

The glyoxylate cycle is required for temporal regulation of virulence by the plant pathogenic fungus *Magnaporthe grisea*

Zheng-Yi Wang,^{1,2} Christopher R. Thornton,¹ Michael J. Kershaw,¹ Li Debao² and Nicholas J. Talbot^{1*}

¹School of Biological Sciences, University of Exeter, Washington Singer Laboratories, Exeter EX4 4QG, UK.

²Biotechnology Institute, Zhejiang University, Hangzhou 310029, China.

Summary

We describe the isolation and characterization of *ICL1* from the rice blast fungus *Magnaporthe grisea*, a gene that encodes isocitrate lyase, one of the principal enzymes of the glyoxylate cycle. *ICL1* shows elevated expression during development of infection structures and cuticle penetration, and a targeted gene replacement showed that the gene is required for full virulence by *M. grisea*. In particular, we found that the prepenetration stage of development, before entry into plant tissue, is affected by loss of the glyoxylate cycle. There is a delay in germination, infection-related development and cuticle penetration in *Δicl1* mutants. Recent reports have shown the importance of the glyoxylate cycle in the virulence of the human pathogenic fungus *Candida albicans* and the bacterial pathogen *Mycobacterium tuberculosis*. Our results indicate that the glyoxylate cycle is also important in this plant pathogenic fungus, demonstrating the widespread utility of the pathway in microbial pathogenesis.

Introduction

The majority of fungi exist as benign saprotrophic degraders of plant and animal material. A small number of species, however, have evolved the ability to invade and colonize living tissue and are the causal agents of a wide variety of diseases in animals and plants. The mechanisms by which fungi bring about diseases in plants and animals are very diverse; animal pathogens invade tissues directly or move within the host via its circulatory

system and, once established in the host, they need to proliferate while contending with both innate immunity and the adaptive immune response. In contrast, plant pathogenic fungi have evolved distinct developmental processes in order to breach tough plant cuticles and invade underlying tissues, and to withstand the extensive battery of chemical and physical defence mechanisms deployed by plants.

Magnaporthe grisea is the causal agent of rice blast, the most serious disease of cultivated rice (Howard and Valent, 1996; Talbot and Foster, 2001), and is typical of many cereal pathogens in infecting its host by means of a specialized infection structure called an appressorium. Appressoria are dome-shaped, melanin-pigmented cells, which develop enormous turgor (Howard *et al.*, 1991) in order to generate an invasive force to rupture the rice leaf cuticle. Having gained entry to the plant tissue, the fungus then grows rapidly, invading plant cells and bringing about observable disease symptoms within 3 days. In heavy infections, disease symptoms can be so severe that whole seedlings will die, whereas in older plants, the fungus can prevent grain-filling or destroy the grain-bearing structures of the plant (Howard and Valent, 1996; Tucker and Talbot, 2001).

The enormous turgor in appressoria is a consequence of the accumulation of very large quantities of glycerol in the cell (de Jong *et al.*, 1997). Glycerol acts as a highly soluble osmolyte, causing rapid influx of water into the appressorium to generate hydrostatic turgor. The biosynthesis of glycerol occurs rapidly during appressorium morphogenesis and involves mobilization of storage reserves from the conidium (Thines *et al.*, 2000). Lipid bodies have been shown to move to the appressorium and undergo aggregation and take-up by vacuoles, before lipolysis at the onset of appressorium maturation (Thines *et al.*, 2000). These observations suggest that glycerol is synthesized predominantly via triacylglycerol lipase activity, which is abundant in mature appressoria, using lipid reserves mobilized from the conidium. One consequence of such rapid lipolysis in *M. grisea* is likely to be the generation of fatty acids, and subsequently, after β -oxidation, of acetyl CoA. We reasoned that *M. grisea* may therefore require a mechanism for using acetyl CoA in order to allow subsequent development of

Accepted 2 December, 2002. *For correspondence. E-mail N.J.Talbot@exeter.ac.uk; Tel. (+44) 1392 264 673; Fax (+44) 1392 264 668.

the pathogen after plant infection. This has prompted us to investigate the role of the glyoxylate cycle in appressorium development.

Here, we report that *M. grisea* *ICL1*, encoding isocitrate lyase, is highly expressed during appressorium development, and especially within mature appressoria and invasive hyphae. Targeted mutation of *ICL1* led to mutants that showed a significant delay in spore germination and disease symptom expression. When considered in conjunction with recent studies showing the significance of isocitrate lyase in two distinct microbial pathogens of human hosts (McKinney *et al.*, 2000; Lorenz and Fink, 2001), our results indicate that the glyoxylate cycle may be of widespread importance to fungal pathogens in both animals and plants.

Results

Magnaporthe grisea *ICL1* is highly expressed during appressorium-mediated plant infection

In order to investigate the role of the glyoxylate cycle in appressorium-mediated plant infection by *M. grisea*, we identified and characterized a putative isocitrate lyase gene. Isocitrate lyase catalyses the production of glyoxylate from isocitrate and is the first step in the glyoxylate pathway that allows acetyl CoA to be converted to pyruvate and, subsequently, via gluconeogenesis, to glucose. *M. grisea* *ICL1* was initially recognized from a collection of expressed sequence tags (ESTs) derived from cDNA clones of an appressorium-stage cDNA library (Rauyaree *et al.*, 2001). The EST sequence was used to design *ICL1*-specific DNA primers, and a 240 bp fragment of the gene was amplified and used to identify a corresponding cDNA and genomic clone spanning the gene locus. DNA sequencing revealed a 1970 bp coding region interrupted by four introns (verified by sequencing of genomic and cDNA clones), which was capable of encoding a 547-amino-acid protein of 60 kDa. The putative *ICL1* gene product showed 85.5% amino acid identity to the isocitrate lyase gene *acu3* of *Neurospora crassa* and 76.3% identity to *acuD* of *Aspergillus nidulans* (Gainey *et al.*, 1992), as shown in Fig. 1. In order to investigate the temporal and spatial pattern of *ICL1* expression during infection-related development, a 1.5 kb promoter fragment upstream of the *ICL1* protein-coding sequence was fused to the green fluorescent protein-encoding gene *sGFP* (Chiu *et al.*, 1996). The *ICL1(p):sGFP* promoter fusion was introduced into a wild-type strain of *M. grisea*, Guy 11, and transformants with a single integration of the plasmid were selected by DNA gel blot analysis (data not shown). Three independent transformants were used to investigate gene expression patterns. Conidia, harvested from plate cultures of a *ICL1(p):sGFP* transformant,

exhibited high levels of GFP fluorescence (Fig. 2A and B). Expression declined initially during germ tube emergence and elongation, before appressorium development (Fig. 2C and D). Once formed, appressoria of *ICL1(p):sGFP* transformants were highly fluorescent, particularly at the onset of turgor generation, 24–48 h after spore germination (Fig. 2E and F). When conidia were incubated on a surface that can be breached by *M. grisea* appressoria, such as sterile onion epidermis, the expression of *ICL1(p):sGFP* was followed throughout the infection process. Appressoria were highly fluorescent during turgor generation (Fig. 2I and J), and emerging penetration pegs also fluoresced brightly (Fig. 2M and N). After cuticle penetration, *M. grisea* formed highly vacuolated, bulbous, branched infection hyphae, which also showed a high level of *ICL1(p):sGFP* expression (Fig. 2O and P). An *M. grisea* transformant expressing *sGFP* under the control of the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter, which gives constitutive expression in most ascomycete fungi (Punt *et al.*, 1987), showed very low levels of *sGFP* fluorescence throughout appressorium formation and invasive growth (Fig. 2G and H).

High levels of *ICL1(p):sGFP* expression were also observed when mycelial cultures were incubated with sodium acetate or olive oil as sole carbon source (Fig. 3). In contrast, no expression was observed when mycelium was grown in glucose-rich medium. In order to measure the induction of *ICL1* expression in response to acetate, an enzyme-linked immunosorbent assay (ELISA) was used to quantify GFP abundance in *M. grisea* transformants. A calibration curve was first generated using purified recombinant GFP to allow subsequent quantification of GFP within mycelial extracts of *ICL1(p):sGFP* transformants (Fig. 3E). Cultures of Guy11 and *ICL1(p):sGFP* transformant ZWI1-4 were then prepared in rich medium, and mycelium was recovered and transferred to minimal growth medium with either glucose or sodium acetate as sole carbon source. Mycelium was removed at four intervals after transfer to these growth conditions, and protein extracts were made (see *Experimental procedures*). GFP was then quantified by ELISA using a monoclonal anti-GFP antibody (Fig. 3F and G). The antibody did not show any cross-reaction with mycelial protein extracts from Guy11 (Fig. 3F). Furthermore, we observed that the *M. grisea* *ICL1* promoter responded to the presence of acetate very rapidly and led to abundant GFP production within 24 h (Fig. 3G). All the observed expression patterns were consistent with results from RNA gel blot analysis of *ICL1* expression (data not shown). We conclude that *M. grisea* *ICL1* is expressed in response to the presence of two carbon compounds as a sole carbon source, and is also highly expressed during appressorium morphogenesis and invasive growth by *M. grisea*.

ICL1 <i>M. grisea</i>	1	MASKNMVNPAVEPSMEDDLFAREVAEVKQWWSDEPRWRYTKRPFTAEOIVSKRGNL
acu3 <i>N. crassa</i>	1	MAANNMVNPAVDPALEDELFAKEVEVKQWWSDSRWRQTKRPFTAEOIVSKRGNL
acuD <i>A. nidulans</i>	1MSYIEEDQRYWDEVAVKNNWWDKSRWRYTKRPFTAEOIVSKRGNL
ICL1 <i>M. grisea</i>	56	KIEYPSNAOSKKLWKILECRFOKRDASYTYGCLEPTMVTQMAKYLDTVYVSGWQS
acu3 <i>N. crassa</i>	56	KIEYASNAOAKKLWKILEDRFAKRDASYTYGCLEPTMVTQMAKYLDTVYVSGWQS
acuD <i>A. nidulans</i>	46	KIEYPSNVQAKKLWGLELERNFNKEASFTYGCLDPTMVTQMAKYLDTVYVSGWQS
ICL1 <i>M. grisea</i>	111	SSTASSSDEPGPDLADYPYTTVPNKVSHLFMAQLFHDRKQORHERLSAPKSEERSKL
acu3 <i>N. crassa</i>	111	SSTASSSDEPGPDLADYPYTTCPNKVCHLFMAQLFHDRKQORERLSPKDOREKL
acuD <i>A. nidulans</i>	100	SSTASSIDEPSDLADYPMNTVPNKVHLLWMAQLFHDRKQORERMTLPKDQRHKV
ICL1 <i>M. grisea</i>	166	QNIDYLRPIIADADTGHGGLTAVMKLTKLFIKGAAGIHIEDQAPGTTKCGHMAG
acu3 <i>N. crassa</i>	166	ANIDYLRPIVADADTGHGGLTAVMKLTKLFIKGAAGIHIEDQAPGTTKCGHMAG
acuD <i>A. nidulans</i>	155	TNVDYLRPIIADADTGHGGLTAVMKLTKLFIKGAAGIHIEDQAPGTTKCGHMAG
ICL1 <i>M. grisea</i>	221	KVLVPISEHINRLVAIRAQADIMGVDLLAIARTDAEATLITTSIDPRDHFILG
acu3 <i>N. crassa</i>	221	KVLVPIQEHINRLVAIRAQADIMGSDLLCIARTDAEATLITTTIDPRDHFILG
acuD <i>A. nidulans</i>	210	KVLVPISEHINRLVAIRAQADIMGTDLLAIARTDSEATLITSTIDPRDHFILG
ICL1 <i>M. grisea</i>	276	CTNPSLQPLADLMTAEQSGKGTGDLQAIEDEWMAKANLKRFFDVAVDVINSSSS
acu3 <i>N. crassa</i>	276	CTNPDLEPLAHLMMKABAEKGTGAQLQAIEDDWLAKADLKRFFDEAVLDVIKAG.K
acuD <i>A. nidulans</i>	265	STNPDLOPLNDLMMWAEQAGKNGAFLQAIEDEWLAKAELKLFNDAVDVAINNS.P
ICL1 <i>M. grisea</i>	331	LRNPKDVAAYKYLQAAKGGK..SNREARAIASSLGVPEIFFDWDSPRTREGYRIRKGG
acu3 <i>N. crassa</i>	331	FSNAKDLAAYKYLQAAVKGKQISNREARAIARQLLGOEIFFDWDSPRTREGYRIRKGG
acuD <i>A. nidulans</i>	320	LPNKKAAIEKYLQOSKGGK..SNLEARAIKAEIAGTDIIFDWEAPRTREGYRIRKGG
ICL1 <i>M. grisea</i>	384	GDCCAINRAIAYAPYADAIWMESKLPDYEQAKEFAEGVHAVYPEQKLAYNLSPSF
acu3 <i>N. crassa</i>	386	GDCDSINRAISYAPYCDAIWMESKLPDYEQAEFAKGRVWVPEQKLAYNLSPSF
acuD <i>A. nidulans</i>	372	GTOCAINRAVAYAPADAIWMESKLPDYKQAKEFADGVHAVWPEQKLAYNLSPSF
ICL1 <i>M. grisea</i>	439	NWKTAMPRDEQETYIRRLAGLGYCWQFITLAGLHTTALISDRFARAYSIVGMRAY
acu3 <i>N. crassa</i>	439	NWKTAMCRDDQETYIRRLAKLGYCWQFITLAGLHTTALISDQFAKAYSIVGMRAY
acuD <i>A. nidulans</i>	427	NWKTAMPRDEQETYIRRLGALGYAWQFITLAGLHTTALISDQFAKAYAKQGMRAY
ICL1 <i>M. grisea</i>	494	GELVQEPPEMELGVDVVKHQKWSGATYVDELQKMTGGVSSTAAMGKGVTEQDFH
acu3 <i>N. crassa</i>	494	GELVQEPPEIDNGVDVVKHQKWSGATYVDELQKMTGGVSSTAAMGKGVTEQDFH
acuD <i>A. nidulans</i>	482	GELVQEPPEMANGVDVVKHQKWSGANVVDNMLKMTGGVSSTAAMGKGVTEQDFKS

Fig. 1. Predicted amino acid sequence of the *M. grisea* ICL1 gene product. Sequences were aligned using the program CLUSTALW (Thompson *et al.*, 1994). Identical amino acids are highlighted on a black background and similar amino acids on a light grey background. Gaps in the alignments are indicated by dashes. Sequences aligned were the predicted products of *M. grisea* ICL1 (GenBank accession AF540383), *Neurospora crassa* acu3 (X62697) and *Aspergillus nidulans* acuD (X62696).

Targeted gene replacement of *M. grisea* ICL1

In order to determine whether ICL1 encodes isocitrate lyase and to characterize its role in *M. grisea*, we carried out a targeted gene replacement. A 5.3 kb *Eco*R1 fragment spanning the ICL1 gene locus was selected, and a 1.8 kb *Xho*I–*Sal*I fragment containing the majority of the protein-coding sequence was removed and replaced with a hygromycin phosphotransferase gene cassette (Carroll *et al.*, 1994) bestowing resistance to hygromycin B (Fig. 4). The resulting construct was introduced into the wild-type rice pathogenic strain of *M. grisea* Guy 11 (Table 1). Transformants were selected and analysed using DNA gel blots (Fig. 4C). Three transformants were identified in which the pre-

dicted gene replacement event had taken place. In these transformants, I-7, I-10 and I-11, a gene probe derived from the protein-coding region of ICL1 failed to hybridize (Fig. 4C). In order to determine whether the glyoxylate cycle was affected in these mutants, the three $\Delta icl1$ transformants were cultured on media containing either sodium acetate or olive oil as sole carbon source, as shown in Fig. 5. In each case, $\Delta icl1$ mutants I-7, I-10 and I-11, failed to grow, whereas the wild-type Guy 11 and two ectopic transformants, I-6 and I-8, grew normally. All strains grew normally on glucose (Fig. 5). We conclude that *M. grisea* ICL1 encodes isocitrate lyase, which is involved in the proliferation of the fungus on two carbon compounds or after lipid metabolism.

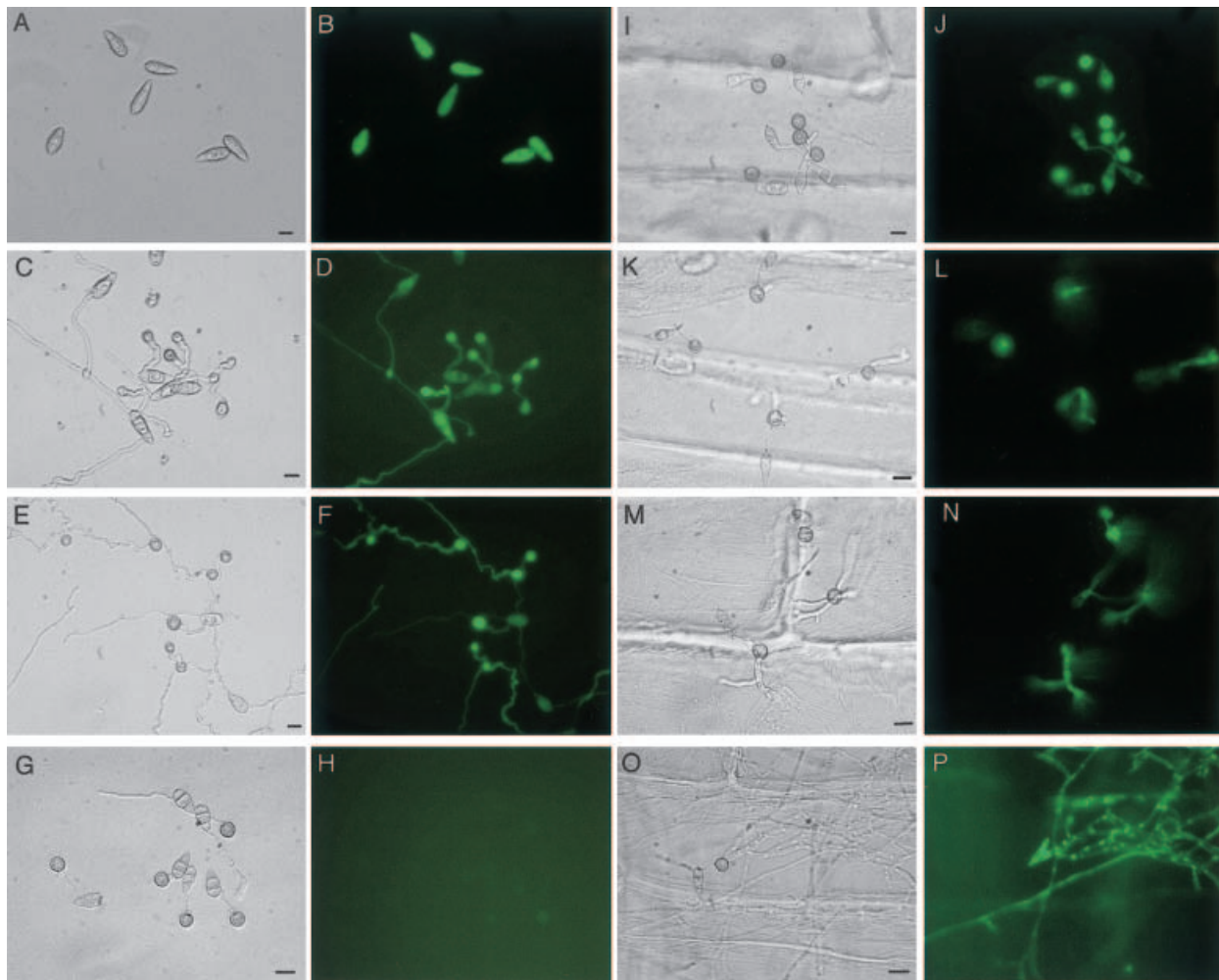


Fig. 2. Expression of *M. grisea ICL1* during conidial germination and appressorium development. A 1.5 kb promoter fragment of *ICL1* was fused to the sGFP green fluorescent protein-encoding allele (Chiu *et al.*, 1996) and introduced into *M. grisea* strain Guy 11. Transformants ZWI-2, 1-4 and 1-6, carrying single-copy integrations of the *ICL1p:sGFP* transgene, were identified, and appressorium development was analysed by epifluorescence microscopy of germinating conidia on an unyielding surface (plastic coverslips, A–H) or a yielding surface (sterile onion epidermis, I–P).

A and B. Three-celled pyriform conidia of *ICL1p:sGFP* transformant ZWI-2, before germination on plastic coverslips (0 h).

C and D. Germ tubes extending from conidia and beginning to form appressoria, 6 h after germination.

E and F. Mature appressoria elaborated from ends of germ tubes, 24 h after germination.

G and H. Control experiment in which a transformant, MJK246, expressing sGFP under control of the *A. nidulans GAPDH* promoter was allowed to undergo appressorium formation for 24 h.

I and J. Appressorium development of transformant ZWI-4 on onion epidermis, 10 h after spore germination.

K and L. Penetration peg formation of ZWI-4, 16 h after spore germination.

M and N. Invasive hyphae formation after 24 h.

O and P. Invasive, secondary hyphae forming after 48 h.

Bar for all frames = 10 μ m.

$\Delta icl1$ mutants show reduced virulence and a delay in the expression of rice blast symptoms

Targeted deletion of *ICL1* did not cause observable effects on hyphal elongation or mycelial growth. There was, however, a reduction in conidiogenesis by $\Delta icl1$ mutants of $\approx 50\%$ compared with the isogenic wild type. To assess the virulence of $\Delta icl1$ mutants, conidial suspensions of uniform concentration were sprayed onto seedlings of a

susceptible rice cultivar, CO-39, and a susceptible barley cultivar, Golden Promise, and the plants were allowed to develop blast symptoms. We observed a reduction in observable disease symptoms on all seedlings inoculated with $\Delta icl1$ mutants I-7 or I-10 compared with plants inoculated with Guy 11 or transformants I-8 or I-30, in which the gene replacement vector had integrated at an ectopic site in the genome (Fig. 6A). The mean lesion density on leaves of rice seedlings inoculated with Guy 11 was

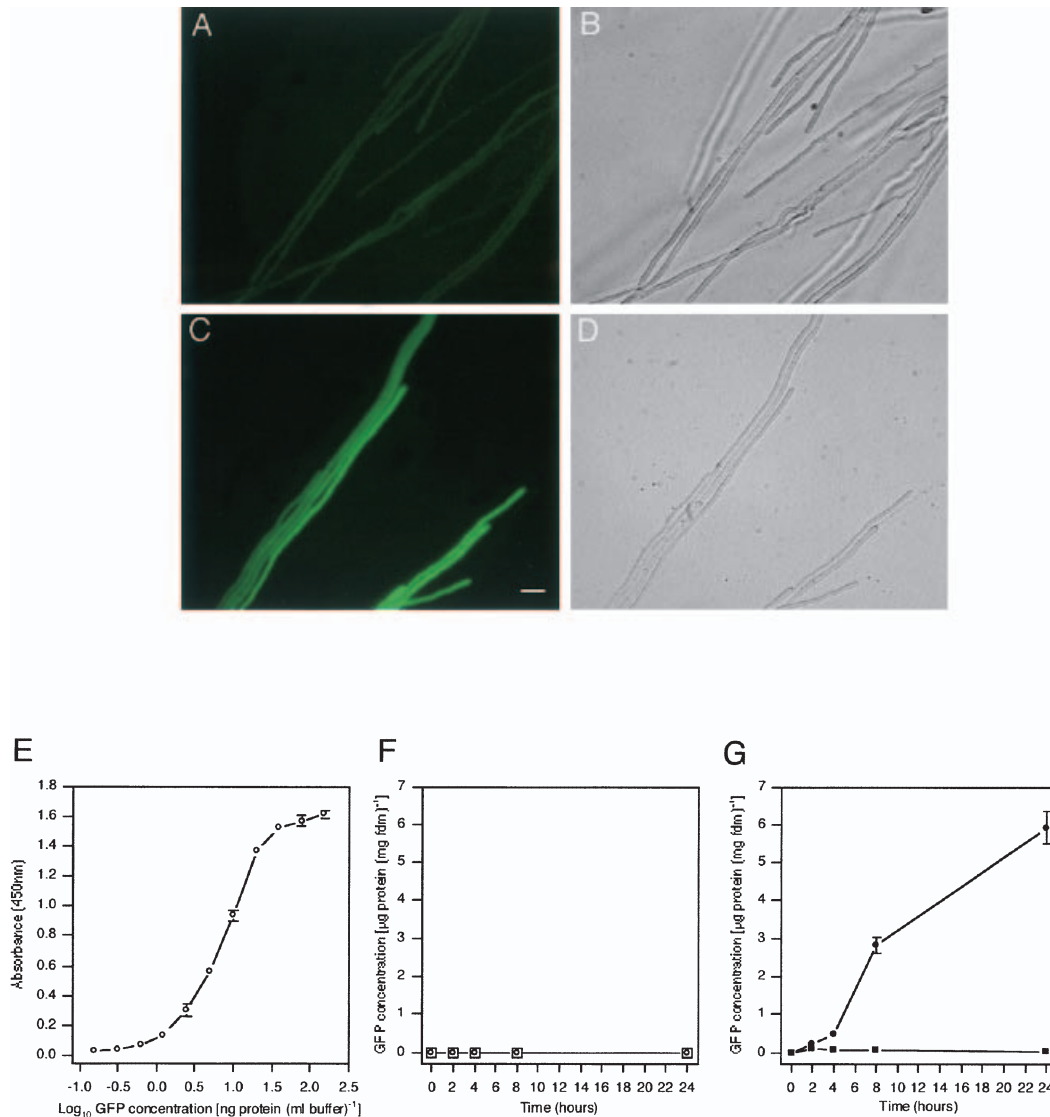


Fig. 3. Expression of *M. grisea* *ICL1* in response to the presence of sodium acetate. The *ICL1p:sGFP* transformant ZWI-4 was incubated in a rich growth medium for 48 h and then transferred to a minimal growth medium for a further 24 h with either glucose (A and B) or sodium acetate (C and D) as sole carbon source. Mycelium was isolated and visualized by epifluorescence microscopy. Bar for all frames = 10 μm. Quantitative analysis of *ICL1p:sGFP* expression was carried out using an enzyme-linked immunosorbent assay (ELISA).

E. A calibration curve was established using a monoclonal anti-GFP antibody (Clontech) and a dilution series of purified recombinant GFP. Cultures of Guy 11 and the *ICL1p:sGFP* transformant ZWI1-4 were prepared and transferred to either glucose- or sodium acetate-containing medium for 24 h (see *Experimental procedures*). The concentration of GFP in mycelial extracts per mg of freeze-dried mycelium [$\mu\text{g (mg FDM)}^{-1}$] was calculated after 0, 2, 4, 8 and 24 h.

F. Graph of GFP concentration in *M. grisea* Guy 11 after transfer of mycelium to glucose (open squares) or sodium acetate (open circles).

G. Graph of GFP concentration in *M. grisea* *ICL1p:sGFP* transformant ZWI1-4 after transfer of mycelium to sodium acetate (black diamonds) or glucose (black squares). Each data point represents the mean of three replications of the experiment. Error bar represents the standard error of the mean.

47.45 ± 3.13 compared with 22.23 ± 5.49 in seedlings inoculated with $\Delta icl1$ mutant I-11 ($P < 0.001$), with a similar reduction observed in barley infections. The most striking difference observed was in the progression of disease symptoms (Fig. 6B–E). In rice seedlings inoculated with Guy 11, rice blast disease symptoms began to appear as

early as 48 h after inoculation with spore suspensions, and they were very obvious after 72 h. In contrast, seedlings inoculated with $\Delta icl1$ mutants did not exhibit rice blast symptoms until 96 h after inoculation (Fig. 6D). To investigate the reasons why plant infection might be delayed and result in less acute disease symptoms, we measured

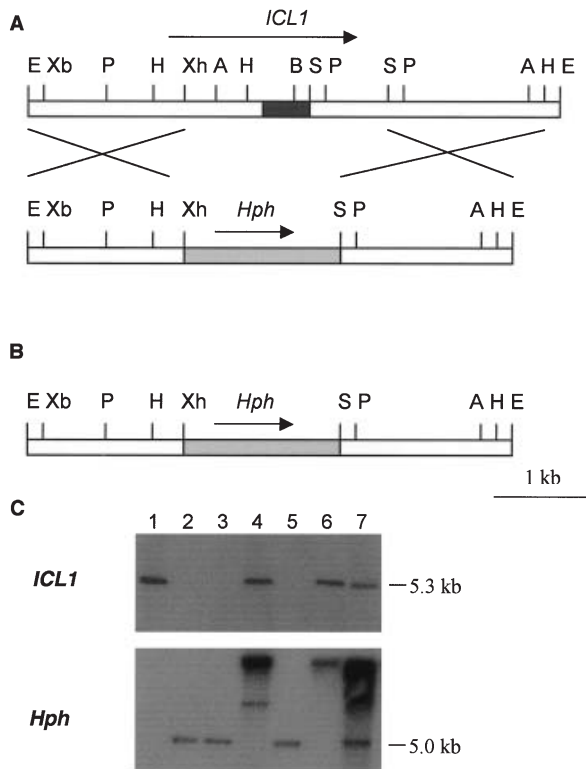


Fig. 4. Organization of the *ICL1* locus and targeted gene replacement.
 A. Restriction map of the wild-type *ICL1* locus showing orientation of the coding region (arrow).
 B. Targeted gene replacement vector pΔJF14 showing mechanism of insertion by homologous recombination and generation of null allele. A, *Apal*; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sal*I; Xh, *Xho*I.
 C. DNA gel blot analysis of *M. grisea* pΔ*icl1* transformants. DNA was digested with *Eco*RI, fractionated, blotted and probed with a 0.8 kb *Xho*I–*Sal*I fragment of *ICL1* deleted by the gene replacement or the 1.4 kb *Hph* cassette. Lane 1, Guy 11; lane 2, Δ*icl1* mutant I-11; lane 3, Δ*icl1* mutant I-10; lane 4, Δ*icl1* mutant I-8; lane 5, Δ*icl1* mutant I-7; lane 6, Δ*icl1* mutant I-5; lane 7, Δ*icl1* mutant I-1.

the ability of Δ*icl1* mutants to perform a number of virulence-associated functions, including completion of the prepenetration stage of development (see Table 2). We found that the emergence of germ tubes after conidial germination was delayed in Δ*icl1* mutants. In Guy 11, $96.19 \pm 1.06\%$ conidia had formed a germ tube 2 h after inoculation onto plastic surfaces compared with $29.06 \pm 16.25\%$ in the Δ*icl1* mutant I-10. By 4 h after inoculation, the rates of germ tube emergence were, however, very similar (Table 2). The formation of appressoria was also substantially delayed in Δ*icl1* mutants compared with Guy 11, which formed appressoria from $74.17 \pm 18.64\%$ of germ tubes within 8 h of germination, compared with only $1.67 \pm 2.89\%$ by Δ*icl1*. The rate of successful appressorium-mediated penetration of plants was determined by allowing conidia to germinate on onion epidermis and form appressoria. The emergence of a penetration hypha at the base of the appressorium and rupture of the epidermis was then assayed by light microscopy (Chida and Sisler, 1987). In Guy 11, $70.5 \pm 3.28\%$ of appressoria had successfully penetrated epidermal layers after 24 h, whereas $44.7 \pm 6.36\%$ of appressoria had completed infection in Δ*icl1* mutant I-11. Consistent with the reduction in cuticle penetration, we observed a delay in turgor generation by appressoria when assayed using an incipient cytorrhysis test. This assay measures the number of cells collapsing after exposure to solute of varying concentrations. Hyperosmotic solutions cause the cells to collapse allowing an assessment of their internal turgor. In 24-h-old appressoria, there was a significant difference in the number of appressoria that were collapsed after incubation in 1 M glycerol between Guy 11 (19.9 ± 8.6) and Δ*icl1* (30.3 ± 9.4). This indicates that appressoria of Δ*icl1* mutants do not develop turgor as rapidly as wild-type *M. grisea* (Table 2). Turgor levels after 48 h of appressorium maturation were almost identical between the Δ*icl1* mutants and Guy 11. Taken together, our results indicate

Table 1. Recombinant *M. grisea* strains used in this study.

Strain name	Genotype/description	Reference
Guy11	Wild-type rice pathogenic strain of <i>M. grisea</i> ^a	Leung <i>et al.</i> (1988)
ZW1-4	<i>ICL1</i> (p): <i>sGFP</i> : <i>ILV1</i> ^{SURb}	This study
ZW1-2	<i>ICL1</i> (p): <i>sGFP</i> : <i>ILV1</i> ^{SUR}	This study
ZW1-6	<i>ICL1</i> (p): <i>sGFP</i> : <i>ILV1</i> ^{SUR}	This study
MJK246	<i>GAPDH</i> (p): <i>sGFP</i> : <i>Hph</i> ^f	Kershaw <i>et al.</i> (1998)
I-7	Δ <i>icl1</i> :: <i>Hph</i>	This study
I-10	Δ <i>icl1</i> :: <i>Hph</i>	This study
I-11	Δ <i>icl1</i> :: <i>Hph</i>	This study
I-6, I-8, I-30	pΔ <i>ICL1</i> transformants of Guy 11 with ectopic integration of vector	This study
C-5, C-6, C-7, C-8	Δ <i>icl1</i> :: <i>Hph</i> : <i>ILV1</i> ^{SUR}	This study

a. Guy 11 was used to generate all recombinant strains used in this study.

b. Sulphonylurea-resistant allele of the *ILV1* acetolactate synthase gene of *M. grisea* used as selectable marker for transformation (Sweigard *et al.*, 1997).

c. Transformant expressing *sGFP* under the control of a constitutive promoter, the glyceraldehyde-3-phosphate dehydrogenase promoter of *A. nidulans* (Punt *et al.*, 1987).

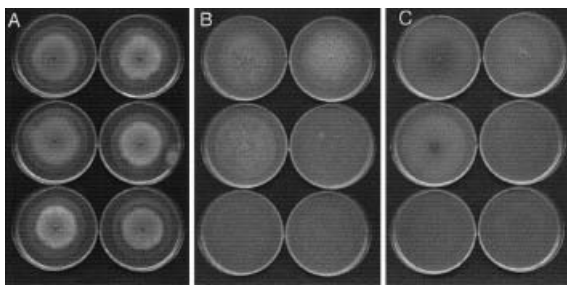


Fig. 5. *M. grisea* $\Delta icl1$ mutants are unable to use acetate or lipids as sole carbon source. Strains were cultured on minimal growth medium with (A) glucose, (B) olive oil and (C) sodium acetate. Plate cultures were grown for 10 days at 24°C. Top row plates are inoculated with Guy 11 (left) and I-8 (right); middle row plates with I-6 (left) and $\Delta icl1$ mutant I-10 (right); bottom row plates with $\Delta icl1$ mutants I-7 and I-11.

that targeted deletion of *ICL1* results in retardation and impairment of a number of virulence-associated processes in *M. grisea*.

Appressorium turgor generation in *M. grisea* is accompanied by movement of lipid droplets into developing appressoria, the coalescence of droplets and take-up by

vacuoles, followed by rapid lipolysis (Thines *et al.*, 2000). To determine whether these processes were affected in $\Delta icl1$ mutants, conidia of Guy 11 and the $\Delta icl1$ mutant I-10 were allowed to form appressoria, and Nile Red staining of triacylglycerol was carried out. We observed abundant lipid droplets in both strains, which degraded during spore germination. Movement of lipid droplets to developing appressoria occurred in the $\Delta icl1$ mutant, but was delayed compared with Guy 11 (Fig. 7). The degradation of lipid was also somewhat delayed. By 8 h after germination, appressoria of the $\Delta icl1$ mutant I-10 possessed abundant lipid bodies, which had already begun to be degraded in Guy 11 (Fig. 7). By 24 h, however, lipid was depleted in both Guy 11 and I-10. We conclude that the absence of the *ICL1* gene delays appressorium maturation and turgor generation and may contribute to the reduction and delay in disease symptom expression by $\Delta icl1$ mutants.

Reintroduction of ICL1 into $\Delta icl1$ mutants restores virulence

To ensure that the phenotypic differences observed in

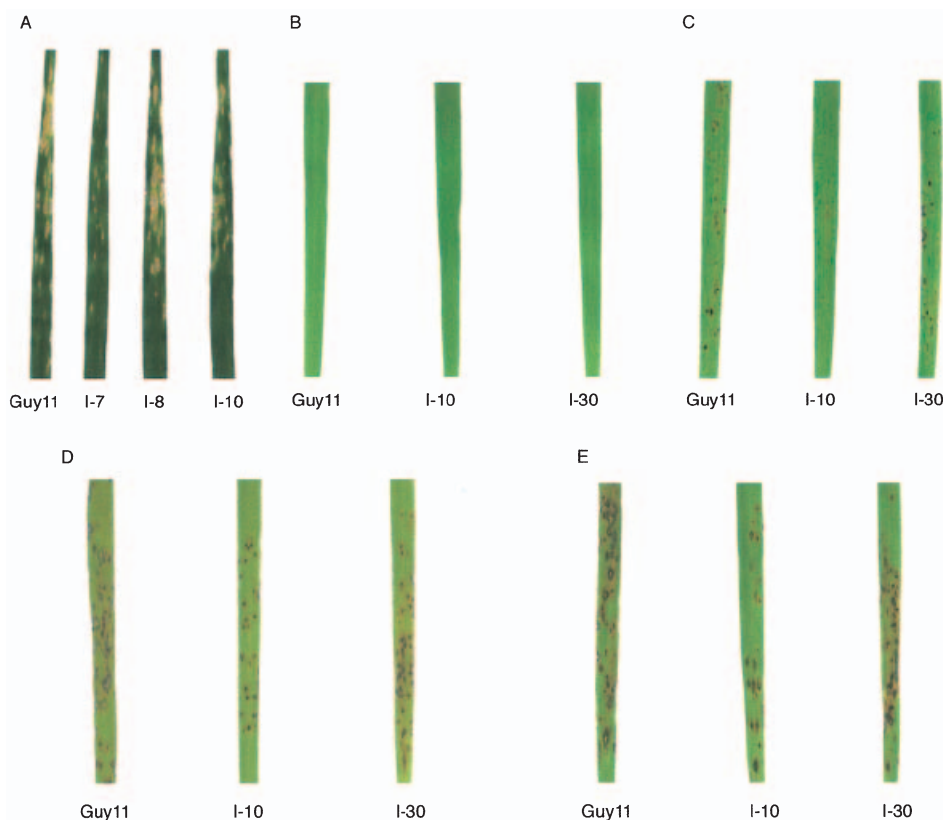


Fig. 6. Rice blast symptoms produced by $\Delta icl1$ mutants of *M. grisea*.

A. Leaves from barley plants of cultivar Golden Promise that were inoculated with suspensions of conidia from the strains indicated at a concentration of 10^4 conidia ml^{-1} . Disease symptoms were allowed to develop for 120 h.

B. Leaves from rice plants of cultivar CO-39 that were inoculated with suspensions of conidia from the strains indicated at a concentration of 10^4 conidia ml^{-1} . Rice blast symptoms were allowed to develop for 48 h, (C) 72 h, (D) 96 h and (E) 120 h.

Table 2. Phenotypic analysis of *Δicl1* mutants of *M. grisea*.

<i>M. grisea</i> strain	Virulence-associated function				
	Conidial germination ^a				
	2 h		4 h		
Guy 11	96.19 ± 1.06% ^a		98.75 ± 0.94% ^a		
I-30	94.28 ± 0.25% ^a		97.53 ± 1.32% ^a		
<i>Δicl1</i> I-10	29.06 ± 16.25% ^b		91.86 ± 3.31% ^b		
	Appressorium formation ^b				
	6 h		8 h	12 h	24 h
Guy 11	16.42 ± 7.20% ^a		58.53 ± 5.40% ^a	95.79 ± 0.46% ^a	97.67 ± 2.00% ^a
I-30	26.33 ± 9.33% ^a		74.17 ± 18.64% ^a	94.58 ± 0.92% ^a	98.00 ± 0.88% ^a
<i>Δicl1</i> I-10	0.00 ± 0.00% ^b		1.67 ± 2.89% ^b	10.17 ± 2.50% ^b	29.47 ± 32.79% ^b
	Appressorium turgor generation (24 h) ^c				
	0.5 M glycerol	1 M glycerol	2 M glycerol	3 M glycerol	4 M glycerol
Guy 11	5.4 ± 2.0% ^a	19.9 ± 8.6% ^a	73.9 ± 15.4% ^a	91.1 ± 7.3% ^a	96.8 ± 4.2% ^a
I-30	5.6 ± 2.2% ^a	20.1 ± 7.9% ^a	70 ± 21.8% ^a	88.7 ± 8.5% ^a	97.0 ± 2.3% ^a
<i>Δicl1</i> I-10	17.9 ± 7.8% ^b	30.3 ± 9.4% ^b	71.9 ± 19.0% ^a	92.8 ± 4.8% ^a	98.2 ± 2.2% ^a
	Appressorium turgor generation (48 h) ^c				
	0.5 M glycerol	1 M glycerol	2 M glycerol	3 M glycerol	4 M glycerol
Guy 11	3.0 ± 1% ^a	9.1 ± 1.3% ^a	63.8 ± 9.9% ^a	91.0 ± 4.7% ^a	97.2 ± 2.3% ^a
I-30	2.0 ± 1.0% ^a	5.6 ± 2.2% ^a	65.0 ± 8.8% ^a	86.1 ± 2.1% ^a	95.8 ± 1.0% ^a
<i>Δicl1</i> I-10	6.7 ± 3.1% ^b	13.3 ± 6.6% ^a	67.5 ± 8.6% ^a	89.4 ± 5.1% ^a	97.6 ± 1.3% ^a
	Appressorium-mediated cuticle penetration ^d				
Guy 11	70.5 ± 3.3% ^a				
I-30	69.8 ± 6.2% ^a				
<i>Δicl1</i> I-10	45.3 ± 3.2% ^b				
<i>Δicl1</i> I-7	44.8 ± 3.9% ^b				
<i>Δicl1</i> I-11	44.7 ± 6.4% ^b				

a. The percentage of conidia that had elaborated a germ tube after incubation in water on plastic coverslips after 4 h or 6 h was recorded ($n = 300$). For all tests, a different letter in the same column indicates that the difference is significant at $P < 0.05$.

b. The percentage of conidia that had formed an appressorium after incubation on hydrophobic plastic surfaces for the times indicated was recorded ($n = 300$).

c. Turgor generation was assessed by incipient cytorrhysis (Howard *et al.*, 1991). The percentage of appressoria that had collapsed after 10 min in glycerol solution was recorded ($n = 300$).

d. The percentage of appressoria that had formed a penetration hypha and ruptured an onion epidermal layer was recorded after 24 h ($n = 300$).

Δicl1 mutants were all associated with the gene replacement event, we reintroduced the *ICL1* gene as a 5.3 kb *EcoRI* fragment carried on a plasmid vector with a gene cassette bestowing sulphonylurea resistance (Carroll *et al.*, 1994). We selected transformants and ensured that they carried a single-copy integration of the *ICL1* gene by DNA gel blot analysis (data not shown). *Δicl1:ICL1* transformants were all able to grow on sodium acetate as sole carbon source and exhibited full virulence, as shown in Fig. 8. All other virulence-associated phenotypes in *Δicl1* mutants were complemented by reintroduction of the gene (data not shown). We conclude that *ICL1* is necessary for full virulence in *M. grisea*.

Discussion

The glyoxylate cycle provides a means for cells to assimilate two-carbon compounds into the tricarboxylic acid cycle (TCA cycle) and channel these via gluconeogenesis to generate glucose. Normally, induction of the glyoxylate cycle indicates that a cell is using lipid metabolism as its predominant source for ATP generation, involving β -oxidation of fatty acids and the production of acetyl CoA.

The acetyl CoA is then channelled through the glyoxylate cycle via isocitrate lyase-mediated production of glyoxylate and the production of malate by malate synthase. Here, we have shown that the rice blast fungus *M. grisea* requires the glyoxylate cycle enzyme isocitrate lyase in order to be fully virulent and produce acute rice blast symptoms.

We observed that *Δicl1* mutants were less virulent than an isogenic wild-type strain of *M. grisea* and were impaired in virulence-associated functions such as germ tube emergence, appressorium development and cuticle penetration. Significantly, all these affected functions are associated with the prepenetration stage of development before the fungus has gained access to nutrients from the host plant. *Δicl1* mutants obviously retain the capacity to cause disease because they appear to be unaffected in their ability to invade and proliferate normally in plant tissue. The virulence defect results instead from a delay in completing the prepenetration stages of development, leading to a lower frequency of appressorium-mediated infection events.

During conidial germination and appressorium formation in *M. grisea*, which occur in a nutrient-free environment, lipid bodies are known to be mobilized from the

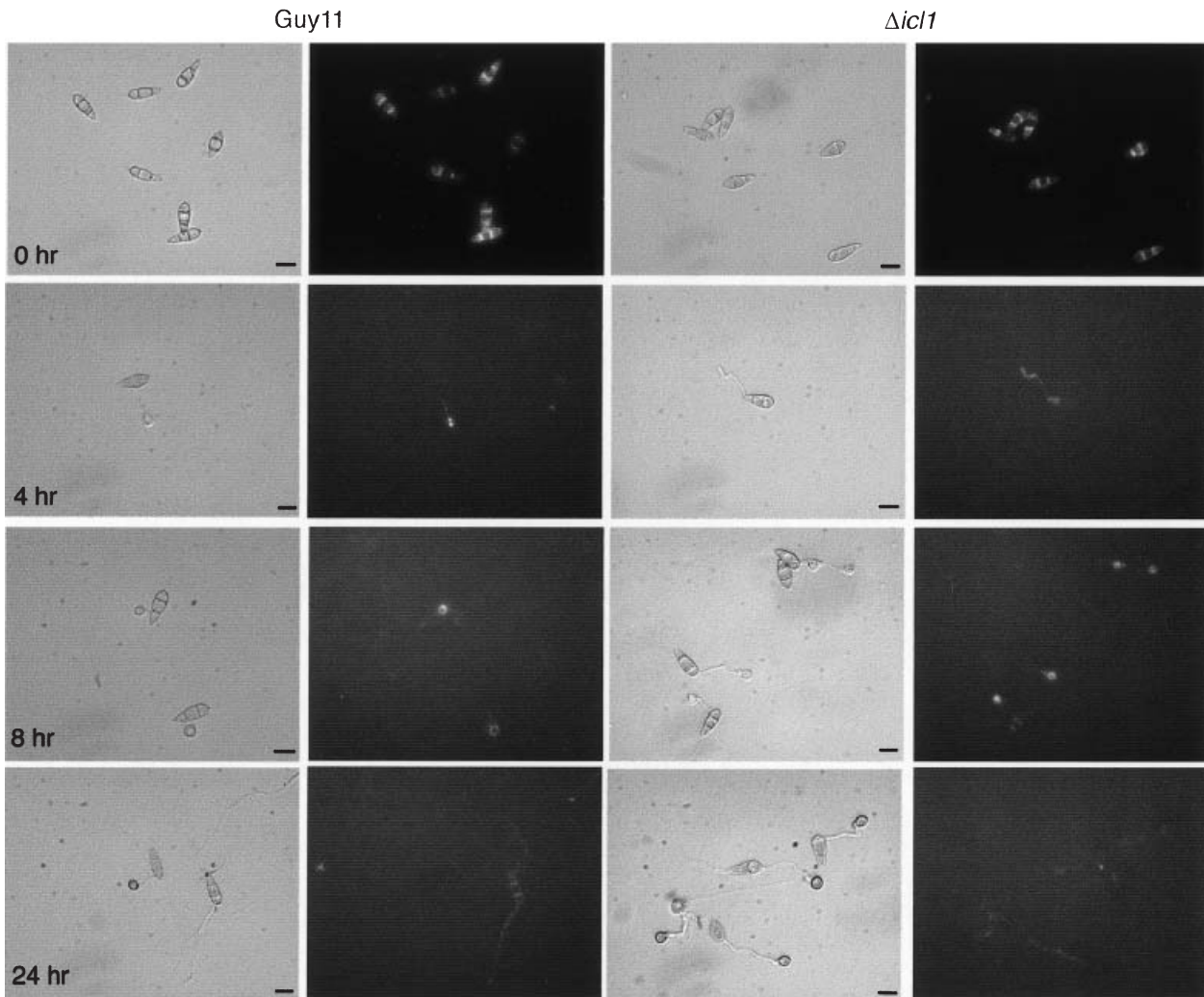


Fig. 7. Cellular distribution of lipid droplets during appressorium morphogenesis by *M. grisea*. Conidia of Guy 11 and $\Delta icl1$ mutant I-10 were incubated in water drops on plastic coverslips and allowed to form appressoria. Samples were removed at 0 h, 4 h, 8 h and 24 h and stained with Nile red for the presence of triacylglycerol. Lipid bodies were visualized by epifluorescence microscopy (right). Lipid bodies migrated to the tips of germ tubes and coalesced within appressoria before disappearing during the onset of turgor generation. The $\Delta icl1$ mutants showed a delay in lipid mobilization from the conidium.

conidium to the germ tube apex (Thines *et al.*, 2000). At the onset of turgor generation, lipid bodies coalesce and are taken up by vacuoles, before rapid lipolysis. Thus, *M. grisea* uses lipid metabolism extensively during appressorium formation. Lipid metabolism may contribute to ATP generation and fuel secondary metabolic pathways, such as melanin biosynthesis, which are critical for appressorium function. However, it also seems likely that lipolysis is important for turgor generation in appressoria via the synthesis of glycerol, which accumulates to very high concentrations in appressoria (de Jong *et al.*, 1997; Thines *et al.*, 2000). A consequence of reliance upon lipid metabolism for turgor generation during the prepenetration stage of development may be induction of the glyoxylate cycle to provide a mechanism of generating glucose.

High-level expression of *ICL1* during conidial germination and appressorium formation indicates that the glyoxylate cycle is stimulated at this time. Significantly, *ICL1* is also highly expressed during penetration peg formation and during the generation of invasive hyphae. The initial stages of plant infection, before glucose acquisition from plant tissue, may therefore be a stage at which lipid metabolism is very important for the proliferation of *M. grisea*.

Recently, it has been shown that *Candida albicans*, the most serious human pathogenic fungus, requires isocitrate lyase in order to be fully virulent in a mouse model of candidiasis (Lorenz and Fink, 2001). A similar requirement was also observed in the pulmonary bacterium *Mycobacterium tuberculosis*, in which isocitrate

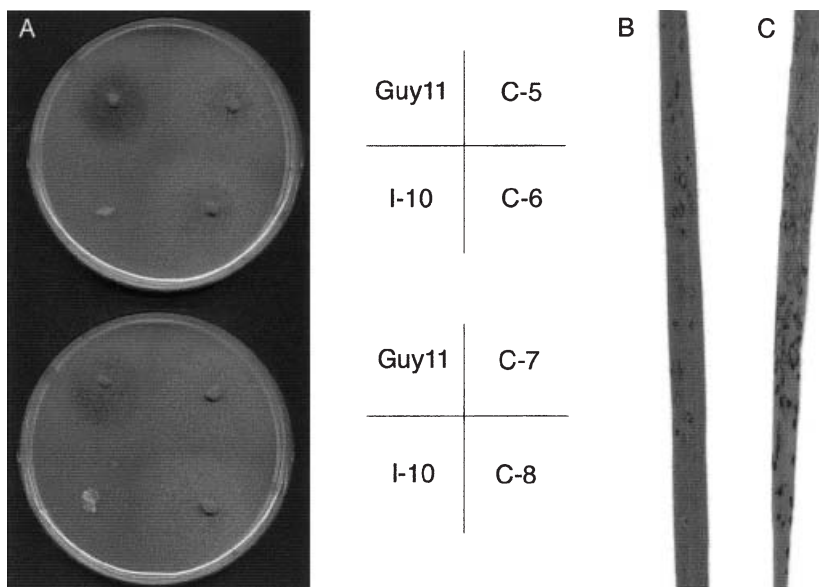


Fig. 8. Complementation of $\Delta icl1$ mutants of *M. grisea* by reintroduction of *ICL1*.

A. *M. grisea* strains were cultured on minimal medium with sodium acetate as sole carbon source.

B. Leaf from rice plant of cultivar CO-39 that was inoculated with a suspension of conidia from $\Delta icl1$ mutant I-10 at a concentration of 10^4 conidia ml^{-1} .

C. Leaf from rice plant inoculated with a conidial suspension of $\Delta icl1::Hph:ICL1:ILV^{SUR}$ transformant C-6 at the same concentration.

lyase mutants show attenuation in virulence (McKinney *et al.*, 2000). Our observation highlights the potential similarities in the metabolic processes required for virulence in diverse microbial pathogens. Clearly, both *M. grisea* and *C. albicans* have co-opted the glyoxylate cycle into fulfilling a specific role in pathogenesis in response to a glucose-deficient environment, the leaf surface in the case of *M. grisea*, and the phagocytic macrophage invaded by *C. albicans*. Isocitrate lyase is also highly expressed during infection by the human pathogenic fungus *Cryptococcus neoformans* (although dispensable for pathogenesis), and to support growth of the intracellular pathogenic bacterium *Rhodococcus equi* on lipids, which it uses in the phagolysosome (Kelly *et al.*, 2002; Rude *et al.*, 2002).

Foliar pathogens of plants need a means of germinating and developing on the leaf surface in the absence of external nutrients, and many species carry out morphogenetic programmes resulting in the development of specific infection structures in this environment. The glyoxylate cycle, which provides a means of coupling lipid and carbon metabolism, may be fundamental to allowing these development to proceed before host invasion. A requirement for *ICL* in virulence has been observed very recently based on the results of an insertional mutant screen in the brassica pathogen *Leptosphaeria maculans*, in which a virulence mutant was found to carry an insertion in an *ICL*-encoding gene (Idnurm and Howlett, 2002). This indicates the potential widespread utilization of this pathway during plant infection by diverse pathogenic fungi and the potential for developing the glyoxylate pathway as a target for disease intervention.

Experimental procedures

Fungal isolates and culture conditions

The wild-type rice pathogenic *M. grisea* strain Guy 11 was used throughout this study. Media composition, fungal growth, nucleic acid extraction and DNA-mediated transformation were all carried out as described previously (Talbot *et al.*, 1993). Restriction digests, gel electrophoresis and DNA gel blot hybridizations were all carried out using standard procedures (Sambrook *et al.*, 1989).

Identification and targeted gene replacement of *M. grisea* *ICL1*

Genomic DNA from *M. grisea* Guy 11 was used for polymerase chain reaction (PCR) amplification with the primers ZW-1 (5'-ACTACCTGCGACCCATTATTGC-3') and ZW-2 (5'-GAGAAGGTCGACACCTGAGTC-3'), designed based on an available *M. grisea* EST sequence (Rauyaree *et al.*, 2001; Soanes *et al.*, 2002). The following PCR conditions were used: 30 cycles of denaturation at 94°C for 10 s, annealing at 58°C for 30 s and extension at 72°C for 3 min. An initial denaturation step of 3 min at 94°C and a final elongation step at 72°C for 10 min were performed. The amplicon was cloned and sequenced and used to identify corresponding genomic and cDNA clones (Talbot *et al.*, 1993). For gene replacement, a 5.3 kb *EcoRI* fragment spanning *ICL1* was cloned into a modified pBluescript vector (Stratagene) from which *XhoI* and *SalI* sites had been removed from the multiple cloning site. The resulting plasmid, pZWICL1, was digested with *XhoI* and *SalI* releasing a 1.65 kb fragment containing the *ICL1* protein-coding sequence. The linearized plasmid was ligated to a 1.4 kb *XhoI*-*SalI*-linked hygromycin phosphotransferase gene cassette to create the gene replacement vector p Δ ICL1. The *EcoRI* insert was liberated and transformed into *M.*

grisea strain Guy 11. For construction of the *ICL1p:sGFP* expression vector, a 1.5 kb promoter fragment from the 5' end of *ICL1* was amplified from pZWICL1 with the primers T3 (5'-AAT TAA CCC TCA CTA AAG GG-3') and ICL1-N (5'-CCA TGT TTT TAG AAG CCA TGG CAC-3'), which introduced an *NcoI* site at the 3' end to allow fusion of the promoter fragment to the start codon of *sGFP*. The amplified DNA fragment was digested with *EcoRI* and *NcoI* and cloned into pMJK80, which contains the *sGFP* allele (Chiu *et al.*, 1996; Kershaw *et al.*, 1998) and *A. nidulans trpC* terminator (Punt *et al.*, 1987). The resulting *ICL1(p):sGFP* fusion was excised as a *EcoRI-XhoI* fragment and cloned into an *M. grisea* transformation vector pCB1532 (Sweigard *et al.*, 1997). For complementation of $\Delta icl1$ mutants, a 5.3 kb *EcoRI* fragment spanning the *ICL1* locus was cloned from pZWICL1 into pCB1532, which carries a selectable marker bestowing resistance to sulphonylurea (Sweigard *et al.*, 1997). The resulting plasmid, pICL1532, was introduced into *M. grisea* $\Delta icl1$ mutant I-10, and transformants were selected on 150 $\mu\text{g ml}^{-1}$ chlorimuron ethyl. For all *M. grisea* transformants, DNA gel blot analysis was carried out to ensure single-copy integration of plasmid DNA. All strains used in the study are given in Table 1.

Quantification of *ICL1p:sGFP* expression by ELISA

Magnaporthe grisea mycelium from Guy 11 and *ICL1(p):sGFP* transformants ZWI1-2, 1-4 and 1-6 was grown in complete medium (Talbot *et al.*, 1993) for 48 h. Mycelium was then removed by filtration and transferred to minimal medium (Talbot *et al.*, 1993) containing glucose or sodium acetate (6 mM) as sole carbon source. Replicate samples of mycelium were removed by filtration through Miracloth (Calbiochem), washed thoroughly with dH_2O , snap frozen in liquid nitrogen and lyophilized. Samples (1 mg) of freeze-dried mycelium (FDM) were placed in Eppendorf tubes, and proteins were extracted by maceration in 1 ml of bicarbonate buffer (pH 9.6) using a hand-held homogenizer. Debris was pelleted by centrifugation at 12 000 *g* for 5 min, and 100 μl of supernatant containing solubilized proteins was transferred to wells of Immulon II HB microtitre plates (95029370; Thermo Labsystems). Extracts were double diluted into 50 μl volume of fresh buffer, and the proteins were immobilized by incubating the plates for 16 h at 4°C in sealed plastic bags. The wells were washed four times with PBST [PBS + 0.05% (v/v) Tween 20 (polyoxyethylene-sorbitan monolaurate); P-1379; Sigma] and once each with PBS and dH_2O and air dried at 23°C in a laminar flow hood. A standard calibration curve of recombinant GFP protein (8360-2; Clontech) was prepared in bicarbonate buffer in microtitre wells as described. For enzyme-linked immunosorbent assays, wells containing immobilized GFP were incubated with mouse anti-GFP monoclonal antibody (mAb) ascites fluid (8371-1; Clontech), diluted 1:2500 in PBST for 1 h followed by goat anti-mouse polyvalent (immunoglobulin classes IgG, IgA and IgM) peroxidase conjugate (A-0412; Sigma) diluted 1:1000 for a further hour. Bound antibody was visualized by incubating wells with tetramethyl benzidine (TMB) substrate solution for 30 min, reactions were stopped by the addition of 3 M H_2SO_4 , and absorbance values were determined at 450 nm with an MRX automated microplate reader (Dynex Technologies).

Wells were given four 5 min rinses with PBST between incubations. Working volumes were 50 μl per well, and incubation steps were performed at 23°C in sealed plastic bags. Using the calibration curve, absorbance values obtained from ELISA of FDM extracts were converted into equivalents of GFP (expressed as μg of protein mg^{-1} FDM). Appropriate absorbance values for conversion to GFP equivalents were determined from each dilution series of extract and concentrations corrected according to dilution factor. This eliminated bias resulting from steric hindrance in ELISA (Thornton, 2001). Each value was converted independently, and the mean was then calculated for each set of replicate values for each treatment at each sampling time.

Phenotypic analysis of $\Delta icl1$ mutants

Vegetative growth was assessed by measurement of colony diameter on plate cultures of *M. grisea* grown on complete medium (Talbot *et al.*, 1993). Conidial development was assessed by harvesting conidia from the surface of 12-day-old plate cultures and determining the concentration of the resulting conidial suspension using a haemocytometer (Corning). To investigate the utilization of carbon sources by $\Delta icl1$ mutants, growth was measured after 12 days on minimal medium agar with 6 mM glucose, sodium acetate or 2 g l^{-1} olive oil as sole carbon source. Appressorium development was assessed by allowing conidia to germinate on hydrophobic plastic coverslips and incubating them in a humid environment for 24 h at 24°C. The frequency of conidial germination and appressorium formation was determined at different time intervals by counting the number of germ tubes and/or appressoria formed from 300 conidia. Each test was repeated three times. Appressorium turgor was estimated using an adaptation of the incipient cytorrhysis (cell collapse) assay (Howard *et al.*, 1991; Dixon *et al.*, 1999). Appressoria were allowed to form on plastic coverslips incubated in a humid chamber for 24 or 48 h, and the surface water was removed carefully with a pipette and replaced with an equal volume of glycerol solution varying in concentration from 0.5 to 5.0 M. The number of appressoria that had collapsed after 10 min was recorded microscopically. The experiments were replicated three times. Lipid mobilization during appressorium development was assessed by Nile red staining, as described previously (Thines *et al.*, 2000). Cuticle penetration was assessed by recording the frequency of penetration peg formation from appressoria on onion epidermis. A 50 μl drop of conidial suspension at a concentration of 1×10^4 conidia ml^{-1} was placed on the surface of onion epidermis and incubated in a humid environment at 24°C for 24 h. The frequency of cuticle penetration was determined microscopically by counting the penetration events from 100 appressoria. The experiments were repeated three times.

Plant infections

Plant infections were performed using 14-day-old seedlings of rice cultivar CO-39 or 10-day-old seedlings of barley cultivar Golden Promise, both of which are very susceptible to blast disease. An *M. grisea* conidial suspension containing 10^4 conidia ml^{-1} was prepared in 0.2% (v/v) gelatin solution.

The suspension was sprayed evenly onto rice plants as described previously (Talbot *et al.*, 1993), and seedlings were incubated in a controlled environment chamber until disease symptoms appeared. Disease lesion densities were recorded from 20 to 30 infected leaves using a 5 cm section of each leaf. Infection assays were carried out three times using 45 plants per assay.

Acknowledgements

This work was funded by a British Council academic links with China award to N.J.T. and L.D., and by a grant (9/P17477) from the Biotechnology and Biological Sciences Research Council to N.J.T.

References

- Carroll, A.M., Sweigard, J.A., and Valent, B. (1994) Improved vectors for selecting resistance to hygromycin. *Fungal Genet Newsl* **41**: 22.
- Chida, T., and Sisler, H.D. (1987) Restoration of appressorial penetration ability by melanin precursors in *Pyricularia oryzae* treated with antipenetrants and in melanin-deficient mutants. *J Pest Sci* **12**: 49–55.
- Chiu, W.L., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H., and Sheen, J. (1996) Engineered GFP as a vital reporter in plants. *Curr Biol* **6**: 325–330.
- Dixon, K.P., Xu, J.-R., Smirnov, N., and Talbot, N.J. (1999) Independent signalling pathways regulate cellular turgor during hyperosmotic stress and appressorium-mediated plant infection by *Magnaporthe grisea*. *Plant Cell* **10**: 2045–2058.
- Gainey, L.D.S., Connerton, I.F., Lewis, E.H., Turner, G., and Balance, D.J. (1992) Characterisation of the glyoxysomal isocitrate lyase genes of *Aspergillus nidulans* (*acuD*) and *Neurospora crassa* (*acu3*). *Curr Genet* **21**: 43–47.
- Howard, R.J., and Valent, B. (1996) Breaking and entering – host penetration by the fungal rice blast pathogen *Magnaporthe grisea*. *Annu Rev Microbiol* **50**: 491–512.
- Howard, R.J., Ferrari, M.A., Roach, D.H., and Money, N.P. (1991) Penetration of hard substrates by a fungus employing enormous turgor pressures. *Proc Natl Acad Sci USA* **88**: 11281–11284.
- Idnurm, A., and Howlett, B.J. (2002) Isocitrate lyase is essential for pathogenicity of the fungus *Leptosphaeria maculans* to canola (*Brassica napus*). *Eukaryotic Cell* **1**: 719–724.
- de Jong, J.C., McCormack, B.J., Smirnov, N., and Talbot, N.J. (1997) Glycerol generates turgor in rice blast. *Nature* **389**: 244–245.
- Kelly, B.G., Wall, D.M., Boland, C.A., and Meijer, W.G. (2002) Isocitrate lyase of the facultative intracellular pathogen *Rhodococcus equi*. *Microbiology* **148**: 793–798.
- Kershaw, M.J., Wakley, G., and Talbot, N.J. (1998) Complementation of the *Mpg1* mutant phenotype in *Magnaporthe grisea* reveals functional relationships between fungal hydrophobins. *EMBO J* **17**: 3838–3849.
- Leung, H., Borromeo, E.S., Bernardo, M.A., and Notteghem, J.L. (1988) Genetic analysis of virulence in the rice blast fungus *Magnaporthe grisea*. *Phytopathol* **78**: 1227–1233.
- Lorenz, M.C., and Fink, G.R. (2001) The glyoxylate cycle is required for fungal virulence. *Nature* **412**: 83–86.
- McKinney, J.D., Honer zu Bentrup, K., Munoz-Elias, E.J., Miczak, A., Chen, B., Chan, W.T., *et al.* (2000) Persistence of *Mycobacterium tuberculosis*. macrophages required the glyoxylate shunt enzyme isocitrate lyase. *Nature* **406**: 735–738.
- Punt, P.J., Oliver, R.P., Dingemans, M.A., Pouwels, P.H., and Van den Hondel, C.A.M.J.J. (1987) Transformation of *Aspergillus* based on the Hygromycin-B resistance marker from *Escherichia coli*. *Gene* **56**: 117–124.
- Rauyaree, P., Choi, W., Fang, E., Blackmon, B., and Dean, R.A. (2001) Genes expressed during early stages of rice infection with the rice blast fungus *Magnaporthe grisea*. *Mol Plant Pathol* **2**: 347–354.
- Rude, T.H., Toffaletti, D.L., Cox, G.M., and Perfect, J.R. (2002) Relationship of the glyoxylate pathway to the pathogenesis of *Cryptococcus neoformans*. *Infect Immun* **70**: 5684–5694.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Soanes, D.M., Skinner, W., Keon, J., Hargreaves, J., and Talbot, N.J. (2002) Genomics of phytopathogenic fungi and the development of bioinformatic resources. *Mol Plant-Microbe Interact* **15**: 421–427.
- Sweigard, J.A., Carroll, A.M., Farrall, L., and Valent, B. (1997) A series of vectors for fungal transformation. *Fungal Genet Newsl* **44**: 52–53.
- Talbot, N.J., and Foster, A.J. (2001) Genetics and genomics of the rice blast fungus *Magnaporthe grisea*: developing an experimental model for understanding fungal diseases of cereals. *Adv Bot Res* **34**: 263–287.
- Talbot, N.J., Ebbole, D.J., and Hamer, J.E. (1993) Identification and characterisation of *MPG1* a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. *Plant Cell* **5**: 1575–1590.
- Thines, E., Weber, R.W.S., and Talbot, N.J. (2000) MAP kinase and protein kinase A-dependent mobilisation of triacylglycerol and glycogen during appressorium turgor generation by *Magnaporthe grisea*. *Plant Cell* **12**: 1703–1718.
- Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673–4680.
- Thornton, C.R. (2001) Immunological methods for fungi. In *Molecular and Cellular Biology of Filamentous Fungi: a Practical Approach*. Talbot, N.J. (ed.). Oxford: Oxford University Press, pp. 227–257.
- Tucker, S.L., and Talbot, N.J. (2001) Surface attachment and pre-penetration stage development by plant pathogenic fungi. *Annu Rev Phytopathol* **39**: 385–417.