the *R. niveus* SAP family. No sequence data are available for the *R. oryzae* enzymes.

In *R. oryzae*, *C. albicans* and *T. album*, these secreted proteinases probably function in providing a nitrogen supply for the organism when inorganic nitrogen in the environment is depleted (Farley & Ikasari, 1992; Ross *et al.*, 1990; Gunkel & Gassen, 1989). Expression of the individual members of the *Tritirachium* gene family has not been examined, but the individual members of the *Candida* SAP gene family are known to be differentially regulated. Sap1–Sap3 are secreted by the yeast form of *C. albicans*, whereas Sap4–Sap6 are expressed by the hyphal form of the organism (Hube *et al.*, 1994). The

regulation of expression of individual *sap* genes has not been examined in any member of the genus *Rhizopus*. However, it is known that in *R. oryzae*, SAP activity is expressed under conditions of either nitrogen or sulphur derepression (Farley & Ikasari, 1992).

The existence of these multigene families and the absence of significant differences in specificity amongst members of the family suggests that analysis of the expression of individual members of the family might contribute to understanding the significance of these multicopy genes. Since *R. oryzae*, unlike *C. albicans*, is not dimorphic and belongs to a different division of the fungal kingdom (the Zygomycota compared to the Ascomycota), as part of our interest in fungal secreted proteinase gene families we have examined how individual members of the *sap* gene family are regulated in *R. oryzae*.

METHODS

Strain, media and growth conditions. *R. oryzae* (CBS 515.94) used in this study was previously described as *Rhizopus oligosporus* USDB 0224 (Lim *et al.*, 1987; Farley & Ikasari, 1992) and was deposited in the Agricultural Research Service (ARS) culture collection as *R. oligosporus* (NRRL 22564).

Exponentially growing mycelium was harvested from submerged cultures incubated with shaking at 30 °C for 12–14 h after inoculation with 10^7 spores per 25 ml minimal medium, which contained 55 mM glucose, 50 mM NH_4Cl , 11.2 mM KH_2PO_4 , 7 mM KCl, 2.1 mM MgSO_4 , and traces of ferrous and zinc chlorides. The mycelium was filtered, washed with treatment medium, transferred to fresh treatment medium and returned to the shaker (Farley & Ikasari, 1992). Treatment media were obtained by modification of minimal medium as follows: nitrogen-free medium did not contain NH_4Cl ; sulphur-free medium contained MgCl_2 in place of MgSO_4 ; medium containing protein as the sole nitrogen source was nitrogen-free medium containing BSA (1%, w/v); and medium containing protein as the sole sulphur source was sulphur-free medium containing BSA (1%, w/v). The concentration of KH_2PO_4 was 33.6 mM except in experiments where the pH of the medium was to be maintained in the range pH 7–8, in which case 33.6 mM K_2HPO_4 was used instead of KH_2PO_4 . For some experiments, as indicated in the text, the concentration of selected components of these treatment media was varied. The stock solution of BSA was 5.6% (w/v), dialysed against at least five changes of distilled water and filter-sterilized (0.2 μm filter).

Primers and probes. Oligonucleotides were synthesized on an Applied Biosystems 380B synthesizer using β -cyanoethyl chemistry. Primers used for cDNA synthesis and in PCRs are indicated in Table 1. Oligonucleotide probes (also shown in Table 1) were radiolabelled using [γ - ^{32}P]dATP and T4 polynucleotide kinase (United States Biochemical).

RNA isolation. Total RNA from *R. oryzae* was prepared using a modification of the guanidine hydrochloride procedure of Logemann *et al.* (1987). Unless indicated otherwise, all operations were carried out at room temperature. Mycelium was harvested by vacuum filtration, pressed between layers of filter paper to remove excess moisture and frozen in liquid nitrogen. Frozen mycelium was lyophilized (this step was essential for successful isolation of intact RNA) and the dry mycelium ground to a fine powder with a mortar and pestle.

Table 1. Primer sequences and gene-specific oligonucleotide probes

Primer/probe name	Sequence (5'–3')
Primers	
RSAPX1[sense]	CTTGA(T/C)TTTGA(T/C)AC(T/C)GG(T/C)TC
RSAPX2[sense]	A(A/G)TA(T/C)ATCTTTGGTGG(T/C)TATGA
RSAPX2[antisense]	TT(A/G)AAGACAACATAGTTGTTCTT
Adaptor primer	GGGGTACCGTCGACATCGA
Poly-T primer	GGGGTACCGTCGACATCGA(T) ₁₇
RSAP3[sense]	GTACCACAATCGGTTCATCCAAG
RSAP3[antisense]	GGCAAAGTCATAATCACCATAACCA
RSAP4[sense]	CACGATCCCGGTCGATAAECTCCAAT
RSAP4[antisense]	GTCACCATAACCGAAGCCAGCGATA
Probes	
<i>sap1</i>	TGGTGGTCAAGTCACCACTAAACTTGG
<i>sap2</i>	CAAAGCTAGAGGTGTCACAGCTAATTGAA
<i>sap3</i>	AGGGATTGTGGTCAAGGAACCGCTGTAT
<i>sap4</i>	TAGCACCATAGGCAGAGGCAACAGAGCG
<i>rpys18</i>	GAAGGTAGTCACGACGAACAACAAGAGT
universal <i>sap</i> probe	CAAGAGAG(T/A)GGT(T/A)CC(A/G)GTGTCACGGATGGC

The dry powder was added to the extraction buffer (8 M guanidine hydrochloride, 20 mM MOPS, 20 mM EDTA, 50 mM β -mercaptoethanol at pH 7.0) at a ratio of 20 mg dry mycelium:1 ml extraction buffer, mixed and centrifuged for 10 min at 16000 g. The supernatant was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, saturated in 0.1 M Tris/HCl, pH 8) and the RNA was precipitated at -20°C overnight by the addition of 0.7 vol. ethanol and 0.2 vol. 1 M acetic acid. The RNA was pelleted (10 min at 9500 g) and washed twice with 3 M sodium acetate, pH 5.5, followed by 70% (v/v) ethanol. The RNA pellet was dissolved in a small volume of water by incubation at 65°C for 5 min and the undissolved material was removed by centrifugation at 16000 g for 5 min.

cDNA synthesis. cDNA used as template in PCR was prepared using the M-MLV reverse transcriptase. In experiments to amplify the *sap* transcript, total RNA prepared from mycelium incubated in nitrogen-free medium for 4 h at 30°C , was treated with proteinase K and DNase prior to cDNA synthesis using the degenerate RSAPX2[antisense] primer. cDNA for the amplification of the *rpys18* transcript was prepared from total RNA, isolated from mycelium incubated in minimal medium containing uric acid (2 mM) as sole source of nitrogen, using the poly-T primer.

DNA isolation. Medium containing 2% (w/v) glucose, 0.3% yeast extract and 1% (w/v) peptone was inoculated with *R. oryzae* spores (10^7 spores per 25 ml medium) and incubated with shaking at 30°C for 20 h. The mycelium was harvested, washed with distilled water, pressed dry and frozen in liquid nitrogen. The frozen mycelium was freeze-dried, ground to a fine powder and stored at -80°C until required. DNA was extracted from the ground mycelium by a modification of the method of Irelan *et al.* (1993). The dry powdered mycelium was suspended in 50 mM Tris/HCl, pH 8, containing 250 mM EDTA and 2.5% (w/v) N-lauroylsarcosine and incubated at 65°C for 5–7 min. The high concentration of EDTA was required to inhibit fungal nucleolytic activity (Haas *et al.*, 1990). Ammonium acetate was added, cell debris was removed and the DNA was precipitated with 2-propanol. The DNA was dissolved in 50 mM Tris/HCl, pH 8, containing 250 mM

EDTA and 1 mg proteinase K ml^{-1} (preincubated at 37°C for 15 min) at 50°C and incubated at this temperature for a further 10 min. Following chloroform/isoamyl alcohol extraction the DNA was incubated with RNase A at 50°C for 10 min. The chloroform/isoamyl alcohol extraction was repeated and the DNA was precipitated sequentially with ethanol (2.5 M ammonium acetate and 3 vols ethanol), followed by PEG (1 M NaCl and 8.2%, w/v, PEG) and finally with ethanol (0.1 M NaCl and 2 vols ethanol) to remove polyphosphates, as described by Rodriguez (1993). The final ethanol precipitation step was repeated once and the DNA was dissolved in 10 mM Tris/HCl, pH 8, containing 1 mM EDTA.

PCR and analysis of products. *sap* cDNA was amplified using *Taq* DNA polymerase in 20 mM Tris/HCl, pH 8.4, containing 50 mM KCl, 2.5 mM MgCl_2 , 200 μM each of dATP, dCTP, dGTP and dTTP and 6.65 μM each of the RSAPX1[sense] and RSAPX2[antisense] primers. Thirty-two cycles of 30 s at 94°C , 30 s at 56°C and 30 s at 72°C were performed, followed by 5 min at 72°C in a Corbett Research FTS-320 thermal sequencer. Products of the reaction were cloned in a T-vector prepared from *EcoRV*-cut pBluescript II KS(+). Clones were screened for those containing insert and randomly selected insert-containing clones were sequenced using an ABI 373A automated sequencer and standard ABI protocols.

sap genomic DNA ($11\text{ ng }\mu\text{l}^{-1}$ final concentration) was amplified using the Expand High Fidelity system (Boehringer Mannheim) and an FTS-320 thermal sequencer (Corbett Research). PCR products were cloned in the T-vector and colonies were transferred to Hybond-N+ membranes. These were hybridized with radiolabelled probe, prepared by random-primed labelling (Feinberg & Vogelstein, 1983) of the cloned *sap1* and *sap2* gene fragments (899 bp *SalI*–*XbaI* fragment), at 60°C overnight. Membranes were washed with $2\times\text{SSC}$, 0.5% SDS followed by $2\times\text{SSC}$, 0.1% SDS at room temperature for 5 min each and then $2\times\text{SSC}$, 0.5% SDS at 60°C for 2 h (medium stringency). This was followed by a higher stringency wash of $0.1\times\text{SSC}$, 0.5% SDS at 65°C for 2 h. Membranes were exposed at -70°C to X-ray film and selected clones were sequenced.

rpys18 cDNA was amplified using *Taq* DNA polymerase in 10 mM Tris/HCl, pH 8.3, containing 50 mM KCl, 1.5 mM MgCl₂, 200 µM each of dATP, dCTP, dGTP and dTTP, and 0.75 µM adaptor primer. Five cycles of 30 s at 94 °C, 30 s at 45 °C and 30 s at 72 °C were performed followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C and 30 s at 72 °C and finally 5 min at 72 °C in a FTS-320 thermal sequencer (Corbett Research). Products of the reaction were cloned in the T-vector and randomly selected insert-containing clones were sequenced.

For the analysis of *sap3* and *sap4* expression, cDNA was amplified using *Taq* DNA polymerase in 10 mM Tris/HCl, pH 9.0, containing 50 mM KCl, and 0.1% Triton X-100, 3.0 mM MgCl₂, 200 µM each of dATP, dCTP, dGTP and dTTP, and 1.0 µM each of either the RSAP3[sense] and RSAP3[antisense] or the RSAP4[sense] and RSAP4[antisense] primers in a MJ Research PTC-200 thermal cycler. For amplification of the *sap3* cDNA, 30 cycles of 5 s at 94 °C and 35 s at 69 °C were performed followed by 5 min at 72 °C. For amplification of the *sap4* cDNA, 30 cycles of 5 s at 94 °C and 30 s at 76 °C were performed followed by 5 min at 72 °C.

Northern blotting, hybridization and autoradiography. For Northern blot analysis, 30 µg total RNA was separated on formaldehyde-agarose gels (Sambrook *et al.*, 1989), transferred to Hybond-N+ membranes (Amersham) using 10 × SSC and fixed in 50 mM NaOH for 5 min. The membrane was washed in 2 × SSC and air-dried. A preliminary gel was run to check RNA loadings; when necessary, the amount of sample used was adjusted to obtain approximately equal amounts of RNA in each lane of the gel. A 0.24–9.5 kb RNA ladder (Gibco-BRL Life Technologies) was used for size determinations. Membranes were prehybridized in Church buffer (Church & Gilbert, 1984) at 40 °C for 1 h and then incubated with radiolabelled probe in fresh Church buffer for at least 16 h. The membranes were washed at room temperature with 2 × SSC, 0.5% SDS and 2 × SSC, 0.1% SDS and then at 42 °C with 0.1 × SSC, 0.5% SDS for 2 h. Membranes were exposed to X-ray film at –70 °C. Because the mRNAs of the three *sap* genes which were expressed under the conditions studied were more or less the same size, separate membranes were prepared for the analysis of each member of the gene family.

Southern blotting, hybridization and autoradiography. For Southern analysis, 5 µg *Eco*RI-digested DNA was separated on 0.8% agarose gels and transferred to Hybond-N+ membranes (Amersham) using 0.4 M NaOH. The membrane was washed in 2 × SSC and air-dried. *Hind*III-digested λ DNA (Gibco-BRL Life Technologies) was used for size determinations. Membranes were prehybridized in Church buffer (Church & Gilbert, 1984) at 60 °C for 1 h and then hybridized with radiolabelled probe, prepared by random-primed labelling (Feinberg & Vogelstein, 1983) of the cloned *sap1* and *sap2* gene fragments (899 bp *Sal*I–*Xba*I fragment), at 60 °C for at least 16 h. Membranes were washed with 2 × SSC, 0.5% SDS followed by 2 × SSC, 0.1% SDS at room temperature for 5 min each and then 1 × SSC, 0.5% SDS at 65 °C for 2 h (low stringency). This was followed by a higher stringency wash of 0.1 × SSC, 0.5% SDS at 65 °C for 2 h. Membranes were exposed to X-ray film at –70 °C.

Computer programs. Editing, analysis and alignment of sequences were carried out using the ABI SeqEd program (version 1.0.3), ESEE (version 1.09d) (Cabot, 1989) and FASTA and BLAST from the Genetics Computer Group (Wisconsin sequence analysis package, version 8). Autoradiograms were scanned with a Bio-Rad GS-670 Imaging Densitometer and the

digital images were processed with Molecular Analyst (version 2.1) and Adobe Photoshop (version 3.0.4) or NIH Image (version 1.60).

RESULTS AND DISCUSSION

Identification of the *R. oryzae sap* gene family

To obtain sequence data for individual members of the *R. oryzae sap* gene family, PCR was used to amplify part of the coding region of the gene. The sequence of the degenerate primers used was based on the sequences of the *R. niveus* gene family (Horiuchi *et al.*, 1988; GenBank accession nos: M19100, X56964, X56965, X56992 and X56993) and the single sequence available for *R. chinensis* (Delaney *et al.*, 1987; GenBank accession no. L33859). For two of the *R. niveus* sequences (X56964 and X56993) the location of the intron, as recorded in the GenBank annotation, is inconsistent with published data (Ashikari *et al.*, 1990) and its use results in a coding sequence which, when translated, bears no resemblance to the sequence of aspartic proteinases except for the N-terminal region. The position of the intron in these two sequences was therefore determined on the basis of published data on exon–intron boundaries in *R. niveus* (Ashikari *et al.*, 1990). In each case the amino acid sequence, deduced from the coding sequence so obtained, was consistent with that of an aspartic proteinase. Three degenerate primers (RSAPX1[sense], RSAPX2[sense] and RSAPX2-[antisense]) were designed based on the nucleotide sequences (Fig. 1).

In the first instance, because *R. oryzae* SAP is known to be secreted by mycelium incubated in nitrogen-free medium (Farley & Ikarashi, 1992), the PCR was performed using cDNA, prepared using RNA isolated from mycelium incubated for 4 h in nitrogen-free medium, and the RSAPX1[sense] and RSAPX2[antisense] primers, which were designed to amplify an 842 bp fragment of the *sap* gene (Fig. 1). The PCR products, which on agarose gel electrophoresis ran as a single band of the expected size, were cloned and three independent clones were sequenced. These provided sequence data for two members of the gene family. That we obtained sequence data from two different transcripts was not unexpected given that, in *R. chinensis*, the SAP activity

Fig. 1. ORFs of *Rhizopus sap* genes. The ORFs of the *R. niveus* *sap* genes are about 1200 nt and consist of two exons (about 330 and 845 nt) separated by a short intron (about 50–80 nt). A *sap* ORF is represented here by the rectangle, with the exons shown in white and the intron in black. The locations of primers used in the PCR are shown by arrows (arrow A, RSAPX1[sense]; arrow B, RSAPX2 [sense]; arrow C, RSAPX2 [antisense]) The location of the gene-specific probes are shown, by bars (1, *sap1*; 2, *sap2*; 3, *sap3*; 4, *sap4*).

comprises two major isoenzymes. However, the sequence data available for *R. niveus* suggested that there might possibly be three additional members to this gene family. Southern analysis was performed using *Eco*RI-digested genomic DNA and PCR-derived fragments of the two genes (842 bp) as probes. *Eco*RI was chosen because it did not cleave either of the cloned gene fragments. At high stringency, the individual probes hybridized as single bands (2.7 kb and 7.9 kb). At lower stringency, additional bands (3.5 kb and 4.7 kb) were detected, suggesting that another member(s) of the gene family remained to be cloned.

To avoid missing members of the gene family which might not be expressed under conditions of nitrogen derepression and to simplify the search, the PCR was repeated using genomic DNA as template and the RSAPX2[sense] and RSAPX2[antisense] primers to amplify a 433 bp fragment from the 3' end of the gene (Fig. 1). Again, the products of the reaction were cloned and 336 clones were screened for other members of the gene family using a mixture of the two cloned genes as a probe. Clones which hybridized strongly at medium stringency but not at high stringency (a total of 106 clones) were identified as possible additional members of the gene family. Randomly selected clones from this subset were sequenced; a third member of the family was identified and used as a probe to narrow the search further. A fourth gene was found in addition to those already identified. In addition, the sequence of the two genes cloned from the first PCR was confirmed for the region of overlap. The four genes are referred to as *sap1*, *sap2*, *sap3* and *sap4*. These four genes accounted for all the clones identified as possible members of the gene family in the first round of screening. However, during the process of screening these 106 clones it was observed that another 45 clones, which had not hybridized strongly to the combined probe during the first round of screening, hybridized at high stringency to the probes used in the third or fourth rounds of screening. Such variability leaves open the possibility that *R. oryzae* possesses an additional gene(s) which has not yet been identified.

All four sequences contained uninterrupted ORFs in the predicted orientation and the deduced amino acid sequences were 70–100% identical to those of the corresponding regions of the *R. niveus* *sap* gene products. Furthermore, all but two of the 169 invariant residues in the corresponding region of the *R. niveus* gene family were also invariant in the *R. oryzae* gene family. This is strong evidence that the sequences are those of the SAPs. The single intron present in the *R. niveus* genes lies 5' of the 433 bp region amplified from *R. oryzae* genomic DNA, so the absence of any intron within the sequence was not unexpected. Within the *R. oryzae* gene family itself there was 77–87% identity at the nucleotide level and 76–92% identity at the amino acid level between the four members of the gene family. This level of identity is comparable to that seen in *R. niveus* (71–83% at the nucleotide level and 69–91% at the amino acid level, over the same region) but not to

Fig. 2. Specificity of the oligonucleotide probes. Plasmids containing the cloned *sap* gene fragments (842 or 433 bp) were digested with *Sal*I and *Xba*I. The DNA fragments were separated by agarose gel electrophoresis and transferred to Hybond-N+ membranes. Membranes were hybridized with radiolabelled oligonucleotide probes as shown, washed at room temperature with $2 \times$ SSC, 0.5% SDS and $2 \times$ SSC, 0.1% SDS and then at 45 °C with $0.1 \times$ SSC, 0.5% SDS for 2 h. Lanes: 1(a, b), cloned *sap1* gene fragments; 2(a, b), cloned *sap2* gene fragments; 3, cloned *sap3* gene fragment; 4, cloned *sap4* gene fragment. The bottom panel shows the corresponding part of an ethidium bromide stained gel.

that seen in *C. albicans* (28–94% at the amino acid level, over the same region). The *C. albicans* family, however, appears to consist of three groups and within these groups the level of identity is comparable to that seen in both *Rhizopus* species.

Selection of probes and quantification of RNA

Random-primed labelling of the cloned gene fragments produced probes which did not discriminate between the members of the gene family. An oligonucleotide probe was therefore designed for each gene (Fig. 1) and shown, in preliminary experiments using Southern blots of the cloned gene fragments, to be specific for that member of the gene family (Fig. 2). In addition, a

degenerate oligonucleotide probe (hereafter referred to as the 'universal *sap* probe') was designed which hybridized to all four genes.

Two *R. oryzae* genes have been cloned: the glucoamylase gene (Ashikari *et al.*, 1986) and the fumarase gene (Friedberg *et al.*, 1995). In addition, sequence for part of the *R. oryzae* arginine-specific carbamoyl-phosphate synthetase gene (*cpsa*) was available (unpublished data; GenBank accession number AF025850). Nothing is known about expression of the glucoamylase gene. Transcription of the fumarase gene is increased under conditions of nitrogen limitation (Friedberg *et al.*, 1995) whereas transcription of the *cpsa* gene is decreased about fivefold in mycelium incubated in either medium containing arginine as the sole nitrogen source or minimal medium containing BSA, compared to mycelium incubated in nitrogen-free medium (not shown). Neither were suitable controls for Northern analysis of *sap* expression. Instead, mRNA levels were measured relative to the rRNAs by loading approximately equal amounts of total RNA in each lane of the gel (Chambers & Sullivan, 1993).

During the course of this work the 3' region of an *R. oryzae* ribosomal protein gene, homologous to the yeast S18 and mammalian S11 genes, was fortuitously amplified by the PCR and cloned. An oligonucleotide probe based on this sequence (referred to as the *rpys18* probe) was used as a positive control with all membranes analysed. Fungal ribosomal-protein mRNA levels are known to respond to carbon or nitrogen limitation (Vierula, 1997; Griffioen *et al.*, 1996) and a similar response was observed in *R. oryzae* (not shown). Hybridization of the *rpys18* probe was therefore used to demonstrate that intact mRNA was present in samples which showed little or no hybridization to *sap* gene probes.

Sizing of the rRNAs and *sap* transcripts

Most fungal small subunit rRNA molecules, including those of the 11 Zygomycetes which have been studied, are between 1701 and 1853 nt (van de Peer *et al.*, 1997). The lengths of the large subunit rRNA from nine species of fungi, including two Zygomycetes, have been determined and they range in size from 3327 to 3484 nt (de Rijk *et al.*, 1997). The sizes of the *R. oryzae* small subunit and large subunit rRNA molecules, estimated from denaturing gel electrophoresis, were 1800 nt and 3400 nt, respectively, both of which are within the range of sizes reported for other fungi.

sap4 transcripts were not detected on Northern blots under any of the conditions examined in this study. Membranes which failed to hybridize to the *sap4* probe did hybridize to the *rpys18* probe (0.76 kb transcript) and to a probe for the *R. oryzae cpsa* gene (1.7 kb transcript). The transcripts of the other three *sap* genes were all about 1.4 kb, which is consistent with the expected size of the coding sequence (1.2 kb) and falls within the range of transcript sizes seen for other fungal proteinase genes having a similar length of coding

sequence (Clark *et al.*, 1997; Hube *et al.*, 1994; Jarai & Buxton, 1994).

pH regulation of *sap* expression

The effect of the pH of the medium on *sap* expression was examined by Northern analysis of RNA extracted from mycelium incubated in medium designed to either allow the pH of the medium to fall from an initial value of about pH 5 to a final value in the range pH 3–4, or maintain the pH of the medium in the range pH 7–8 (Fig. 3). *sap* transcripts were present in mycelium incubated in nitrogen-free medium, sulphur-free medium and medium containing BSA as the sole nitrogen source, and medium containing BSA as the sole sulphur source provided the pH of the medium was allowed to fall below pH 4.5 (Fig. 3, lanes 3–6). However, when the pH of the medium was prevented from falling below pH 7.0 no *sap* transcripts were detected (Fig. 3, lanes 9–12). The ribosomal protein gene transcript was present regardless of the pH of the medium, demonstrating that intact mRNA was present in the samples obtained from the high-pH media. On an overexposed autoradiograph (not shown), *sap* transcripts were also detectable in the RNA extracted from mycelium incubated in minimal medium or in minimal medium containing BSA but only when the pH of the medium was allowed to fall below pH 4.5.

Taken together, the following two observations show that *sap* expression is regulated by the pH of the medium per se: (1) provided the pH of the medium is less than pH 4.5, *sap* transcripts are present even in minimal medium and (2) under conditions in which *sap* transcripts are abundant even in the absence of exogenous protein, high pH turns off *sap* expression. A similar result was obtained for the *A. niger pepA* and *pepB* genes (Jarai & Buxton, 1994), whereas the *C. albicans* SAP4–SAP6 genes were only expressed when the pH of the medium was pH 6 or higher (Hube *et al.*, 1994). Proteins involved in modulating gene expression in response to the pH of the environment have been described in *A. niger* (Tilburn *et al.*, 1995; MacCabe *et al.*, 1996) and *Saccharomyces cerevisiae* (Li & Mitchell, 1997). Similar proteins may be responsible for the pH-dependent regulation of *sap* expression in *R. oryzae*. Expression of the *C. albicans* SAP2 gene is also affected by the pH of the medium, but in this case it is suggested that the effect is indirect in that the pH of the medium affects the rate at which exogenous protein is hydrolysed to produce peptides which induce SAP2 expression (Hube *et al.*, 1994).

Identical results were obtained using either the universal *sap* probe or a combination of the four gene-specific *sap* probes. Furthermore, the ratio of the amount of *sap* transcript relative to rRNA for the mycelium incubated in nitrogen-free medium to the amount of *sap* transcript relative to rRNA for the mycelium incubated in medium containing protein as the sole source of nitrogen was 1:2 for both the universal probe and the combination of the four gene-specific probes, which suggests that the *sap*

Fig. 3. pH regulation of *sap* gene expression. Mycelium was grown in minimal medium, transferred to treatment medium and incubated with shaking at 28 °C for 5 h. Total RNA was extracted and equal amounts (30 µg) were subjected to denaturing gel electrophoresis. The lanes show RNA samples extracted from mycelium incubated in various media. Lanes: 1, minimal medium at low pH; 2, minimal medium containing BSA at low pH; 3, nitrogen-free medium at low pH; 4, medium containing BSA as the sole nitrogen source at low pH; 5, sulphur-free medium at low pH; 6, medium containing BSA as the sole sulphur source at low pH; 7, minimal medium at high pH; 8, minimal medium containing BSA at high pH; 9, nitrogen-free medium at high pH; 10, medium containing BSA as the sole nitrogen source at high pH; 11, sulphur-free medium at high pH; 12, medium containing BSA as the sole sulphur source at high pH. Northern blots were hybridized with radiolabelled oligonucleotide probes as indicated.

Fig. 4. Northern blot analysis of *sap1*, *sap2* and *sap3* expression in low-pH medium. Northern blots were prepared as described in the legend to Fig. 3 and probed with radiolabelled gene-specific oligonucleotides as shown. The lanes show RNA samples extracted from mycelium incubated in various media. Lanes: 1, minimal medium; 2, minimal medium containing BSA; 3, nitrogen-free medium; 4, medium containing BSA as the sole source of nitrogen; 5, sulphur-free medium; 6, medium containing BSA as the sole source of sulphur. The *rpys18* probe hybridized to all the RNA samples.

genes identified in this study account for all the *sap* transcript detectable under these conditions. A similar result was obtained with the mycelium incubated in sulphur-free medium and medium which contained protein as the sole source of sulphur.

Expression of the individual *sap* genes

Northern analysis using the individual gene-specific probes showed that at low pH expression of *sap1*, *sap2* and *sap3* was repressed by the presence of inorganic

nitrogen and sulphur in the medium, whereas all three genes were expressed in mycelium incubated under conditions of nitrogen or sulphur derepression. No *sap1* or *sap2* transcript was detected in mycelium incubated in minimal medium and the *sap3* transcript was only detectable on an overexposed autoradiograph (Fig. 4, lane 1). All three transcripts are easily detectable in mycelium incubated in sulphur-free medium (Fig. 4, lane 5) and all three are present in mycelium incubated in nitrogen-free medium, although there was only a low level of the *sap1* transcript (Fig. 4, lane 3). This result is

Fig. 5. RT-PCR analysis of *sap3* and *sap4* expression in low-pH medium. (a) Specificity of the primers. The PCR was performed using the *Sall*-*Xba*I fragments described in Fig. 2 as template and either the RSAP3[sense] and RSAP3[antisense] primers (left-hand panel) or the RSAP4[sense] and RSAP4[antisense] primers (right-hand panel). Lanes: 1, cloned *sap1* gene fragment; 2, cloned *sap2* gene fragment; 3, cloned *sap3* gene fragment; 4, cloned *sap4* gene fragment. (b) RNA (3 µg), prepared as described in the legend to Fig. 3, was treated with DNase and used for cDNA synthesis with the RSAPX2[antisense] primer. One twenty-fifth of the cDNA synthesis reaction was used as template for the PCR with either the RSAP3[sense] and RSAP3[antisense] primers (left-hand panel) or the RSAP4[sense] and RSAP4[antisense] primers (right-hand panel). A longer exposure of the right-hand panel is also shown below, but the faint band present in lane 6 (right-hand panel) is not visible in the photograph. The lanes show RNA samples extracted from mycelium incubated in various media. Lanes: 1, minimal medium; 2, minimal medium containing BSA; 3, nitrogen-free medium; 4, medium containing BSA as the sole source of nitrogen; 5, sulphur-free medium; 6, medium containing BSA as the sole source of sulphur.

consistent with previous measurements of SAP activity (Farley & Ikasari, 1992) and establishes that at low pH either nitrogen or sulphur derepression is sufficient to turn on expression of these three *sap* genes.

Farley & Ikasari (1992) observed that the level of SAP activity after 5 h incubation in treatment medium was almost the same for nitrogen-free and sulphur-free medium, even though secretion of SAP activity in sulphur-free medium begins about 2 h later than in nitrogen-free medium. The higher rate of accumulation of SAP activity in sulphur-free medium can be explained by the higher level of *sap* transcripts observed in mycelium incubated in sulphur-free medium compared to mycelium incubated in nitrogen-free medium (Fig. 4; fourfold higher for *sap3* and seven- to ninefold higher for *sap1* and *sap2*). In addition, that the *sap3* transcript levels are increased fourfold whereas the *sap1* and *sap2* transcripts are increased ninefold and sevenfold, respectively, indicates that there are differences in the promoter regions of these members of the *sap* gene family.

Transcripts for all three *sap* genes were also detected with exogenous protein as the sole nitrogen or sulphur source (Fig. 4, lanes 4 and 6). However, consistent with previous measurements of SAP activity (Farley & Ikasari, 1992) which showed negligible levels of proteinase activity in minimal medium containing BSA, exogenous BSA alone was not sufficient to turn on expression of these genes to any significant extent. The *sap2* transcript was not detected in mycelium incubated in minimal medium containing BSA (Fig. 4, lane 2) and the *sap1* and *sap3* transcripts were only detected on an

overexposed autoradiograph. Expression of the *sap1*, *sap2* and *sap3* genes is, therefore, controlled at the level of transcription in a hierarchical manner in which pH occupies the higher position and the second level in the hierarchy is nitrogen or sulphur derepression. At both levels of control, *sap1*, *sap2* and *sap3* are co-ordinately controlled, i.e., the signals which turn on or off expression of any one of these genes turn on or off all three of them. From these experiments, however, it is not possible to determine the relative proportion of each *sap* gene transcript in the total pool of *sap* gene transcripts. A similar hierarchy of transcriptional control of expression of the *A. niger pepA* and *pepB* genes has been described by Jarai & Buxton (1994).

sap4 transcripts were not detected on Northern blots but analysis, following Li *et al.* (1981), of the number of nucleotide substitutions per site at each position of the codons in the partial sequence of *sap4* compared to that for the other three *sap* genes did not provide any evidence that *sap4* was a pseudogene. RT-PCR was used to determine whether or not any *sap4* transcript was present in mycelium incubated at low pH at levels below the detection limit of Northern analysis (Fig. 5). *sap3* transcripts were also analysed to provide a positive control. Under the conditions used for the PCR, the primers for amplification of the *sap4* cDNA were completely specific for *sap4* (Fig. 5a). The primers for amplification of the *sap3* cDNA also amplified *sap4*; however, the amplification of *sap4* was less efficient and Northern analysis had shown that under derepression conditions the level of any *sap4* transcripts would be insignificant in comparison to the level of *sap3* transcripts. The relative yields of the *sap3* and *sap4* PCR

products were consistent with the results of the Northern analysis, which had shown that *sap4* transcripts were less abundant than *sap3* transcripts. The yields of PCR products for *sap3* (Fig. 5b) were also consistent with the pattern of *sap3* expression determined by Northern analysis, in that the *sap3* transcript had been shown to be present in all the samples analysed and levels of the *sap3* transcript were higher under derepressed than repressed conditions. *sap4* transcripts were present in mycelium under all the conditions examined, as evidenced by the presence of product of the expected size in the PCR. However, there was less PCR product and therefore less *sap4* transcript present in the samples from mycelium incubated under conditions of nitrogen or sulphur derepression (fourfold and eightfold less product, respectively) than in mycelium incubated in minimal medium (Fig. 5b, right-hand panel; compare lanes 1, 3 and 5). The pattern of expression of *sap4* is in contrast to that of *sap1*, *sap2* and *sap3*, which suggests that there are major differences in the promoter region of the *sap4* gene compared to those of the other three members of the *sap* gene family.

Role of exogenous protein in *sap* expression

Under nitrogen or sulphur derepression conditions, the addition of exogenous BSA to the medium increases the amount of *sap* transcript relative to rRNA levels in the mycelium (Fig. 4; compare lanes 4 and 6 with lanes 3 and 5). Similar results were obtained for *A. niger* (Jarai & Buxton, 1994). The ratio of the amount of *sap* transcript relative to the rRNA for mycelium incubated in nitrogen-free medium, to the amount of *sap* transcript relative to the rRNA for the mycelium incubated in medium containing protein as the sole nitrogen source, is 1:13 for *sap1* and 1:5 for both *sap2* and *sap3*. Similarly, for sulphur-free medium and medium containing protein as the sole sulphur source, the ratio is 1:1 for *sap2* and 1:2 for both *sap1* and *sap3*. It might be expected that the amount of SAP activity in the medium would increase as a consequence of the higher levels of *sap* transcripts, but this is not the case. The amount of SAP activity relative to the mass of mycelium present in the medium is either the same (nitrogen derepression conditions) or reduced (sulphur derepression conditions) (Farley & Iksari, 1992). It is possible, therefore, that in addition to transcriptional activation of *sap* gene expression under derepression conditions, translation of the *sap* transcript and/or secretion of SAP protein is regulated, or autodegradation of the secreted enzyme is occurring.

The addition of exogenous protein to these derepression media may, however, be having a more general metabolic effect rather than a specific effect on *sap* gene expression. In particular, addition of exogenous protein to the medium may have metabolic consequences other than direct induction of *sap* gene expression. The effect of exogenous BSA on *sap* expression under sulphur derepression conditions can be mimicked (and even exceeded) by the addition of glucose to the medium, equivalent, on a molar basis, to the amino acids available

Fig. 6. Northern analysis of *sap* expression on various sulphur derepression media. A Northern blot was prepared as described in the legend to Fig. 3 and probed with radiolabelled probes as shown. The lanes show RNA samples extracted from mycelium incubated in various media. Lanes: 1, sulphur-free medium containing 55 mM glucose; 2, sulphur-free medium containing 171 mM glucose; 3, medium containing 55 mM glucose and 1% (w/v) BSA as the sole source of sulphur. The *rpys18* probe hybridized to all three RNA samples.

Fig. 7. Northern analysis of *sap* expression on various nitrogen derepression media. A Northern blot was prepared as described in the legend to Fig. 3 and probed with radiolabelled probes as shown. The lanes show RNA samples extracted from mycelium incubated in various media. Lanes: 1, nitrogen-free medium containing 55 mM glucose; 2, medium containing 55 mM glucose, and 0.1 mM glycine, 0.2 mM aspartate and 0.3 mM glutamine as the sole sources of nitrogen; 3, nitrogen-free medium containing 171 mM glucose; 4, medium containing 171 mM glucose, and 0.1 mM glycine, 0.2 mM aspartate and 0.3 mM glutamine as the sole sources of nitrogen; 5, medium containing 55 mM glucose and 1% (w/v) BSA as the sole source of nitrogen. The *rpys18* probe hybridized to all five RNA samples.

from hydrolysis of the BSA (Fig. 6). Under nitrogen derepression conditions, glucose is without effect, but the effect of exogenous BSA on *sap* expression under these conditions can be mimicked by the addition of aspartate, glycine and glutamine in amounts equivalent to, or less than, those provided by hydrolysis of the BSA (Fig. 7). These results suggest that exogenous protein

might affect the level of *sap* transcripts indirectly by the provision of metabolic intermediates, which might be substrates for energy production or precursors in the synthesis of nucleotides, rather than directly by induction of *sap* gene expression.

Functional significance of the *sap* gene family

A number of reasons for the existence of gene families within a particular organism have been suggested. These include the provision of duplicates of essential genes to provide a safety net for the cell, the provision of an array of enzymes having slightly different specificities, the provision of enzymes which can be independently regulated under different environmental or physiological conditions and the provision of multiple copies of a gene to achieve a higher level of expression of the gene product than would be possible from a single copy of the gene.

SAPs would not appear to be essential for growth of *R. oryzae*, since the fungus is able to utilize a range of alternative nitrogen sources such as ammonium salts, urea, uric acid and xanthine. Since there is 69–76% identity between the amino acid sequences of the proteinases from *R. oryzae* and *R. chinensis*, the substrate-binding sites of the *R. oryzae* enzymes may be identified by analogy with those of the *R. chinensis* enzymes. The sequence data obtained in this study provides information on all but two residues (positions 15 and 16; *R. chinensis* numbering) defining the substrate-binding site in SAP1 and SAP2 and for 11 of the 26 subsite-defining residues in SAP3 and SAP4. There are four differences amongst the *R. oryzae* isoenzymes (positions 222, 225, 278 and 294), of which two may be significant (the Phe/Tyr substitution at position 278 and the Asp/Tyr substitution at position 294). However, since similar differences between the *C. albicans* isoenzymes have no effect on substrate specificity (Cutfield *et al.*, 1995; Smolenski *et al.*, 1997), the differences observed for the *R. oryzae* isoenzymes are unlikely to result in significant differences in specificity between these isoenzymes. This is a common feature of all known fungal proteinase gene families.

sap1, *sap2* and *sap3* are co-ordinately expressed at low pH under conditions of either nitrogen or sulphur derepression. *sap4* is independently regulated, but represents only a minor fraction of the total pool of *sap* transcript under all the conditions examined in this study. The presence of three genes (*sap1*, *sap2* and *sap3*) encoding proteinases with almost identical substrate-binding sites and, by implication, specificity, which are all expressed under identical conditions, suggests that these genes exist to provide amplified *sap* expression under conditions of low pH and nitrogen or sulphur derepression.

Long-term survival of the fungus in the absence of its preferred nitrogen source (ammonium salts), which may be the most abundant nitrogen source available only in laboratory media, is dependent on the fungus finding a

secondary nitrogen supply. In this situation, *R. oryzae* responds by the production of catabolic enzymes involved in two different nitrogen-assimilation pathways [protein assimilation (Farley & Ikasari, 1992) and purine assimilation (P. C. Farley & S. Santosa, unpublished)]. Attempts to induce nitrite reductase activity under these conditions have been unsuccessful (A. U. Dewi, A. S. Krave & P. C. Farley, unpublished), suggesting that *R. oryzae*, like *R. oligosporus* (Sorenson & Hesseltine, 1966), is unable to utilize nitrate as a secondary nitrogen source. That overall expression of the *sap* genes (protein assimilation pathway) is amplified by turning on multiple copies of the gene suggests that protein is a particularly important secondary nitrogen source for this fungus and reflects how much protein needs to be degraded to obtain sufficient nitrogen for growth (about 6×10^{20} peptide bonds have to be hydrolysed to provide 1 mmol nitrogen from protein). This may be the case, not only in *R. oryzae*, but also in the other fungi which amplify expression of a secreted proteinase by turning on multiple members of a gene family.

In the case of *R. oryzae*, which has been used for centuries in the fermentation of soya beans to produce tempe, protein would be the main nitrogen source available to the fungus during this fermentation. The turning on and amplification of *sap* gene expression, together with the existence and expression of enzymes involved in purine but not nitrate assimilation, fits perfectly with its use in the tempe fermentation, whether this is a consequence of centuries of selection for these traits or part of the reason for it being suitable for this fermentation process in the first place.

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