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## Identification of three ubiquitin genes of the rice blast fungus *Magnaporthe grisea*, one of which is highly expressed during initial stages of plant colonisation

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**Abstract** Differential cDNA screening was used to identify genes expressed during the colonisation of rice leaves by the pathogenic fungus *Magnaporthe grisea*. This led to the identification of a gene, called *UEP1*, which encodes a ubiquitin extension protein. *UEP1* was highly expressed 48 h after initial fungal infection of rice leaves when *M. grisea* is proliferating in the leaf epidermis but not yet causing disease symptoms. *UEP1* appeared to be down-regulated after this time despite further extensive growth of the fungus throughout the leaf tissue. To investigate the potential role of ubiquitin in fungal pathogenesis we subsequently isolated *UEP3* and *PUB4*, encoding a second ubiquitin extension protein and a polyubiquitin respectively. *UEP1* was expressed abundantly during active growth of *M. grisea* in axenic culture but was down-regulated by starvation-stress. *UEP3* showed a similar pattern of expression to *UEP1* during the growth of *M. grisea* in culture and after environmental stress, but was not highly expressed during plant colonisation. *PUB4* was highly expressed after environmental stress, but was not highly expressed during plant colonisation. *UEP1* was found to be present in a much-higher copy number per haploid genome compared to *UEP3* and *PUB4*. The restricted high-level expression of *UEP1* suggests that *M. grisea* undergoes rapid ribosomal biogenesis and protein turnover during initial plant-tissue colonisation, which is regulated by a specific *UEP1*-encoded component of the *M. grisea* ubiquitin gene family.

**Key words** *Pyricularia oryzae* · Ribosomal biosynthesis · Gene amplification · Plant infection · Fungal pathogenesis

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### Introduction

*Magnaporthe grisea* (Hebert) Barr is an ascomycete plant pathogen capable of infecting more than 50 grass species (Ou 1985; Rossman et al. 1990). These include a large number of forage grasses and important food crops such as wheat, barley and rice. *M. grisea* is best known as the causal agent of rice blast, the most serious disease of cultivated rice. Rice blast routinely causes between 11 and 30% losses to the world rice harvest and is responsible for sporadic recurrent epidemics throughout South East Asia and South America (Baker et al. 1997). The fungus has therefore been intensively studied and has become a tractable organism for classical and molecular genetic analysis. These efforts have led to rapid progress in studying the fundamental basis of fungal infection by *M. grisea* (for reviews see Talbot 1995; Howard and Valent 1996).

Infection of rice by *M. grisea* begins when a conidium carried by a dew drop, lands on a leaf, and attaches itself to the leaf surface with an adhesive released from the conidial apex (Hamer et al. 1988). Once attached, the spore germinates by producing a germ tube which grows parallel to the leaf surface, before forming a terminal differentiated cell, the appressorium, in response to the leaf-surface environment (Hamer et al. 1988; Lee and Dean 1994). The appressorium is a heavily melanised cell which allows the fungus to penetrate the rice leaf epidermis directly (Howard and Ferrari 1989). To do this the appressorium generates enormous turgor pressure (Howard et al. 1991) due to the accumulation of high concentrations of glycerol within the cell (de Jong et al. 1997). This draws water into the appressorium developing a hydrostatic turgor which is focused and translated into a mechanical force, allowing a narrow penetration peg to rupture the leaf cuticle. Once in the plant, infection hyphae ramify through the underlying epidermal and mesophyll cells and about 72 h later necrotic lesions become visible on the surface of rice leaves. Between 5 and 7 days following rice infection, aerial conidiophores differentiate from within the coalescing le-

sions releasing thousands of new conidia to perpetuate the cycle (Ou 1985).

Progress in understanding the genetic basis of pathogenicity in *M. grisea* has been made predominantly by investigating the early stages of plant infection and, in particular, appressorium formation (Howard and Valent 1996). A number of genes, for example, are now known to be required for appressorium elaboration and function, including *PMK1*, encoding a MAP kinase required for appressorium development (Xu and Hamer 1996), and *CPKA*, encoding a cAMP-dependent protein kinase required for appressorium function (Mitchell and Dean 1995; Xu et al. 1997). Other genes, meanwhile, are required for efficient appressorium elaboration, such as *MPG1*, encoding a cell-wall hydrophobin involved in leaf-surface perception (Talbot et al. 1993, 1996), and *NPR1* and *NPR2*, which regulate this function (Lau and Hamer 1996).

Studies aimed at understanding the later stages of pathogenesis have, however, been largely at the cytological level (Heath et al. 1990) and, as a result, very little is known about the physiology and underlying molecular biology of plant tissue-colonisation. This means that a number of important questions regarding the ability of *M. grisea* to proliferate within living plant tissue, and bring about disease, remain unanswered. To address these questions we have employed a differential cDNA screening approach to identify *M. grisea* genes expressed during growth *in planta*. In this study we report the identification of a novel ubiquitin-encoding gene, *UEP1*, which is highly expressed during the initial stages of plant-tissue colonisation by *M. grisea*. In a wider investigation, to determine the role of ubiquitin in plant colonisation, we also report the isolation and characterisation of two other ubiquitin-encoding genes designated *UEP3* and *PUB4*. *UEP3* shows a similar pattern of expression to *UEP1* during growth of *M. grisea* in axenic culture, while *PUB4* has a distinct expression pattern; but neither gene is highly expressed during pathogenesis.

## Materials and methods

**Fungal material and infection assays.** All fungal isolates used in this study are stored in the laboratory of N. J. T. (University of Exeter), and the fertile rice pathogenic strain Guy-11 was employed (Valent et al. 1991). The fungus was grown in complete medium (CM) as described by Talbot et al. (1993a). For experiments where nutritional conditions were altered, the fungus was grown in minimal medium with either no added nitrate (-N) or glucose (-C). Rice infections were performed using cultivar CO-39, a dwarf rice cultivar which is very susceptible to blast (Valent et al. 1991). Plants were maintained in a controlled environment chamber (Convion, Whatman lab. Sales Ltd., Kent) with 14 h light (27°C, 85% relative humidity) and 10 h dark (21°C, 85% relative humidity) according to Valent et al. (1991). Plants were infected using an artist's airbrush (Badger airbrush, Franklin Park, Illinois, USA). A conidial suspension ( $10^5$  conidia  $\text{ml}^{-1}$ ) was produced by flooding agar plates with 0.2% (v/v) gelatin solution and the suspension was sprayed evenly onto rice plants. The plants were placed in plastic bags for 24 h, well-watered, and incubated until disease symptoms were apparent 96–144 h later.

**Differential cDNA screening.** A  $\lambda$ -Gem 4 (Promega) directional cDNA library constructed from blast-infected rice-leaf mRNA (Tal-

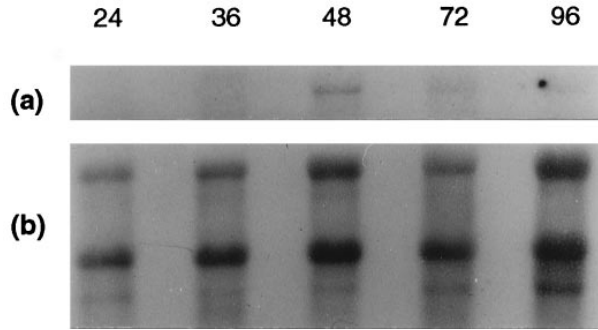
bot et al. 1993a) was screened at high density with radiolabelled cDNA probes derived from infected rice-leaf RNA (+) and *M. grisea* mycelium RNA (-). Plaques showing stronger hybridisation to the infected rice-leaf cDNA probe were picked and single-plaque purified. Hybridisation conditions and high-stringency washes were as previously described (Talbot et al. 1993b). Plaques selected from the differential cDNA screen were Southern blotted to subtract any rice cDNAs inadvertently selected. *M. grisea* cDNAs were screened by Northern blots and a sub-set chosen for further analysis. The pHM8 cDNA was selected from this collection for characterisation. Isolation of other members of the ubiquitin gene family was carried out by screening a *M. grisea* conidiospore cDNA library (kindly obtained from JinRong Xu and John Hamer, Purdue University) with pHM8 at low stringency (42°C). One-hundred plaques were selected showing various levels of hybridisation to pHM8 and classified by high-stringency hybridisations and genomic Southern blots into three groups. DNA sequence analysis of a subset of cDNAs identified pHM300 representing *UEP3* and pHM400 representing *PUB4*, in addition to cDNAs representing *UEP1*.

**Nucleic acid analysis.** RNA was extracted from *M. grisea* according to the method of Timberlake (1980). Total RNA was extracted from rice tissue using a guanidine thiocyanate method (Sambrook et al. 1989). Poly (A)<sup>+</sup> enrichment was carried out by oligo-dT cellulose chromatography (Pharmacia) according to the manufacturer's instructions. For RNA gel-blots, 5  $\mu\text{g}$  of total RNA was routinely fractionated in 1.2% MOPS-formaldehyde gels and transferred to Hybond-N (Amersham). Gel-electrophoresis, digestion with restriction enzymes and Southern-hybridisations were all carried out using standard procedures (Sambrook et al. 1989). DNA hybridisation probes were labelled by the random primer method (Feinberg and Vogelstein 1983) using a Prime-It kit (Stratagene) according to the manufacturer's instructions. Genomic reconstruction experiments were performed using genomic sub-clones of *UEP1*, *UEP3* and *PUB4*. The genome size of *M. grisea* was assumed to be 38 Mb (Skinner et al. 1993; Talbot et al. 1993b; Orbach et al. 1996). The pHM8 (*UEP1*) cDNA hybridised to a 4.4-kb *EcoRI* fragment of genomic DNA. Therefore, assuming a genome size of 38 Mb, this represents  $1.16 \times 10^{-4}$  of the haploid genome. Thus 1  $\mu\text{g}$  of genomic DNA will contain 0.116 ng of this *EcoRI* fragment if *UEP1* is present as one copy per haploid genome. A 4.4-kb genomic sub-clone of *UEP1*, designated pHMG-1, was selected and a Southern blot prepared with *EcoRI*-digested pHMG-1 in concentrations equivalent to 1, 2, 3, 4, 5, 10, and 20 copies of *UEP1*. This was hybridised with a 4.4-kb *EcoRI* fragment of pHMG-1. The resulting hybridisation signal was compared to that obtained with 1  $\mu\text{g}$  of genomic DNA included on the same gel. The same procedure was repeated for *UEP3* and *PUB4*. For *UEP3* a 6.5-kb *SaII* genomic DNA fragment was sub-cloned to create pHMG-U3, while for *PUB4* a 9.0-kb *EcoRI* genomic DNA fragment was sub-cloned to create pHMG-U4. DNA sequence analysis was carried out using the dideoxy chain-termination method of Sanger et al. (1977) with a Sequenase 2.0 kit (Amersham-USB). Manual and automated DNA sequencing was carried out from both single- and double-stranded DNA templates. Automated sequencing was carried out using dye-labelled terminator cycle sequencing with the Thermo-sequenase kit (Amersham-USB). Samples were processed using a Perkin Elmer ABI 377 (Royal Devon and Exeter NHS Trust, Exeter, Devon, UK). Isolated cDNAs were routinely sub-cloned from pGEM-1 to pBluescript (Stratagene) and nested deletions made using Exonuclease III (Promega). Genomic clones of the ubiquitin-encoding genes were isolated by screening a Guy-11 genomic library constructed from strain Guy-11 in  $\lambda$  GEM11 (Talbot et al. 1993a). Densitometric analysis of autoradiographs was performed on scanned images with a Power Macintosh 8200/120 computer using the public domain NIH Image programme (developed at the U. S. National Institutes of Health and available on the Internet as <http://rsb.info.nih.gov/nih-image/>).

**Exposure of *M. grisea* to environmental stress.** Procedures were adapted from the experiments to examine cellular stress and ubiquitin gene expression of Finley et al. (1987). Guy-11 mycelium was grown for 2 days at 25°C and then subjected to: (1) exposure to ultra-

violet radiation (302 nm), without maintaining the temperature, for 6 min followed by a 15-min recovery time at 25 °C (total UV intensity of 0.09 J cm<sup>-2</sup>), (2) cold shock by incubation at 4 °C for 20 min and then recovery at 25 °C for 15 min, (3) heat shock by incubation at 42 °C for 20 min, (4) heat shock by incubation at 42 °C for 20 min

with a 15-min recovery time at 25 °C, and (5) heat shock by incubation at 42 °C for 20 min with a 30-min recovery time at 25 °C. Total RNA was extracted from the mycelium and an equally loaded RNA gel-blot prepared. This blot was hybridised with pHM8, pHM300, pHM400, and pMG-1 (rDNA) to ensure equal loading and transfer of RNA.



**Fig. 1** Temporal analysis of *UEP1* expression during colonisation of rice tissue by *M. grisea*. Total RNA (5 µg per lane) was extracted from infected rice leaves at selected times following infection: 24 h, 36 h, 48 h, 72 h and 96 h. The RNA was fractionated under denaturing conditions and blotted onto Hybond-N membranes. (a) RNA gel-blot probed with pHM8 cDNA (*UEP1*). (b) RNA gel-blot probed with an 8-kb fragment of pMG-1 containing the cytoplasmic rRNA-encoding repeat unit (rDNA) as a loading control

**Results**

**Identification of *UEP1*, a gene expressed during the pathogenesis of *M. grisea***

A cDNA library constructed from blast-infected rice leaves was differentially screened to identify genes preferentially expressed during *in planta* growth (Talbot et al. 1993 a). One of the identified cDNAs, designated pHM8, was characterised in detail. Examination of the expression of pHM8 *in planta* was first undertaken using RNA gel-blot analysis. Total RNA was extracted from rice leaves at five time points following the infection of plants with a compatible *M. grisea* isolate. The times selected were 24 h, 36 h, 48 h, 72 h and 96 h subsequent to the initial inoculation of rice leaves with conidial suspensions of the fungus. A 600-bp

**Fig. 2a, b** Nucleotide sequence of *M. grisea UEP1* showing extensive homology to a number of ubiquitin fusion genes. **a** The CEP52 genes encode a single copy of ubiquitin, fused to a carboxy terminal 52 amino-acid protein. In *S. cerevisiae* this class of fusion protein is cleaved at glycine-76 to generate mature ubiquitin shown by <sup>1</sup>. A putative nuclear localisation signal is underlined. A putative zinc-finger RNA-binding site is indicated in bold by C...C at codons 91, 96, 99, 110, and 115 (Özkaynak et al. 1987). **b** The predicted aminoacid sequence of *UEP1* was aligned with known ubiquitin carboxy extension proteins (CEP52) from a number of organisms: *Cryptococcus neoformans* (U16992), *Acanthamoeba castellanii* (630455), *Drosophila melanogaster* (103436), *Caenorhabditis elegans* (L31492), *Oryza sativa* (485518), *Nicotiana tabacum* (83594) and *Saccharomyces cerevisiae* (A29456). The sequence shows 97% identity to *S. cerevisiae UB11*. *UEP1* has the Genbank accession number AF056623 indicates identical sequence

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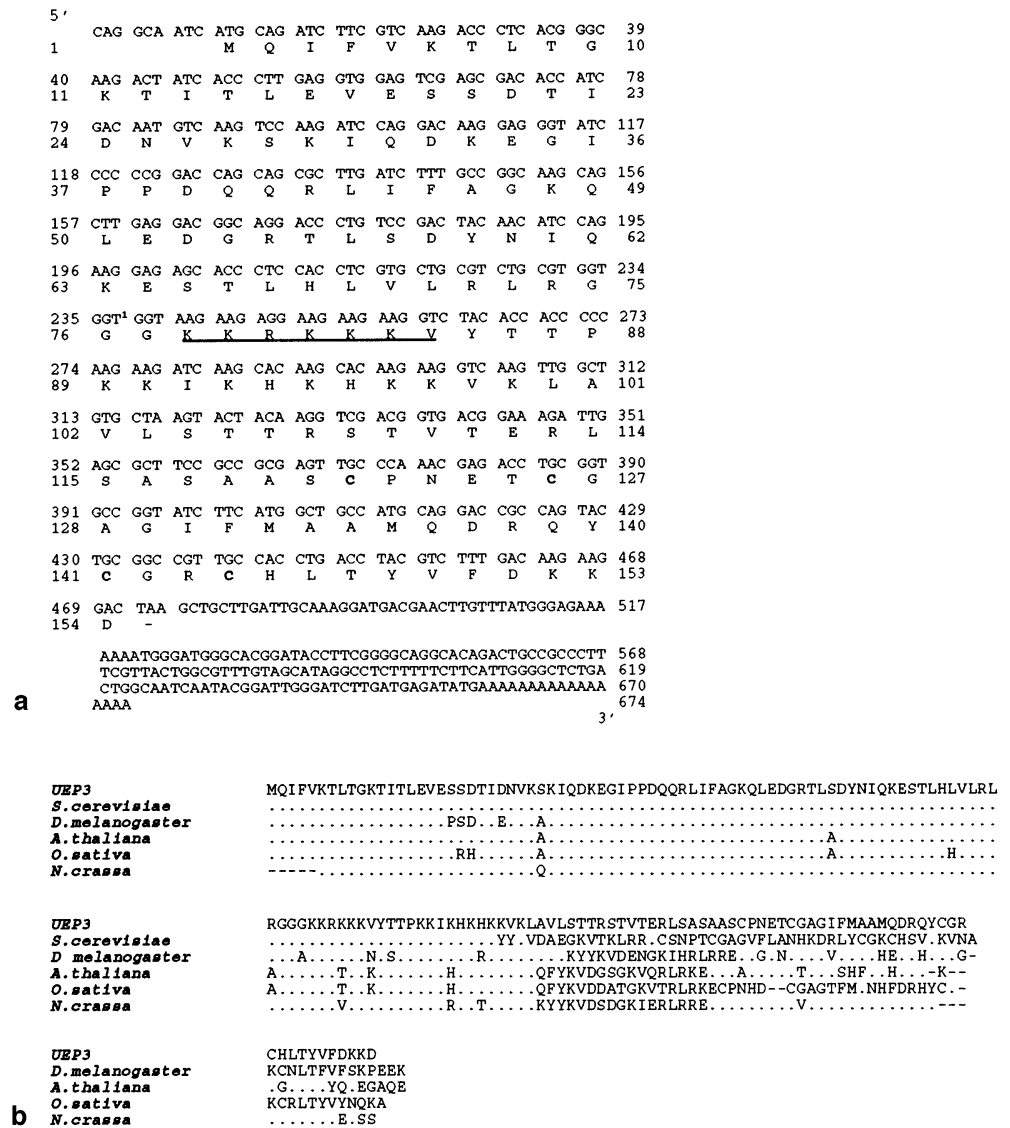
5'
1  GCC GCC AAG ATG CAG ATC TTC GTG AAG ACT CTG ACT GGC 39
   10
40  AAG ACC ATC ACC TTG GAG GTC GAG TCC TCG GAC ACG ATC 78
   23
79  GAC AAT GTG AAG TCC AAG ATT CAG GAC AAG GAG GGA ATT 117
   36
118 CCC CCG GAC CAA CAG CGT CTT ATC TTT GCT GGC AAG CAG 156
   49
157 CTG GAG GAC GGC CGT ACT CTC TCC GAC TAC AAC ATC CAG 195
   62
196 AAG GAG TCG ACC CTC CAC TTG GTC CTC CGC CTG CGT GGT 234
   75
235 GGT1 ATC ATC GAG CCC AGC TTG AAA GCT CTG GCC AGC AAG 273
   88
274 TTC AAC TGC GAC AAG CAG ATT TGC CGC AAG TGC TAC GCT 312
   101
313 CGT CTC CCA CCC CGT GCC ACA AAC TGC AGG AAG AGA AAG 351
   114
352 TGT GGA CAC ACG AAC CAG CTT CGC CCC AAG AAG AAG CTC 390
   127
391 AAA TAA ACGATTTACCCCTCATCAGCGTCTTTGGCGTCACGTTTTTCGG 439
   128  K -
          TTCGCGATCGAGAGTACAGGGAGGGGTTATTTTCGGAAAAGCTTTGGCGCAC 490
          AGGGAAGCCAGGGACATTTGACAAGTCAAGGACTTTGTCGCTGCCATACAT 541
          CCAAAGAGCCTCAGAACATGATTCATGAATTTTTCATCAAATACCTGA 592
          TACCTTGAAAAAATAAAAAAAAAAAAAA 3' 620
    
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a
UEP1 MQIFVKTLTGKTTITLEVSSDITDNVSKSIQDKKEGIPDPQQLIFAGKQLEDGRTLSDYNIQKESTLHLV
C.neoformans .....K.K..SS.I...A..K
A.castellanii .....V...R.Y...VV...H...K...S.HW...
D.melanogaster .....RI...Q.Y...M...H...K...N...
C.elegans .....RQ...Q.Y...S...K...SSE.I...
O.sativa .....Q...R.Y.Q...M...H...V...K...S...I.N
N.tabacum .....M...R.Y.Q...M...H...V...R...K...S...I...
S.cerevisiae .....Y...SV...

b
UEP1 LRLRGGIIEPSLKALASKFNCDKQICRKYARLPPRATNCRRLCGHTNQLRPKPKL
C.neoformans .....Y..E.....K.K..SS.I...A..K
A.castellanii .....V...R.Y...VV...H...K...S.HW...
D.melanogaster .....RI...Q.Y...M...H...K...N...
C.elegans .....RQ...Q.Y...S...K...SSE.I...
O.sativa .....Q...R.Y.Q...M...H...V...K...S...I.N
N.tabacum .....M...R.Y.Q...M...H...V...R...K...S...I...
S.cerevisiae .....Y...SV...
    
```

**Fig. 3a, b** Nucleotide sequence of *M. grisea* *UEP3* showing extensive homology to a number of ubiquitin fusion genes. **a** The CEP76 genes encode a single copy of ubiquitin, fused to a carboxy terminal 76 amino-acid protein. In *S. cerevisiae* this class of fusion protein is cleaved at glycine-76 to generate mature ubiquitin and this position is shown by <sup>1</sup>. A putative nuclear localisation signal is *underlined*. A putative zinc-finger RNA-binding site is indicated in *bold* by C...C at codons 121, 126, 141, and 144 (Özkaynak et al. 1987). **b** The predicted amino-acid sequence of *UEP3* was aligned with known ubiquitin carboxy extension proteins (CEP76) from a number of organisms: *Drosophila melanogaster* (S23988), *Oryza sativa* (485519), *Saccharomyces cerevisiae* (70658), *Neurospora crassa* (U01220), *Arabidopsis thaliana* (99772). The predicted *UEP3* amino-acid sequence is 56% identical to *UBI3* of *S. cerevisiae*. *UEP3* has the Genbank accession number AF056624 indicates identical sequence



transcript was identified by the hybridisation of pHM8 to total RNA and was most abundant 48 h following plant inoculation (Fig. 1a). The transcript was only barely visible subsequent to this time, despite there being a much greater biomass of *M. grisea* present as the infection proceeded (Talbot et al. 1993a). This pattern of expression was observed in an RNA gel-blot analysis of two independent pathogenicity assays. The time of maximal accumulation of the pHM8 transcript corresponds to a period of rapid plant colonisation by *M. grisea*, but is prior to symptom development (Talbot 1995). During this stage of pathogenesis the fungus proliferates rapidly through the first two to three epidermal cells encountered after appressorial penetration and is characterised by a bulbous, determinate, growth pattern (Heath et al. 1990) Subsequent to this stage of development, the hyphae narrow and extend through the leaf epidermis and mesophyll before lesions develop 96 h after initial plant infection.

DNA sequence analysis of pHM8 revealed an open reading frame (ORF) putatively encoding a 128 amino-acid

protein of 14.6 kDa. This ORF was followed by a 3' untranslated region of 173 bases and a 21-nucleotide poly-(A) tail (Fig. 2a). Based on amino-acid sequence alignment, the translational product was predicted to be a ubiquitin fusion gene showing 97% amino-acid identity to the *UBI1* gene of *Saccharomyces cerevisiae* (Fig. 2b). This is a highly conserved gene encoding a ubiquitin extension protein in which a 76 amino-acid ubiquitin peptide is joined to a 52 amino-acid carboxy extension protein (CEP-52). The gene corresponding to the pHM-8 cDNA clone encodes a ubiquitin extension protein and was therefore designated *UEP1*. The 52-residue tail of the fusion protein is basic in nature, containing 31% glycine and arginine residues. The carboxy terminus consists of a stretch of highly basic amino acids (Fig. 2b) which strongly resembles a motif known to be required for localisation of proteins to the nucleus (Dingwall and Laskey 1986). The carboxy terminus also contains a cysteine-rich zinc-finger motif originally identified within a 5 S RNA gene-specific transcription factor TFIIIA in *Xenopus* oocytes (Miller et al. 1985)

which may be involved in nucleic acid binding (Vincent 1986).

Ubiquitin normally functions by being covalently attached to other proteins and targeting them for proteolysis. Its main and best known function is, indeed, as an essential component of the proteasome pathway (Hilt and Wolf 1996). Ubiquitin has, however, also been implicated in a number of essential processes including cell-cycle regulation, ribosomal biogenesis, DNA repair, and the response of cells to extracellular stress (Hershko et al. 1983; Finley and Varshavsky 1985; Finley and Chau 1991; Rechsteiner 1991). In order to investigate the potential role of ubiquitin in the pathogenesis of *M. grisea* and the specific function of *UEP1*, two strategies were adopted. First, a search was undertaken to identify other ubiquitin-encoding genes with potentially complementary functions. Secondly, regulation of the ubiquitin gene family in response to environmental factors was studied.

#### Identification of the ubiquitin gene family of *M. grisea*

Ubiquitin is encoded by three types of genes in most eukaryotes studied so far (Jentsch 1992). In *S. cerevisiae* the first type is represented by *UBI1*, *UBI2* and *UBI3* which each encode a single copy of ubiquitin fused to an unrelated tail protein. These are known as ubiquitin carboxy extension proteins (CEP) and *UEP1* clearly belongs to this group. Secondly, there are poly-ubiquitin genes which encode multiple copies of ubiquitin fused together in a head-to-tail configuration. In *S. cerevisiae* this function is served by *UBI4* (Finley et al. 1987). Finally, a third type of gene has been identified in certain mammalian and viral systems, but is absent from *S. cerevisiae*. These genes encode a number of ubiquitin-like proteins of unknown function (Jentsch 1992; Casteels et al. 1996).

Using the *S. cerevisiae* ubiquitin genes as a guide to the likely organisation of the *M. grisea* gene family, we carried out a search for other ubiquitin-encoding genes of *M. grisea*. Low-stringency Southern hybridisation with pHM8 was used to identify ubiquitin-encoding genes from a *M. grisea* conidiospore cDNA library plated at high density. Using this method, *M. grisea* sequence-homologues of the *UBI3* and *UBI4* genes of *S. cerevisiae* were identified based on the high conservation of the ubiquitin-sequence components of these genes. The identified cDNAs were designated *UEP3* and *PUB4* (for Ubiquitin Extension Protein and Poly Ubiquitin, respectively).

*UEP3* putatively encodes a 17.3-kDa protein consisting of two parts; a 76-amino-acid ubiquitin protein and a 76-amino-acid carboxy terminal fusion protein (Fig. 3). *UEP3* showed strong homology to *UBI3* of *S. cerevisiae* and the *ubi/crp-6* gene of *Neurospora crassa*. The ubiquitin component was found to be 100% identical at the amino-acid level to that of *S. cerevisiae* *UBI3*. The fusion protein contained a putative nuclear localisation signal in the C-terminal CEP76 domain (amino acids 79–84) and a putative zinc-finger domain (Fig. 3). The C-terminal sequences

were initially highly homologous, but in all cases diverged significantly after amino acid 96 (Fig. 3).

*PUB4* putatively encodes a 42.5-kDa protein and, in common with the other polyubiquitin-encoding genes, this is composed of five copies of ubiquitin linked head-to-tail in a spacerless configuration (Fig. 4). Very strong homology was found between *PUB4* and the *S. cerevisiae* *UBI4*, the maize polyubiquitin gene *MubC5*, and the polyubiquitin gene from *N. crassa*.

The expression of *M. grisea* ubiquitin-encoding genes is influenced by environmental stress

Recently it has become apparent that a number of *M. grisea* genes expressed during pathogenesis are also regulated by nutrient starvation (Talbot et al. 1993; Lau and Hamer 1996). Nutrient deprivation may, therefore, be one of the environmental cues for fungal development, both during the pre-penetration phase of development – when conidia germinate in dew drops on the plant surface (Talbot et al. 1993a; Jellitto et al. 1994) – and much later during disease-symptom outbreak (Talbot et al. 1997). To determine whether expression of the ubiquitin genes is regulated by nutrient starvation, total RNA was extracted from a *M. grisea* mycelium exposed to nitrogen-starvation stress for 48 h, as well as *M. grisea* mycelium exposed to glucose starvation for 48 h, and an RNA gel-blot analysis was carried out. The *UEP1* transcript was most abundant when the fungus was maintained under high nutrient conditions, whereas starvation for either glucose or nitrate caused a decrease in transcript abundance (Fig. 5a). *UEP3* showed a similar decrease in transcript abundance following starvation stress. In contrast, *PUB4* was constitutively expressed with only a very slight increase in expression after starvation stress. A control hybridisation experiment was carried out using the *MPG1* gene (Fig. 5a) which showed expression only after starvation for either glucose or nitrate (Talbot et al. 1993a). We conclude that *UEP1* and *UEP3* are most highly expressed, under conditions of nutrient availability, while *PUB4* is constitutively expressed.

To investigate the regulation of *UEP1*, *UEP3* and *PUB4* in greater detail, *M. grisea* mycelium was also subjected to a number of treatments known to induce stress proteins (Finley et al. 1987), and an RNA gel-blot analysis was carried out. *M. grisea* was subjected to three environmental stresses; heat shock, UV shock and cold shock. A control experiment was carried out by extracting RNA from *M. grisea* grown in complete medium, which was not subjected to any stress treatment, for 2 days. All three ubiquitin genes were highly expressed in axenic culture and only small differences in expression were observed after stress exposure. *UEP1* and *UEP3* expression decreased very slightly after exposure of *M. grisea* to ultraviolet radiation (UV), cold stress and heat shock, and increased slightly upon recovery from stress treatment (Fig. 5b). *PUB4* was also highly expressed during the growth of *M. grisea* in culture, although the transcript was most abundant follow-

ing exposure of the fungus to environmental stress. Exposure to UV, heat, and cold stress all caused a slight increase in *PUB4* transcript abundance and a 15–30 min recovery time after heat stress led to a further slight increase. We found that starvation stress has a greater effect on *M. grisea* ubiquitin gene expression than the other environmental stresses tested.

Strikingly, we also found that, although expression of *UEP1*, *UEP3* and *PUB4* was easily detected during fungal growth in culture, the *UEP3* and *PUB4* transcripts were not detected *in planta* by RNA gel-blot analysis even after overloading the RNA gels (data not shown). This suggests that either *UEP3* and *PUB4* are not expressed during *in planta* growth or, more likely, that their expression is at too low a level to be detected by RNA gel-blot hybridisation with total rice-leaf RNA.

*UEP1* is present at more than one copy per haploid genome

To determine whether all members of the *M. grisea* ubiquitin gene family are present in a single copy per haploid genome, reconstruction experiments were carried out to determine the gene copy number for *UEP1*, *UEP3* and *PUB4* (Fig. 6). *UEP3* and *PUB4* both hybridised weakly to genomic DNA, suggesting that they are present at a lower copy number (1–2 copies per haploid genome). The *UEP1* gene, however, hybridised with equal intensity to *M. grisea* genomic DNA and to a genomic sub-clone of *UEP1* which was adjusted in concentration to represent ten copies of the gene per haploid genome. This indicates that *UEP1* is present at a much higher copy number than the other ubiquitin-encoding genes. The simple hybridisation pattern observed suggests that *UEP1* is present at a single site and is tandemly repeated. Mapping of a *UEP1*-associated RFLP in 36 random ascospore progeny from a cross, however, suggested that *UEP1* may be present at more than one site in the genome. This is based on the fact that the RFLP did not segregate as a single Mendelian marker and showed linkage to RFLP markers on more than one linkage group (data not shown). Based on this analysis we conclude that *UEP1* has been amplified in the *M. grisea* genome and is present at approximately ten copies per haploid genome.

**Discussion**

The *M. grisea UEP1* gene was identified by differential cDNA screening and represents a gene abundantly expressed during plant colonisation by the fungus. The expression of this gene appeared to be highest during the initial stages of plant infection at a stage prior to the appearance of rice blast disease symptoms. During this period the fungus proliferates rapidly through the leaf epidermis and takes on a determinate growth pattern as it grows intracellularly and from cell to cell (Heath et al. 1990). The fun-

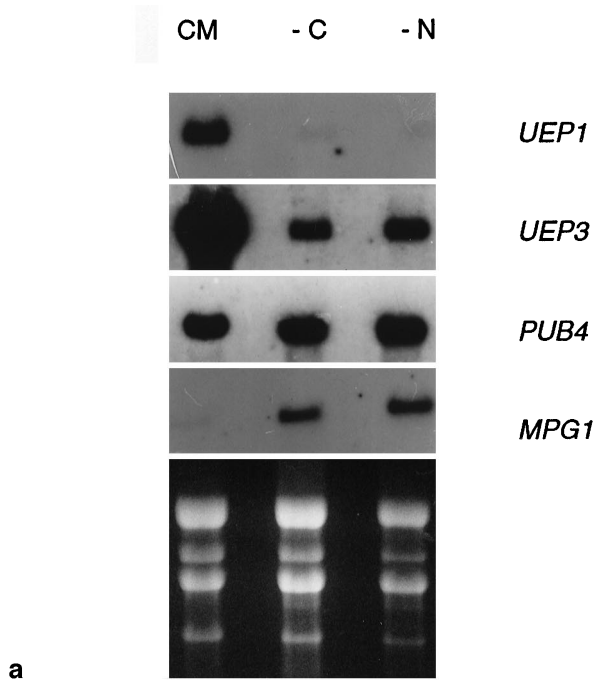
5'	ATG	CAG	ATC	TTC	GTG	AAG	ACT	CTC	ACT	GGC	AAG	ACC	ATC	39
1	M	Q	I	F	V	K	T	L	T	G	K	T	I	13
40	ACC	CTC	GAG	GTG	GAG	TCT	AGC	GAT	ACC	ATC	GAC	AAC	GTC	78
14	T	L	E	V	E	S	S	D	I	I	D	N	V	26
79	AAG	TCG	AAG	ATC	CAG	GAC	AAG	GAG	GGT	ATC	CCT	CCG	GAC	117
27	K	S	K	I	Q	D	K	E	G	I	P	F	D	39
118	CAA	CAG	CGT	CTT	ATT	TTT	GCC	GGC	AAG	CAA	CTC	GAG	GAC	156
40	Q	Q	R	L	I	F	A	G	A	Q	L	E	D	52
157	GGT	CGG	ACA	CTT	TCC	GAC	TAC	AAC	ATC	CAG	AAG	GAG	AGT	195
53	G	R	T	L	S	D	Y	N	I	I	K	E	S	65
196	ACT	CTC	CAC	CTC	GTG	CTC	CGC	CTC	CGT	GGT	GGC <sup>4</sup>	ATG	CAG	234
66	T	L	H	L	V	L	R	L	R	G	G	M	Q	78
235	ATT	TTC	GTC	AAA	ACC	CTC	ACT	GGC	AAG	ACG	ATT	ACC	TTG	273
79	I	F	V	K	T	L	T	L	G	K	T	I	T	91
274	GAG	GTC	GAG	TCC	TCC	GAT	ACC	ATC	GAC	AAT	GTA	AAG	TCG	312
92	E	V	E	S	S	D	T	I	D	N	V	K	S	104
313	AAG	ATC	CAG	GAC	AAG	GAG	GGT	ATT	CCT	CCG	GAC	CAA	CAA	351
105	K	I	Q	D	K	E	G	I	P	P	D	Q	L	117
352	CGC	CTT	ATT	TTC	GCT	GGA	AAG	CAG	CTG	GAG	GGC <sup>4</sup>	ATG	CAG	390
118	R	L	I	F	A	G	K	Q	L	E	D	G	R	130
391	ACC	CTT	TCC	GAC	TAC	AAT	ATC	CAG	AAG	GAA	ACG	ACC	CTC	429
131	T	L	S	D	Y	N	I	Q	K	E	S	T	L	143
430	CAT	TTG	GTC	CTT	CGT	CTC	GGT	GGC <sup>4</sup>	ATG	CAG	ATT	TTT	468	
144	H	L	V	L	R	L	R	G	G	M	Q	I	F	156
469	GTC	AAG	ACT	TTG	ACG	GGC	AAG	ACC	ATC	ACG	TTG	GAG	GTC	507
157	V	K	T	L	T	G	K	T	I	T	I	E	V	169
508	GAG	TCT	TCA	GAC	ACG	ATT	GAC	AAT	GTT	AAA	TCC	AAG	ATC	546
170	E	S	S	D	T	I	D	N	V	K	S	K	I	182
547	CAA	GAC	AAG	GAG	GGT	ATT	CCT	CCG	GAC	CAA	CAG	CGA	CTG	585
183	Q	D	K	E	G	I	P	D	Q	R	L	R	L	195
586	ATC	TTC	GCT	GGA	AAG	CAG	TTG	GAG	GAT	GGA	CGC	ACG	CTG	624
196	I	F	A	G	K	Q	L	E	D	G	R	T	L	208
625	TCC	GAC	TAC	AAC	ATC	CAA	AAG	GAA	TCT	ACT	CTG	L	TTG	663
209	S	D	Y	N	I	Q	K	E	S	T	C	H	L	221
664	GTC	CTG	CGT	GGT	GGT <sup>4</sup>	ATG	CAA	ATT	TTT	GTC	AAG	ACG	CIT	702
222	V	L	R	G	G	M	Q	I	F	V	K	T	L	234
703	ACC	GGC	AAA	ACC	ATC	ACA	CTC	GAA	GTT	GAA	TCT	TCA	GAC	741
235	T	G	K	T	I	T	I	E	V	E	S	S	D	247
742	ACA	ATC	GAC	AAC	GTC	AAA	TCA	AAG	ATT	CAA	GAC	AAG	GAG	780
248	T	I	D	N	V	K	S	K	I	Q	D	K	E	260
781	GGT	ATC	CCG	CCT	GAC	CAA	CAA	CGT	TTG	ATC	TTT	GCG	GGC	819
261	G	I	P	P	D	Q	Q	R	L	I	F	A	G	273
820	AAG	CAG	CTG	GAG	GAT	GGA	AGA	ACC	CTG	TCT	GAT	TAC	AAT	858
274	K	Q	L	E	D	G	R	T	L	S	D	Y	N	286
859	ATT	CAG	AAG	GAG	TCG	ACT	CTG	L	C	CTT	GTC	CTT	CGC	897
287	I	Q	K	E	S	T	L	H	V	L	R	R	L	299
898	CGG	GGT	GGA <sup>4</sup>	ATG	CAA	ATT	TTT	GTC	AAG	ACG	CTA	ACC	GGC	936
300	R	G	G	M	Q	I	F	V	K	T	L	T	G	312
937	AAA	ACC	ATC	ACA	CTG	GAA	GTG	GAA	TCT	TCA	GAC	ACA	ATT	975
313	K	T	I	T	L	E	V	E	S	S	D	T	I	325
976	GAC	AAT	GTC	AAA	TCA	AAG	ATT	CAA	GAC	AAG	GAG	GGT	ATC	1014
326	D	N	V	K	S	K	I	Q	D	K	E	G	I	338
1015	CCG	CCG	GAC	CAA	CAA	CGT	TTG	ATC	TTT	GCG	GGC	AAG	CAG	1053
339	P	P	D	Q	Q	R	L	I	F	A	G	K	Q	351
1054	CTG	GAG	GAT	GGA	AGA	ACC	CTG	TCT	GAT	TAC	AAT	ATT	CAG	1092
352	L	E	D	G	R	T	L	S	D	Y	N	I	Q	364
1093	AAG	GAG	TCG	ACT	CTG	CAC	CTT	GTG	CTT	CGT	CTT	CGT	GGC	1131
365	K	E	S	T	L	H	L	V	L	R	L	R	G	377
1132	GGT	CAG	TAG	ATAGACTTTGGCAAAGACACGCTAGTCTGATGACTTCGA	1179									
378	G	Q	-											
	CGTTGGGACATGCAATTGGTGTTCCTTTGGGTTTGGTTCAGTCAGGAACGGG	1230												
	ATGATATGAATGGATGTTAACTTCTACTACACATACTTTGACACTAGCAT	1281												
	AGCGGTGTCAAAAATTCAACAATTTGAGTAAAAA	1326												

a

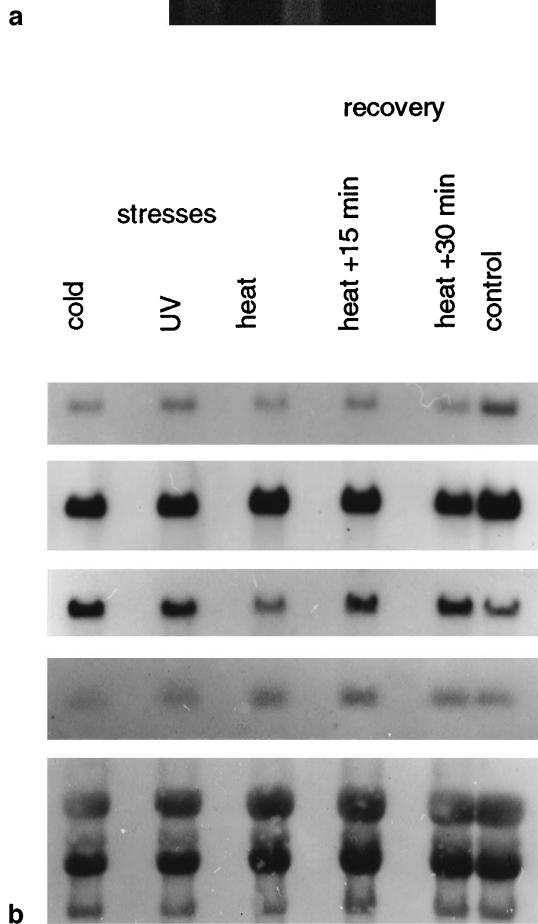
Fig. 4a

gus is likely to have spread only to three or four epidermal cells at each site of infection at this time (Heath et al. 1990), and therefore only a small fraction of the leaf RNA sample used for RNA gel-blot analysis is likely to be composed of *M. grisea* transcripts. This suggests that *UEP1* is very highly expressed at this time. The reduction in *UEP1* tran-

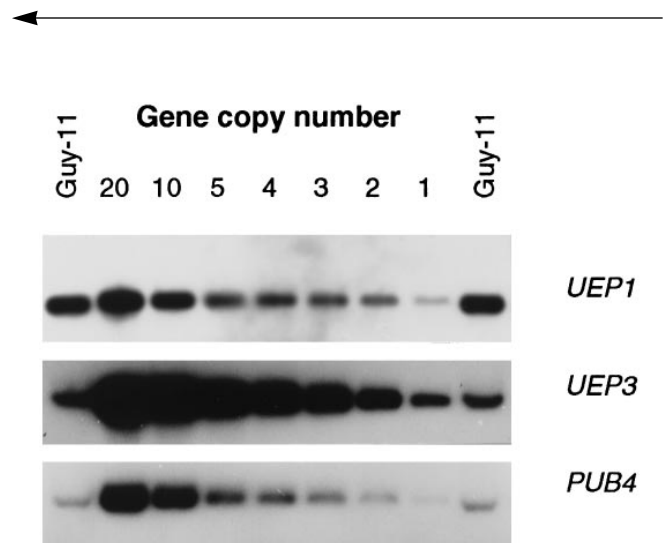




**Fig. 5a, b** RNA gel blot analysis of *UEP1* during environmental stress. **a** RNA was extracted from *M. grisea* mycelium maintained under high nutrient conditions (labelled CM), mycelium starved for nitrate for 48 h (labelled -N), and mycelium starved for glucose (labelled -C). Total RNA (5 µg per lane) was fractionated under denaturing conditions, blotted onto Hybond-N membranes and probed with the following: pHM8 cDNA (*UEP1*), pHM300 cDNA (*UEP3*), pHM400 cDNA (*PUB4*) and a 3.75-kb *XbaI/HindIII* fragment of pNT800 (*MPG1*). The ethidium bromide-stained gel of the corresponding RNA samples is shown below. **b** *M. grisea* was grown in shake culture for 48 h in complete medium at 24°C and then subjected to conditions known to induce stress-related proteins (Finley et al. 1987). The stress treatments used were: cold shock at 4°C for 20 min, UV exposure for 6 min, heat shock at 42°C for 20 min, 42°C for 20 min, 15-min recovery at 25°C, 42°C for 20 min, and a 30-min recovery at 25°C. RNA from an untreated mycelium used as a control. Total RNA (5 µg per lane) extracted from the fungal mycelium after stress treatment was fractionated under denaturing conditions, blotted onto Hybond-N and probed with the following: pHM8 cDNA (*UEP1*); pHM300 (*UEP3*); pHM400 cDNA (*PUB4*); a 6.4-kb *SalI* fragment of pCB573 (*ILV1*); an 8-kb fragment of pMG-1 containing the cytoplasmic rRNA-encoding repeat unit (rDNA)



before symptom development and subsequent conidiation (Talbot et al. 1993a). This suggests that prolonged expression of both *UEP1* and *UEP3* would be required to facilitate ribosome biogenesis throughout fungal growth. It seems very likely, therefore, that both genes are expressed



**Fig. 6** *UEP1* is present in more than one copy in the *M. grisea* genome. For each member of the gene family a genomic sub-clone was isolated (see Materials and methods) and the DNA concentration adjusted to correspond with the amount a single-copy gene would represent in 1 µg of *M. grisea* genomic DNA, assuming a genome size of 38 Mb (Hamer et al. 1988). A DNA concentration series was prepared to represent 1, 2, 3, 4, 5, 10 and 20 copies of the gene and the DNA fractionated by electrophoresis and then Southern blotted. Hybridisations were carried out with (a) a 4.4-kb *EcoRI* fragment from the *UEP1* genomic sub-clone, pHMG-1 (b) a 6.5-kb *SalI* fragment from the *UEP3* genomic sub-clone, pHMG-U3 and (c) a 9.0-kb *EcoRI* fragment from the *PUB4* genomic sub-clone, pHMG-U4. Hybridisation signals were compared with two control lanes containing 1 µg of *EcoRI*-digested *M. grisea* genomic DNA from strain Guy-11

throughout pathogenesis to fulfil this function, but perhaps not at a high-enough level to be detected by RNA gel-blots of infected plant material. The very high expression of *UEP1* during a particular stage of plant colonisation, however, may suggest that *UEP1* also has an alternate function.

Genomic reconstruction experiments imply that *UEP1* is present at approximately ten copies per haploid genome.

This differs markedly from *S. cerevisiae* where all four members of the ubiquitin family are present as single-copy genes (Özkaynak et al. 1987). The fact that there are multiple copies of *UEP1* in the genome may contribute a gene-dosage effect to allow for the extraordinarily high expression of *UEP1* during pathogenesis.

To-date the only record of ubiquitin being expressed by a plant pathogenic fungus is the expression of a polyubiquitin gene by the late blight pathogen *Phytophthora infestans* during growth in potato leaves (Pieterse et al. 1991). The high-level expression of this stress-related ubiquitin during pathogenesis suggested that *P. infestans* is subject to starvation-related stress during *in planta* growth. This is in marked contrast to the situation in *M. grisea* where *UEP1* is highly expressed *in planta* but the polyubiquitin gene, *PUB4*, is not. This suggests that *M. grisea* is not subjected to starvation stress during initial plant colonisation when growth is rapid (Talbot et al. 1993a). Perhaps starvation-stress only influences gene expression subsequently, just prior to symptom outbreak (Talbot et al. 1993a, 1997).

A consequence of the processing of the *UEP1* gene product during ribosome biogenesis is the release of free ubiquitin. It may be that the assimilative stage of fungal development (Heath et al. 1990), just after appressorium-mediated infection and before rapid colonisation of leaf tissue, requires extensive protein turnover as the fungus adjusts to intracellular growth in living plant tissue. A requirement for extra free ubiquitin during this assimilatory stage of development may therefore explain why *UEP1* is highly expressed at this time. Another potential role for *UEP1*, however, may be in a regulatory capacity. Ubiquitins have been implicated in the degradation of mitotic cyclins, as well as several cell-surface receptors and transcriptional regulators (Ciechanover 1994). The morphological differentiation associated with the growth of *M. grisea* in rice-leaf tissue (Heath et al. 1990; Bourrett and Howard 1990; Talbot 1995) suggests that growth of the pathogen in living plant tissue may require a rapid adjustment of metabolic activity in order for the fungus to survive and proliferate. Consistent with a regulatory role, it has been noted that ubiquitin fusions encoded by *UBI1*, *UBI2* and *UBI3* resemble post-translationally formed ubiquitin-protein conjugates which are intermediates in ubiquitin-dependent protein degradation (Finley et al. 1989). They may therefore interact directly with components of the ubiquitin proteolytic pathway, exerting a regulatory effect (Hilt and Wolf 1996). It is possible that in *M. grisea* the definitive ubiquitin pathway has been modified to accommodate pathogenic growth.

The restricted high-level expression of *UEP1* suggests that it has been co-opted by *M. grisea* into serving an important role in the early stages of pathogenesis. Although normally considered to fulfil a 'housekeeping' function, ubiquitin is intimately associated with the ability of cells to respond to environmental change. Understanding the function of *UEP1* may therefore shed light on the adaptive mechanisms employed by fungi in order to survive and grow in living plant tissue.

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## References

- Baker B, Zambryski P, Staskawicz B and Dinesh-Kumar SP (1997) Signalling in plant-microbe interactions. *Science* 276:726–733
- Bourrett TM, Howard RJ (1990) In vitro development of penetration structures in the rice blast fungus *Magnaporthe grisea*. *Can J Bot* 68:329–342
- Casteels D, Kas K, Merregaert J (1996) Ubiquitin and the ubiquitin-like proteins. *Protein Peptide Lett* 3:343–349
- Ciechanover A (1994) The ubiquitin-proteasome proteolytic pathway. *Cell* 79:13–21
- Dingwall C, Laskey RA (1986) Protein import into the cell nucleus. *Annu Rev Cell Biol* 2:367–390
- Feinberg AP, Vogelstein B (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 132:6–13
- Finley D, Chau V (1991) Ubiquitination. *Annu Rev Cell Biol* 7:25–69
- Finley D, Varshavsky A (1985) The ubiquitin system: functions and mechanisms. *Trends Biochem Sci* 10:343–346
- Finley D, Özkaynak E, Varshavsky A (1987) The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation, and other stresses. *Cell* 48:1035–1046
- Finley D, Bartel B, Varshavsky A (1989) The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. *Nature* 338:394–401
- Hamer JE, Howard RJ, Chumley FG, Valent B (1988) A mechanism for surface attachment in spores of a plant pathogenic fungus. *Science* 239:288–290
- Heath MC, Valent B, Howard RJ, Chumley FG (1990) Correlations between cytologically detected plant-fungal interactions and pathogenicity of *Magnaporthe grisea* toward weeping lovegrass. *Phytopathology* 80:1382–1386
- Hershko A, Heller H, Elias S, Ciechanover A (1983) Components of a ubiquitin-protein ligase system. *J Biol Chem* 258:8206–8214
- Hilt W, Wolf DH (1996) Proteasomes: destruction as a programme. *Trends Biochem Sci* 21:96–102
- Howard RJ, Ferrari MA (1989) Role of melanin in appressorium formation. *Exp Mycol* 13:403–418
- Howard RJ, Valent B (1996) Breaking and entering – host penetration by the fungal rice blast pathogen. *Magnaporthe grisea*. *Annu Rev Microbiol* 50:491–512
- Howard RJ, Ferrari MA, Roach DH, Money NP (1991) Penetration of hard substrates by a fungus employing enormous turgor pressures. *Proc Natl Acad Sci USA* 88:11281–11284
- Jelitto TC, Page HA, Read ND (1994) Role of external signals in regulating the pre-penetration phase of infection by the rice blast fungus *Magnaporthe grisea*. *Planta* 194:471–477
- Jentsch S (1992) The ubiquitin-conjugation system. *Annu Rev Genet* 26:179–207
- Jong JC de, McCormack BJ, Smirnoff N, Talbot NJ (1997) Generation of enormous turgor by accumulation of molar concentrations of glycerol in a plant pathogenic fungus. *Nature* 389:244–245
- Lau GW, Hamer JE (1996) Regulatory genes controlling MPG1 expression and pathogenicity in the rice blast fungus *Magnaporthe grisea*. *Plant Cell* 8:771–781
- Lee Y-H, Dean RA (1994) Hydrophobicity of contact surface induces appressorium formation in *Magnaporthe grisea*. *FEMS Microbiol Lett* 115:71–75

- Mitchell TK, Dean RA (1995) The cAMP-dependent protein kinase catalytic sub-unit is required for appressorium formation and pathogenesis by the rice blast fungus *Magnaporthe grisea*. *Plant Cell* 7: 1869–1878
- Miller J, McLachlan AD, Klug A (1985) Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus* oocytes. *EMBO J* 4: 1609–1614
- Ohmachi T, Giorda R, Shaw DR, Ennis HL (1989) Molecular organization of developmentally regulated *Dictyostelium discoideum* ubiquitin cDNAs. *Biochemistry* 28: 5226–5231
- Orbach MJ, Chumley FG, Valent B (1996) Electrophoretic karyotypes of *Magnaporthe grisea* pathogens of diverse grasses. *Mol Plant-Microbe Interact* 9: 261–271
- Ou SH (1985) Rice diseases. Commonwealth Mycological Institute CAB, Kew, Surrey, pp 109–201
- Özkaynak E, Finley D, Varshavsky A (1984) The yeast ubiquitin gene: head-to-tail repeats encoding a polyubiquitin precursor protein. *Nature* 312: 663–666
- Özkaynak E, Finley D, Solomon MJ, Varshavsky A (1987) The yeast ubiquitin genes: a family of natural gene fusions. *EMBO J* 6: 1429–1439
- Pieterse CMJ, Risseuw EP, Davidse LC (1991) An *in planta* induced gene of *Phytophthora infestans* codes for ubiquitin. *Plant Mol Biol* 17: 799–811
- Rechsteiner M (1991) Natural substrates of the ubiquitin proteolytic pathway. *Cell* 66: 615–618
- Redman KL (1994) The smaller protein formed as a ubiquitin fusion in *Drosophila* is processed from ubiquitin and found on the 60 S ribosomal subunit. *Insect Biochem Mol Biol* 24: 191–201
- Rossmann AY, Howard RJ, Valent B (1990) *Pyricularia grisea*, the correct name for the rice blast fungus. *Mycologia* 82: 509–512
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
- Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain inhibitors. *Proc Natl Acad Sci USA* 74: 5463–5467
- Skinner DZ, Budde AD, Farman ML, Smith JR, Leung H, Leong SA (1993) Genome organization of *Magnaporthe grisea*: genetic map, electrophoretic karyotype, and occurrence of repeated DNAs. *Theor Appl Genet* 87: 545–557
- Talbot NJ (1995) Having a blast: exploring the pathogenicity of *Magnaporthe grisea*. *Trends Microbiol* 3: 9–16
- Talbot NJ, Ebbole DJ, Hamer JE (1993a) Identification and characterisation of *MPG1*, a gene involved in pathogenicity of the rice blast fungus, *Magnaporthe grisea*. *Plant Cell* 5: 1575–1590
- Talbot NJ, Salch Y, Ma M, Hamer JE (1993b) Karyotype variation within clonal lineages of the rice blast fungus, *Magnaporthe grisea*. *Appl Envir Microbiol* 59: 585–593
- Talbot NJ, Kershaw MJ, Wakley GE, Vries OMH de, Wessels JGH, Hamer JE (1996) *MPG1* encodes a fungal hydrophobin involved in surface interactions during infection-related development of *Magnaporthe grisea*. *Plant Cell* 8: 985–999
- Talbot NJ, McCafferty HRK, Ma M, Moore K, Hamer JE (1997) Nitrogen starvation of the rice blast fungus *Magnaporthe grisea* may act as an environmental cue for disease symptom expression. *Physiol Mol Plant Pathol* 50: 179–195
- Timberlake WE (1980) Developmental gene regulation in *Aspergillus nidulans*. *Dev Biol* 78: 497–510
- Valent B (1990) Rice blast as a model system for plant pathology. *Phytopathology* 80: 33–36
- Valent B, Farrall L, Chumley FG (1991) *Magnaporthe grisea* genes for pathogenicity and virulence identified through a series of backcrosses. *Genetics* 127: 87–101
- Vincent A (1986) TFIIIA and homologous genes – the finger proteins. *Nucleic Acids Res* 14: 4385–4391
- Xu JR, Urban M, Sweigard JA, Hamer JE (1997) The CPKA gene of *Magnaporthe grisea* is essential for appressorial penetration. *Molec Plant-Microbe Interact* 10: 187–194