

Peroxisomal carnitine acetyl transferase is required for elaboration of penetration hyphae during plant infection by *Magnaporthe grisea*

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Summary

Magnaporthe grisea, the causal agent of rice blast disease, invades plant tissue due to the action of specialized infection structures called appressoria, which are used to breach the leaf cuticle and allow development of intracellular, infectious hyphae. In this report we demonstrate that peroxisomal carnitine acetyl transferase (CAT) activity is necessary for appressorium function, and in particular, for the elaboration of primary penetration hyphae. The major CAT activity in *M. grisea* is encoded by the *PTH2* gene, which shows elevated expression in response to acetate and lipid, and is regulated by the cyclic AMP response pathway. Furthermore, a Pth2–GFP fusion protein colocalizes with a peroxisomal marker protein. Targeted deletion of *PTH2*, generated mutants that were completely non-pathogenic, lacked CAT activity and were unable to utilize a range of lipid substrates. The impairment of appressorium function in $\Delta pth2$ was associated with a delay in lipid reserve mobilization from germ tubes into developing infection cells, and abnormal chitin distribution in infection structures. Addition of glucose to $\Delta pth2$ mutants partially restored the ability to cause rice blast disease and lipid reserve mobilization. Taken together, our findings provide evidence that Pth2 plays a role in the generation of acetyl CoA pools necessary for appressorium function and rapid elaboration of penetration hyphae during host infection.

Introduction

In order to cause diseases in plants, pathogenic microorganisms have to traverse the plant cuticle to gain entry to

underlying tissue. Pathogenic fungi have evolved the ability to breach plant cuticles using either a battery of coordinately regulated cell wall-degrading enzymes (Tonukari *et al.*, 2000), or by generating enormous invasive forces by means of specialized structures known as appressoria (Bechinger *et al.*, 1999; Tucker and Talbot, 2001). The blast fungus, *Magnaporthe grisea* causes the most serious disease of cultivated rice and uses an appressorium to break through the tough outer cuticle of a rice leaf (Dean, 1997; Talbot, 2003). The *M. grisea* appressorium is a dome-shaped cell, which differentiates from the end of a fungal germ tube soon after a spore has germinated on the leaf surface (Bourett and Howard, 1990). The appressorium is bounded by a melanin-rich cell wall and accumulates glycerol to very high concentrations, allowing generation of hydrostatic turgor and production of a substantial invasive force that is required to rupture the leaf cuticle (Howard *et al.*, 1991; Howard and Valent, 1996; de Jong *et al.*, 1997). A narrow penetration hypha is then formed at the base of the appressorium and enters a rice epidermal cell by invagination of the plasma-lemma (Bourett and Howard, 1990; Heath *et al.*, 1990). Epidermal cells subsequently become filled with invasive hyphae and the fungus soon proliferates throughout the rice leaf, ultimately killing plant cells and producing the characteristic necrotic lesions associated with rice blast disease.

Initial development of the rice blast fungus occurs on the leaf surface in the absence of external nutrients. Understanding the physiology of appressorium function and plant infection therefore requires a knowledge of how storage products in the spore are mobilized and metabolized, to bring about formation of an appressorium and successful plant infection. Previously, it was reported that *M. grisea* spores mobilize lipid bodies to the developing appressorium under control of the Pmk1 MAP kinase pathway and that rapid lipolysis occurs within the mature infection cell at the onset of turgor generation (Thines *et al.*, 2000). Consistent with this idea, triacylglycerol lipase activity has been measured in mature *M. grisea* appressoria and is regulated by the cAMP response pathway (Thines *et al.*, 2000; Weber *et al.*, 2001). Mutants lacking cAMP-dependent protein kinase A are non-pathogenic because they form misshapen appressoria that do not degrade lipid and are

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non-functional (Xu *et al.*, 1997; Thines *et al.*, 2000). Furthermore, recent evidence has shown that isocitrate lyase activity is required for efficient appressorium function, indicating a role for the glyoxylate cycle in plant infection by *M. grisea* (Wang *et al.*, 2003).

An important consequence of lipolysis and fatty acid β -oxidation in the appressorium is the generation of acetyl CoA, which must be transported to distinct cellular compartments for subsequent utilization. The transfer of acetyl CoA across intracellular membranes is catalysed by carnitine acetyl transferases (CATs), which convert acetyl CoA units to acetyl-carnitine for transport across the membrane (Elgersma *et al.*, 1995; van Roermund *et al.*, 1995). CATs are a member of the carnitine acyltransferase family that also comprise the carnitine palmitoyltransferases and carnitine octanoyltransferases and are highly conserved throughout eukaryotes (Schulz, 1991). Interestingly, a forward genetic screen for pathogenicity mutants of *M. grisea* previously identified a gene, *PTH2*, as potentially encoding a CAT (Sweigard *et al.*, 1998). We therefore decided to investigate the role of *PTH2* in fungal pathogenesis, in order to determine how this enzyme contributes to appressorium development and why this is so critical to successful plant infection.

In this report, we demonstrate that *PTH2* encodes the major CAT in *M. grisea* and is necessary for appressoria to function correctly. We also show that Pth2 is regulated by the cyclic AMP response pathway and is located in peroxisomes, which are abundant in appressoria. Pth2 contributes to effective lipid reserve mobilization during appressorium maturation and is necessary for penetration hypha formation and host invasion. Our results indicate that intracellular transport of peroxisomally generated acetyl CoA within the appressorium is critical to plant infection by the rice blast fungus.

Results

A previous insertional mutagenesis screen identified a large number of pathogenicity mutants, one of which, *pth2*, appeared to result from inactivation of a putative CAT-encoding gene (Sweigard *et al.*, 1998). In order to analyse the role of CATs in the fungus, we first identified the complete *PTH2* locus (MG01721.4) in the *M. grisea* genome sequence (Dean *et al.*, 2005). *PTH2* encodes a 614 amino acid protein of 69.2 kDa which showed the highest similarity to the *CT-CAT2* gene of *Candida tropicalis* (44% amino acid identity), and *CAT2* of *Saccharomyces cerevisiae* (40% identity), and contains a previously unreported SKL type I peroxisomal targeting sequence at its C-terminus. *M. grisea* also possesses a second putative CAT-encoding gene, MG0681.4, which was most closely related to *FacC* from *Neurospora crassa* and *Aspergillus nidulans* (50% identity).

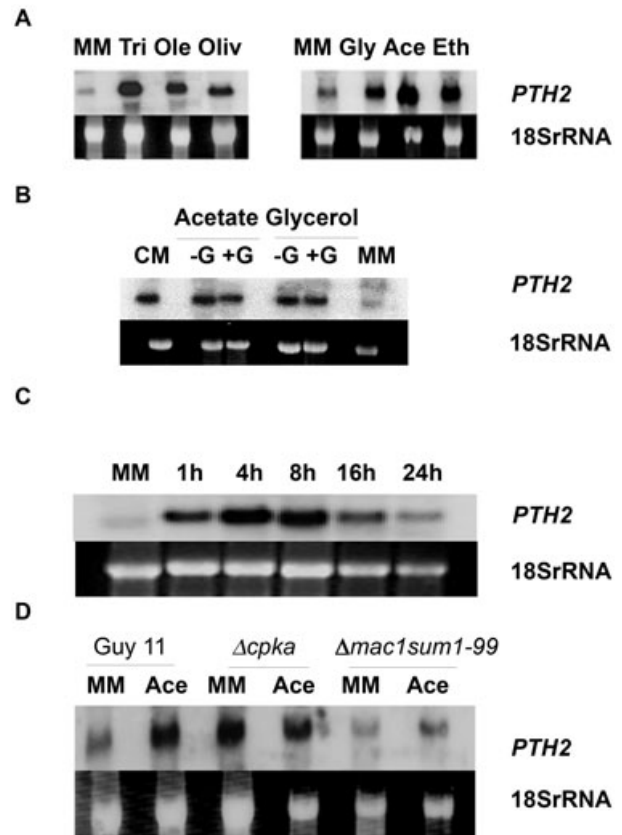


Fig. 1. Gene expression analysis of *PTH2*.

A. RNA was extracted from mycelium of Guy 11 grown in minimal medium with glucose (MM), minimal medium without glucose and replaced with 50 mM triolein (Tri), or oleic acid (Ole), or glycerol (Gly), or sodium acetate (Ace), or ethanol (Eth) for 24 h. All RNA gel blots were probed with the 1.5 kb *PTH2* polymerase chain reaction (PCR) amplicon.

B. RNA gel blot of *PTH2* expression in presence (+G) or absence (-G) of glucose in mycelial cultures of Guy 11 grown in sodium acetate or glycerol.

C. RNA gel blot of Guy 11 mycelium grown in minimal medium containing sodium acetate. RNA was removed at time intervals of 1 h, 4 h, 8 h, 16 h and 24 h and harvested.

D. RNA gel blot of Guy 11, $\Delta cpka$ and $\Delta mac1sum1-99$ mycelium grown in minimal medium with glucose (MM) and minimal medium with sodium acetate (Ace).

PTH2 is regulated by the cAMP response pathway

To explore the role of *PTH2* in *M. grisea* and its potential function in lipid metabolism, we decided to examine *PTH2* expression during growth of the fungus on lipids and fatty acids. Elevated expression of a 2.1 kb *PTH2* transcript was observed when the fungus was grown on ethanol, acetate, olive oil, oleic acid, triolein, or glycerol as sole carbon sources compared with growth on glucose, as shown in Fig. 1A. Elevated expression of *PTH2* was associated with substrate induction as opposed to glucose repression because the presence of glucose in the medium did not affect *PTH2* transcript levels when the fungus was exposed to acetate or glycerol (Fig. 1B). This

is in contrast to the regulation of *FacC* expression in *A. nidulans*, for example, which is subject to glucose repression (Stemple *et al.*, 1998). Substrate induction of *PTH2* expression was also rapid, with elevated transcript levels observed as soon as 1 h after transfer to acetate-containing growth medium, with peak transcript 8 h after transfer to acetate (Fig. 1C). To study the genetic control of *PTH2*, we examined mutant strains of *M. grisea* affected in the operation of the cyclic AMP response pathway. Acetate-induction of *PTH2* expression was examined in a $\Delta cpkA$ mutant, which lacks the catalytic subunit of PKA (Mitchell and Dean, 1995; Xu *et al.*, 1997), and a $\Delta mac1sum1-99$ mutant which lacks the regulatory subunit of PKA and therefore shows constitutive cAMP-independent PKA activity (Adachi and Hamer, 1998). *PTH2* expression was de-repressed in the presence of glucose in the $\Delta cpkA$ mutant (Fig. 1D), while, conversely, in the $\Delta mac1sum1-99$ mutant, *PTH2* was not elevated in expression either in the presence or absence of acetate (Fig. 1D). Taken together, these findings indicate that *PTH2* expression in mycelium is normally repressed in the presence of glucose, which requires the cAMP-dependent PKA signalling pathway.

PTH2 encodes a CAT required for lipid metabolism

To investigate the function of *PTH2*, a targeted gene replacement was performed in which an 80 bp *EagI*-*NcoI* fragment of the *PTH2* gene was removed and replaced with a 1.4 kb hygromycin phosphotransferase gene cassette (Fig. 2A). The gene disruption construct was introduced into a rice pathogenic wild-type strain of *M. grisea*, Guy 11, and hygromycin-resistant transformants selected and analysed by DNA gel blot. The wild-type *PTH2* allele was contained in a 13 kb *HindIII* fragment (Fig. 2B, lane 1), while the gene replacement event was verified by the presence of a 14.5 kb *HindIII* fragment (Fig. 2B, lanes 2, 3, 4 and 6). Other transformants showed hybridization to both *HindIII* fragments indicating ectopic insertion of the gene replacement vector (Fig. 2B, lanes 5 and 7). Growth tests revealed that $\Delta pth2$ mutants were unable to utilize acetate, triglycerides (triolein) or long chain fatty acids (olive oil) as sole carbon sources (Fig. 2C), consistent with loss of carnitine metabolism.

To determine whether *PTH2* encodes a CAT, protein extracts were made from *M. grisea* mycelium grown in the presence of sodium acetate, and CAT activity was assayed (Kawamoto *et al.*, 1978) as shown in Fig. 2D. The wild-type *M. grisea* strain Guy 11 showed CAT activity of 25 nmol min⁻¹ mg⁻¹ protein compared with a level of 2.4 nmol min⁻¹ mg⁻¹ protein observed in a $\Delta pth2$ mutant. Reintroduction of the *PTH2* gene into a $\Delta pth2$ mutant restored CAT activity to normal levels (Fig. 2D).

Pth2 is a peroxisomal CAT

Carnitine acetyl transferases can be present in both peroxisomes and mitochondria. In mammalian cells, fatty acid β -oxidation, for example, occurs in both mitochondria and peroxisomes (Schulz, 1991) and involves fatty acid chain shortening which generates acetyl CoA and medium chain acyl-CoAs, which are subsequently transported into the mitochondria to undergo complete oxidation to CO₂ and H₂O (Bieber, 1988). In the yeast *S. cerevisiae*, however, β -oxidation occurs exclusively in peroxisomes and the carnitine shuttle transfers activated acetyl groups from the peroxisome or cytosol to mitochondria (Schmalix and Bandlow, 1993; van Roermund *et al.*, 1995). Characterization of an acetylcarnitine carrier-like protein in the mitochondrial membrane of *Arabidopsis thaliana* indicates that this pathway also operates in higher plants (Lawand *et al.*, 2002). Peroxisomal citrate synthase, however, provides the major exit route from fatty acid metabolism in oilseed plants, in contrast to the dependency on a peroxisomal carnitine shuttle that occurs in animals and in yeast (Pracharoenwattana *et al.*, 2005). In filamentous fungi, such as *M. grisea*, the situation is not yet clear, but it appears that peroxisomal and mitochondrial β -oxidation both operate in some species, such as *A. nidulans* (Maggio-Hall and Keller, 2004). Studies in *S. cerevisiae* have, however, shown that *CAT2* encodes both peroxisomal and mitochondrial CAT isoforms which contain both an N-terminal amphiphilic α -helix that acts as a mitochondrial targeting signal and the tripeptide, AKL, a functional variant of the type I peroxisomal targeting signal (Gould *et al.*, 1989; Swinkels *et al.*, 1992).

To determine the subcellular location of Pth2 we constructed and expressed a Pth2-GFP fusion protein in *M. grisea* under the control of its native promoter. This gene fusion was fully functional and able to complement all $\Delta pth2$ mutant phenotypes (data not shown). GFP expression was observed with a punctate distribution in vegetative hyphae grown in axenic culture and then transferred to medium containing 50 mM sodium acetate, and within conidia (Fig. 3). To investigate the cellular distribution in more detail we introduced a FoxA-RFP fusion protein to the same Pth2-GFP transformant of *M. grisea*. FoxA is an *A. nidulans* gene that encodes a multifunctional fatty acid β -oxidation enzyme containing a type-1 peroxisomal-targeting signal (Maggio-Hall and Keller, 2004). The construct used contains the PTS-containing C-terminus of foxA fused downstream of RFP (Maggio-Hall and Keller, 2004). Colocalization of the foxA-RFP and Pth2-GFP fusion proteins was observed in intracellular vesicles, which accumulated at the cortex of cells (Fig. 3). We also noted some very diffuse background Pth2-GFP fluorescence in hyphae of the fungus. We conclude that Pth2 is located predominantly in per-

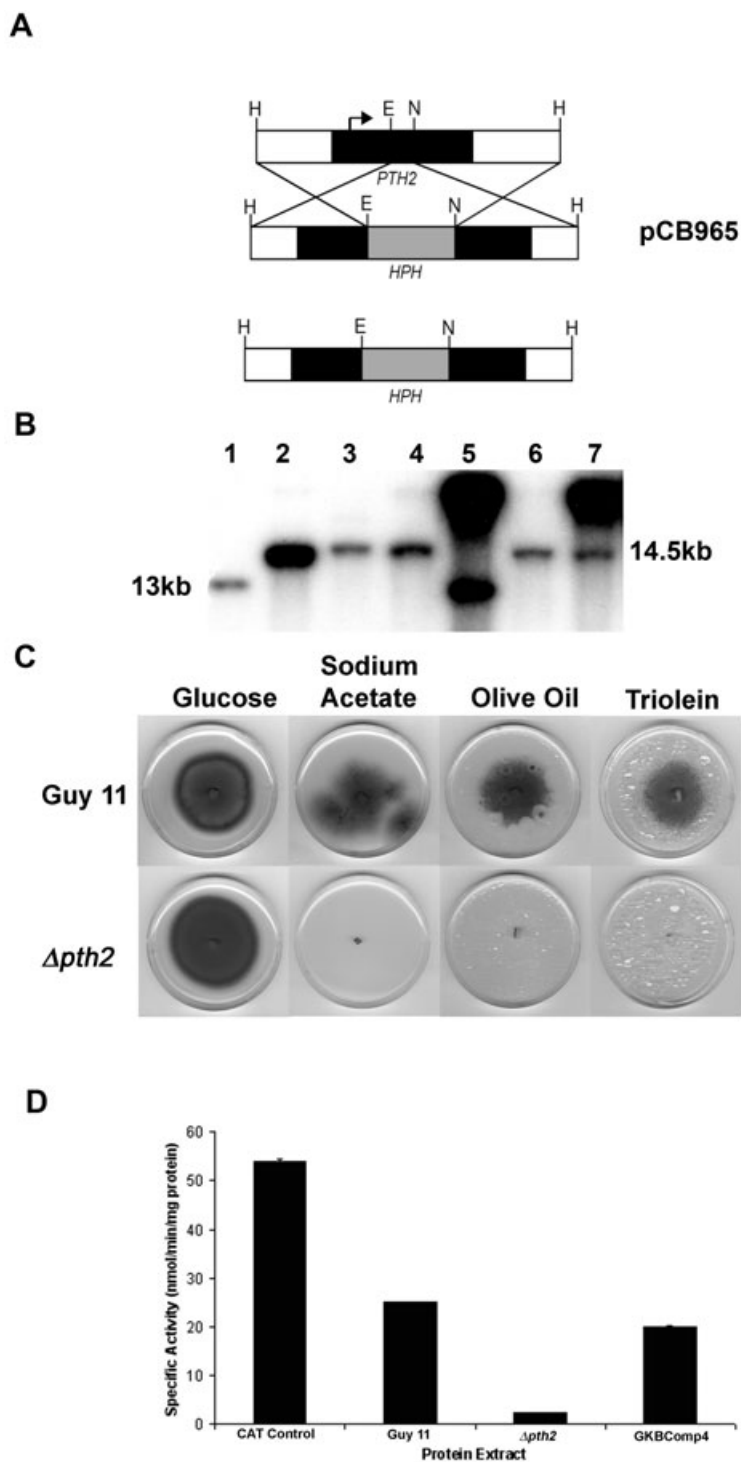


Fig. 2. Targeted gene replacement of *Magnaporthe grisea* *PTH2*.

A. Restriction map and orientation of the *PTH2* locus. A HindIII fragment spanning the locus was subcloned and an 80 bp EagI–NcoI fragment of *PTH2* was removed and replaced with a 1.4 kb EagI–NcoI fragment hygromycin B resistance gene cassette (Carroll *et al.*, 1994). E, EagI; H, HindIII; N, NcoI.

B. DNA gel blot analysis of pPth2:Hph transformants. Genomic DNA was prepared from the wild-type strain Guy 11 (lane 1), the ectopic integration transformants, Ect-31, Ect-35 (lanes 5 and 7), and four Δ pth2::Hph mutant transformants, pth2-1, pth2-27, pth2-33, pth2-49 (lanes 2, 3, 4 and 6 respectively). Blot was probed with the 1.5 kb *PTH2* PCR amplicon.

C. Growth tests of Guy 11 and Δ pth2-1 mutant cultured on minimal medium agar containing 50 mM glucose, or sodium acetate, olive oil, or triolein for 10 days at 24°C.

D. Bar charts of carnitine acetyltransferase (CAT) enzymatic activity. Average CAT specific activity is expressed as nanomoles of CoA-SH produced per minute per milligram of protein. Mycelium from Guy 11, Δ pth2-1 and GKBComp4 (a complemented Δ pth2-1::*PTH2*:Sur transformant) was grown in complete medium for 24 h and then transferred to minimal medium containing 50 mM sodium acetate for 24 h before harvesting. The control experiment utilized purified CAT from pigeon breast muscle (CAT Control). The error bar shows standard deviation from three biological replications of the experiment.

oxisomes although it is possible that a small cytosolic pool of the protein also exists.

PTH2 is required for cuticle penetration and infection of the leaf epidermis

The role of *PTH2* in rice blast disease was investigated by

inoculating seedlings of the blast-susceptible rice cultivar CO-39 and the barley cultivar Golden Promise, with a Δ pth2 mutant. No rice blast disease symptoms were observed, as shown in Fig. 4, consistent with previous analysis of a *pth2* insertion mutant (Sweigard *et al.*, 1998). Furthermore reintroduction of the *PTH2* gene restored fully the ability to cause rice blast disease (Fig. 4). We

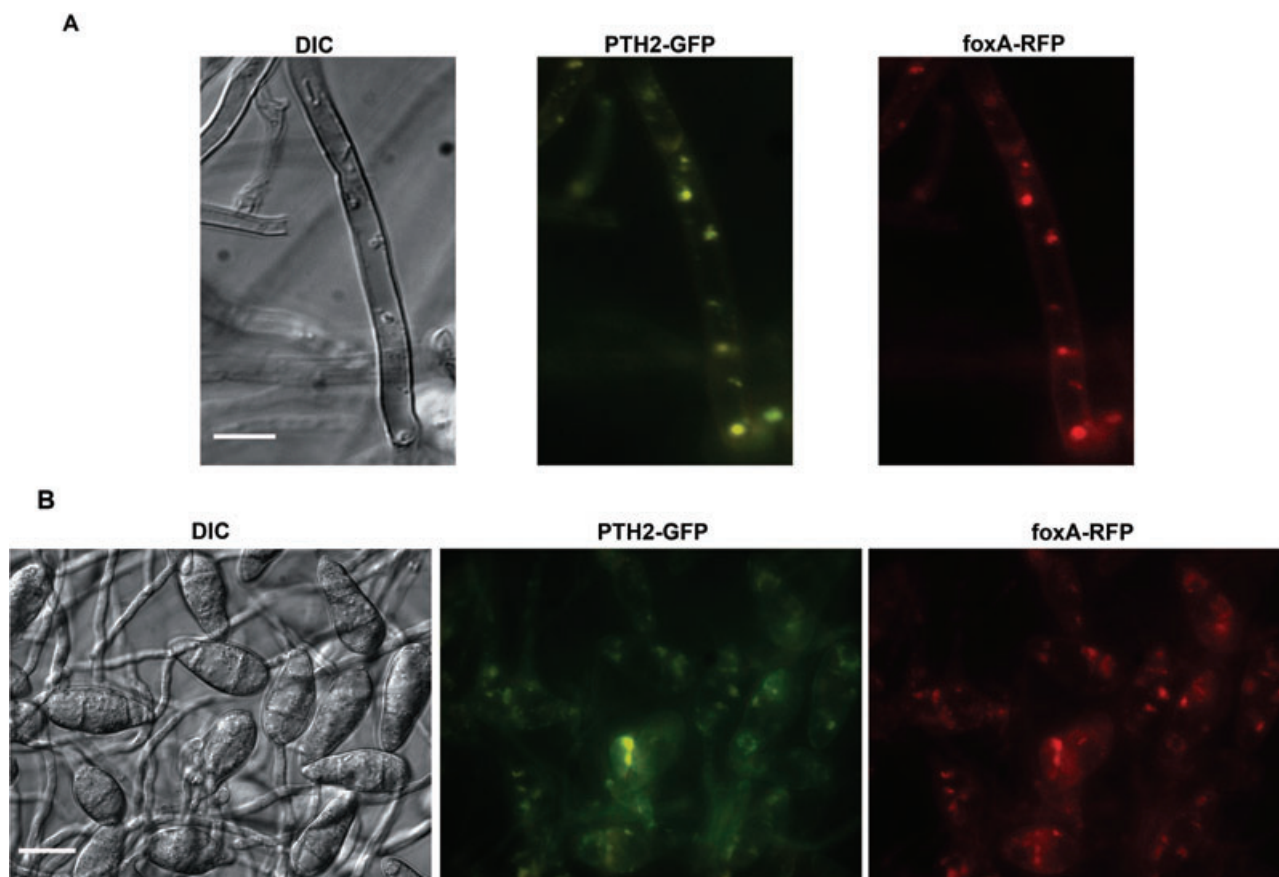


Fig. 3. Intracellular localization of the Pth2–GFP fusion protein and colocalization with foxA–RFP.

A. Mycelium of a transformant expressing Pth2–GFP and foxA–RFP was incubated in minimal medium supplemented with 50 mM sodium acetate for 24 h. Hyphae were viewed under differential interference contrast (DIC) and epifluorescence. Bar = 5 μ m.

B. Conidia were harvested from 10-day-old complete media growth plates and GFP fluorescence and RFP fluorescence were observed within the peroxisomes. Bar = 10 μ m.

therefore conclude that *PTH2* encodes the major CAT activity in *M. grisea*, which is essential for development of rice blast disease.

To investigate the reasons why $\Delta pth2$ mutants fail to cause rice blast disease, a detailed examination of virulence-associated functions was undertaken. We found that conidiogenesis was reduced 10-fold (6×10^5 conidia ml^{-1}) in $\Delta pth2$ mutants compared with Guy 11 (3×10^6 conidia ml^{-1}), but spores germinated normally and developed appressoria. To determine the extent to which these infection structures were functional in the $\Delta pth2$ mutant, appressoria of the $\Delta pth2$ and Guy 11 were allowed to form on intact rice leaf surfaces and those in which the cuticle was first removed by abrasion. Disease symptoms were then recorded 72–102 h after inoculation. We found that no disease lesions were observed on non-abraded leaves of either rice or barley seedlings inoculated with $\Delta pth2$ mutant, as shown in Fig. 4B. However, small disease lesions were observed on abraded leaf sections inoculated with the $\Delta pth2$ mutant (Fig. 4B),

although these were much less developed than those formed by Guy 11. This suggests that the initial events of plant infection are most likely to be affected by loss of Pth2 because the capacity for growth in rice leaf tissue is still present in $\Delta pth2$ mutants, albeit at a much-reduced rate compared with the isogenic wild type. Further examination to visualize invasive hyphae within rice epidermal cell layers was performed by epifluorescence microscopy using aniline blue (Fig. 4C and D). The fluorochrome shows a strong association with β -1–3 glucans (Hood and Shew, 1996) and has been utilized to analyse glucan composition of fungal cell walls (van Sengbusch *et al.*, 1983). Rice leaves were inoculated with conidia from Guy 11 and $\Delta pth2$ mutant and the fungus allowed to develop for 4 days. At this time, Guy 11 penetration hyphae were observed ramifying throughout the rice leaf tissue as shown in Fig. 4C. In contrast, in the $\Delta pth2$ mutant, no penetration hyphae (Fig. 4D) were observed indicating that rice cuticle penetration and formation of penetration hyphae are affected by loss of Pth2.

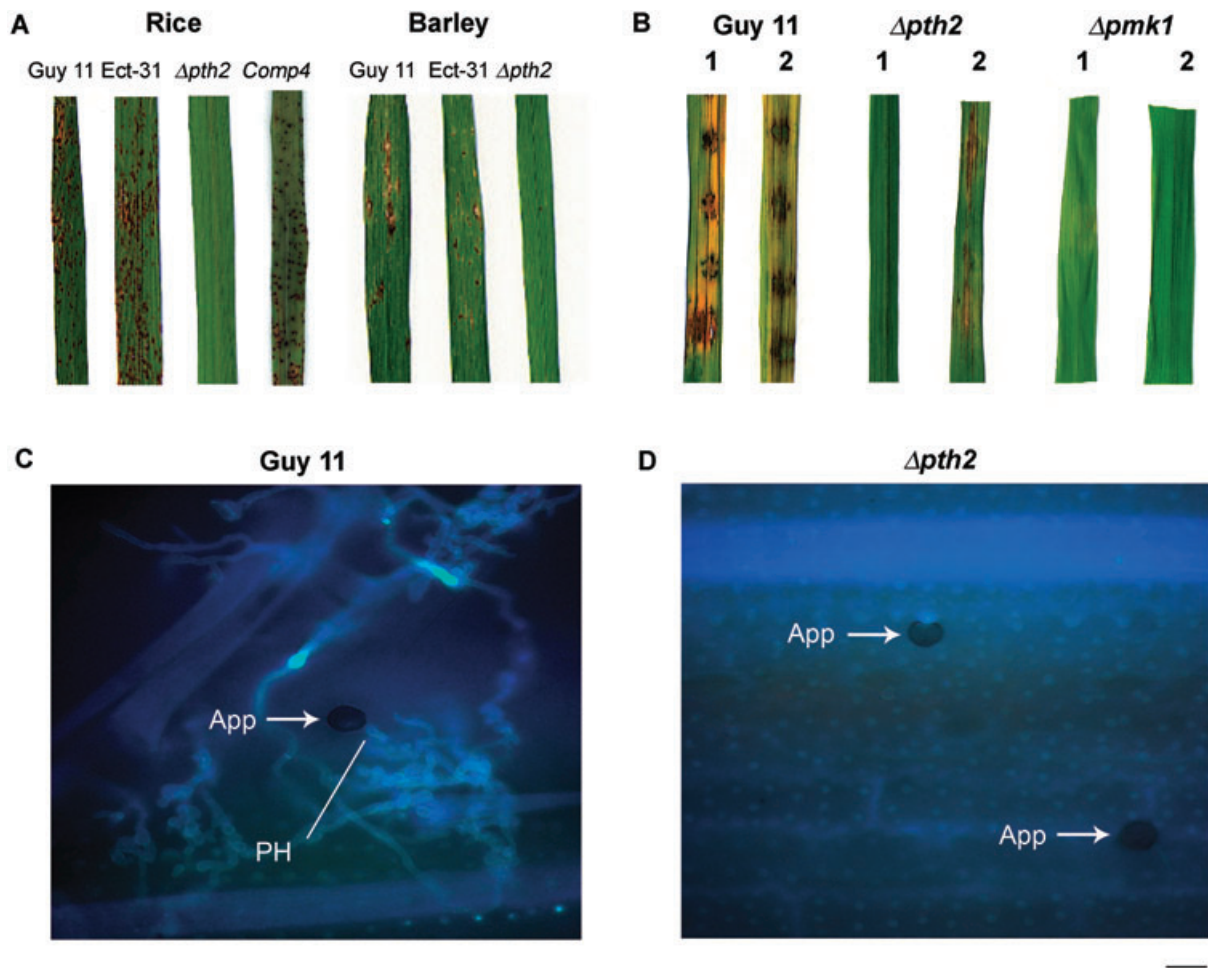


Fig. 4. Rice blast symptoms produced by $\Delta pth2$ mutants and assessment of appressorium-mediated penetration.

A. Rice and barley leaves inoculated with the wild-type strain, Guy 11, ectopic integration transformant, Ect-31, $\Delta pth2$ -1 mutant and PTH2-complemented transformant Comp4.

B. Rice leaf wounding assay to remove the cuticle by abrasion. Conidial suspensions of Guy 11, $\Delta pth2$ -1 mutant and $\Delta pmk1$ were incubated onto non-abraded (1) and abraded (2) rice leaf sections.

C. Aniline blue staining to visualize invasive hyphae of Guy 11 after 96 h development within rice leaves. App, appressorium; PH, penetration hyphae. Bar = 10 μ m.

D. Aniline blue staining to visualize invasive hyphae of $\Delta pth2$ -1 mutant after 96 h development on rice leaves. App, appressorium. Bar = 10 μ m.

Epidermal penetration assays were performed with the $\Delta pth2$ mutant and Guy 11 by inoculating onion epidermal strips with a concentration of 1×10^4 conidia ml^{-1} . Emergence of penetration hyphae from the base of the appressorium and epidermal rupture was then determined and recorded (Chida and Sisler, 1987). In $\Delta pth2$ infections, $50 \pm 7\%$ appressoria produced penetration hyphae after 24 h compared with $92 \pm 3.5\%$ in Guy 11 infections. After 48 h, $61 \pm 4\%$ of the $\Delta pth2$ mutant had successfully penetrated. These observations indicate that $\Delta pth2$ mutant appressoria retain the capacity for production of polarized penetration pegs which are able to breach a highly yielding surface, such as an onion epidermal strip, but are reduced in their ability to elaborate penetration

hyphae to breach the tough outer cuticle of the rice leaf and enter leaf tissue.

PTH2 is dispensable for turgor generation within the appressorium

The inability of $\Delta pth2$ mutants to undergo rice cuticle penetration suggested that appressorium function may be compromised by loss of CAT. Appressorium-mediated penetration in *M. grisea* is a turgor-driven process associated with substantial glycerol accumulation in the infection cell (de Jong *et al.*, 1997). Glycerol may be generated through the action of triacylglycerol lipases, which would also liberate fatty acids in the appressorium (Thines *et al.*,

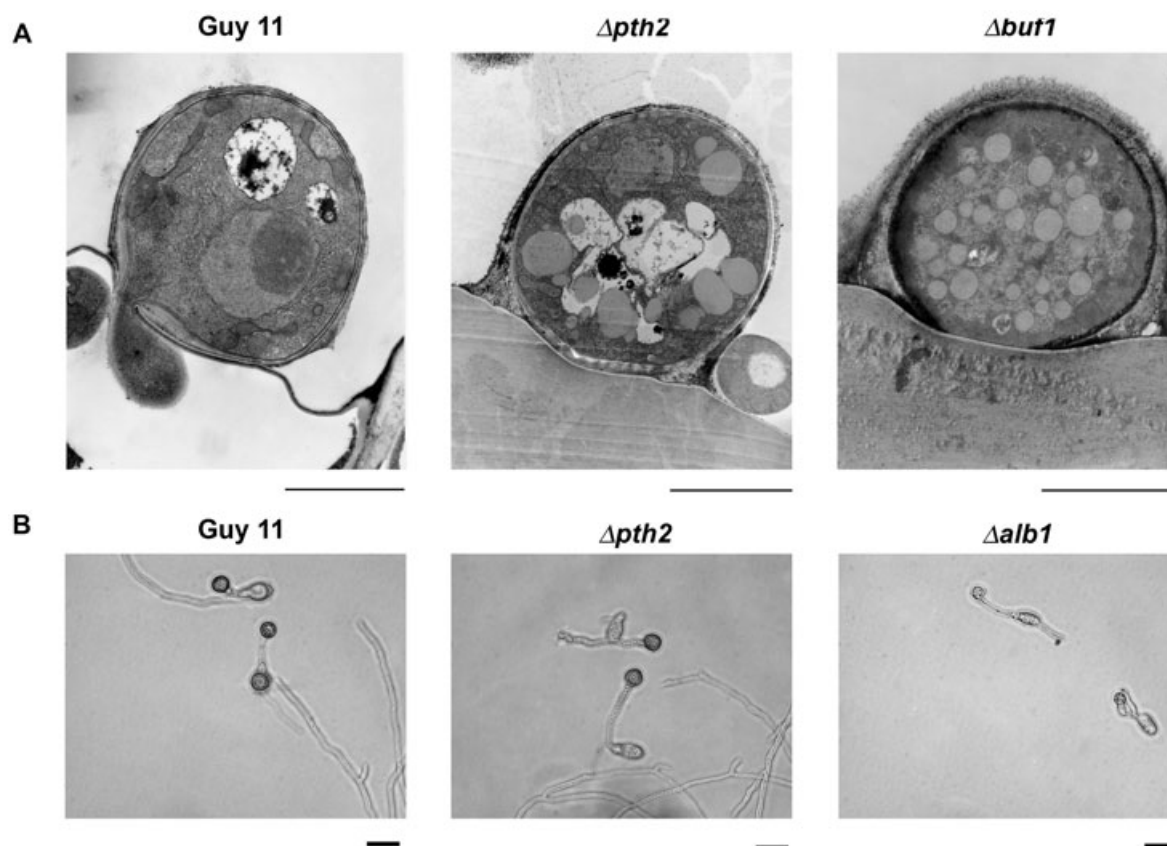


Fig. 5. Ultrastructural analysis of *M. grisea* appressoria during turgor generation.

A. Appressoria of Guy 11, $\Delta pth2$ -1 and melanin-deficient $\Delta buf1$ mutant were allowed to form on sterile onion epidermis. In Guy 11, a distinct, thin melanin layer is visible just outside the plasma membrane of the Guy 11 appressorium. In contrast, the $\Delta buf1$ mutant has a thick, diffuse microfibrillar layer of melanin precursors. In the $\Delta pth2$ -1 mutant, a thinner melanin layer is seen within the appressorium. Bar = 5 μ m.

B. Conidia of Guy 11, $\Delta pth2$ -1 and melanin-deficient $\Delta alb1$ mutant were allowed to form appressoria on glass coverslips and visualized by phase contrast microscopy. Bar = 20 μ m.

2000). In order to test whether Pth2 is required for turgor generation in the appressorium, incipient cytorrhysis assays were performed in which the rate of appressorium collapse was determined in the presence of hyperosmotic concentrations of glycerol. We observed no significant difference in appressorial turgor between the $\Delta pth2$ mutant ($61.2 \pm 4.9\%$ collapsed appressoria in the presence of 2 M glycerol) and Guy 11 ($56.4 \pm 0.4\%$ collapsed appressoria at 2 M glycerol) indicating that *PTH2* is not needed for generation or maintenance of appressorium turgor.

Appressorial melanin biosynthesis is reduced, but not severely compromised by absence of PTH2

We reasoned that one potential reason why $\Delta pth2$ mutants might be affected in appressorium function might be due to a requirement for peroxisomal acetyl-CoA as a substrate for dihydroxynaphthalene melanin biosynthesis. In *M. grisea*, melanin deposition in the appressorium cell wall is essential for appressorium function (Howard and

Valent, 1996; Money and Howard, 1996) and Pth2 could generate cytoplasmic acetyl CoA, which is channeled into the pentaketide melanin biosynthesis pathway. To explore this possibility, light and transmission electron microscopy were performed in a $\Delta pth2$ mutant, Guy 11 and the melanin-deficient, non-pathogenic mutants *alb1* and *buf1*, which lack polyketide synthase and trihydroxynaphthalene reductase respectively (Howard and Valent, 1996). Electron microscopy of appressorium cross-sections showed a thin, distinct layer of melanin deposited just outside the plasma membrane of appressoria of Guy 11 (Fig. 5A) in comparison to a thick, diffuse layer of microfibrillar melanin precursors present within the $\Delta buf1$ mutant, as shown in Fig. 5A. In $\Delta pth2$ mutants, a melanin layer was observed, but was reduced in thickness when compared with Guy 11. Appressorium melanization could, however, be readily observed by phase contrast microscopy in both Guy 11 and $\Delta pth2$ mutants (Fig. 5B).

To test whether the reduction in melanization in $\Delta pth2$ mutants was significant, we tested whether addition of a 20 μ g ml⁻¹ scytalone solution could restore appressorium

function to $\Delta pth2$ in rice leaf cuticle penetration assays. Scytalone is a melanin biosynthetic intermediate, which has previously been shown to restore the ability of *alb1* mutants to produce disease lesions (Howard and Valent, 1996). We observed no restoration of lesion formation in $\Delta pth2$ mutants by addition of scytalone (data not shown). Based on this experiment, and the fact that $\Delta pth2$ mutant appressoria still generate turgor, we conclude that the reduction in melanin production observed in $\Delta pth2$ mutants is not the primary reason for their lack of pathogenicity.

Lipid mobilization is impaired in developing appressoria of the $\Delta pth2$ mutant

During appressorium maturation, glycogen and lipid reserves move into the developing infection structures. Lipid bodies then coalesce and are taken-up by vacuoles by autophagocytosis resulting in rapid lipolysis (Thines *et al.*, 2000; Weber *et al.*, 2001). To determine whether these processes are altered in $\Delta pth2$ mutant, conidia were germinated on plastic coverslips and appressorium development allowed to occur over a 48 h period. Infection structures were then stained with Nile Red solution to visualize lipid bodies, as shown in Fig. 6. Large numbers of lipid bodies were present within conidia, germ tubes and developing appressoria of Guy 11 (Fig. 6). After 8 h, lipid bodies were predominantly mobilized to appressoria and between 12 and 24 h, lipid bodies became depleted at the onset of turgor generation (Fig. 6A). In contrast, in a $\Delta pth2$ mutant, very few lipid bodies were present in appressoria after 8 h indicating a delay in lipid body mobilization to incipient appressoria (Fig. 6A). Quantitative analysis revealed that large number of lipid bodies were still present in germ tubes of the $\Delta pth2$ mutant after 24 h incubation (Fig. 6B). We can conclude that in $\Delta pth2$ mutants, lipid bodies are not metabolized within appressoria at the same rate as in a wild-type *M. grisea* strain.

Addition of glucose can partially remediate the $\Delta pth2$ mutant phenotype

During plant infection, rapid cell wall biosynthesis occurs at the apex of penetration hyphae enabling fungal proliferation in plant tissue (Bourett and Howard, 1990). Because the fungus is in a nutrient-free environment on the leaf surface, the generation of precursors for cell wall biosynthesis must result from degradation of conidial storage products. One potential role for Pth2-generated acetyl-CoA might therefore be as a substrate for the glyoxylate cycle and gluconeogenesis as a means of generating glucans and chitin required for cell wall biosynthesis. To investigate this idea, rice cuticle penetration assays were performed using a $\Delta pth2$ mutant in the presence of

2.5% glucose or sucrose solution (Fig. 7). The ability to cause rice blast symptoms was partly restored to the $\Delta pth2$ mutant in the presence of either glucose or sucrose, although the mutant was still reduced in disease lesion formation when compared with the wild-type strain (Fig. 7B). Interestingly, lipid mobilization to the appressorium in a $\Delta pth2$ mutant was also restored to near normal levels, and penetration hyphae were observed invading rice tissue when the fungus was incubated in the presence of glucose (Fig. 7). It seems likely that glucose take-up by the fungus can partly restore the functional competence of appressoria, and in particular their ability to form penetration hyphae.

Pth2 mutants produce penetration hyphae that are chitin-deficient

The partial restoration of rice cuticle penetration and invasive growth of $\Delta pth2$ mutants in the presence of glucose, suggested that cell wall biosynthesis might be affected in the mutant. We reasoned that take-up of glucose likely provides an alternative Pth2-independent route for glucan and chitin synthesis. To investigate whether cell wall biosynthesis by the fungus is affected by loss of Pth2, we incubated a $\Delta pth2$ mutant on onion epidermis, a highly yielding surface upon which we had previously shown that the mutant could elaborate penetration hyphae (data not shown). The fungus was allowed to form appressoria and then stained with calcofluor white, which binds to chitin in the cell wall. Calcofluor white fluorescence was observed in the cell walls of appressoria of both Guy 11 and $\Delta pth2$, but was not observed in penetration hyphae of $\Delta pth2$ mutant even after 48 h of development (Fig. 8). Vegetative hyphae of $\Delta pth2$ mutants stained normally with calcofluor white (data not shown). These findings provide evidence that chitin synthesis during invasive hypha formation is impaired in the $\Delta pth2$ mutant.

Discussion

Carnitine acetyl transferase is a critical enzyme for directing acetyl CoA to the correct intracellular compartment for subsequent utilization (Elgersma *et al.*, 1995). In the budding yeast *S. cerevisiae*, where fatty acid β -oxidation occurs only in peroxisomes, Cat2 provides a means for peroxisomally generated acetyl CoA to be transferred to the cytoplasm, while Cat1 channels acetyl CoA into mitochondria for oxidation via the tricarboxylic acid cycle (Schmalix and Bandlow, 1993; van Roermund *et al.*, 1995).

In this study we have shown that *PTH2* encodes the major CAT activity in *M. grisea*, based on enzymatic assays and the inability of *pth2* mutants to utilize acetate or lipids as a sole carbon source. The inability of $\Delta pth2$

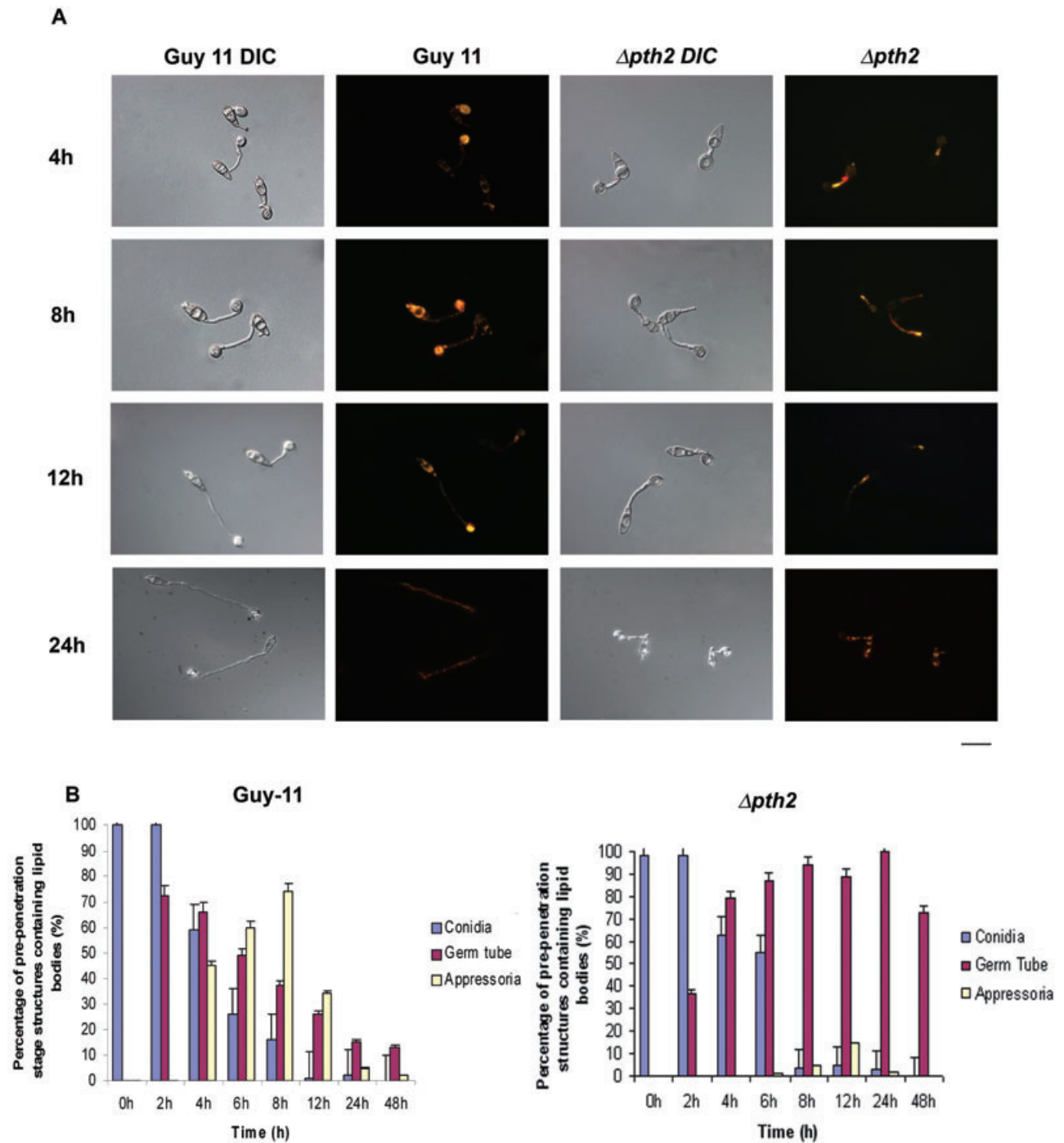


Fig. 6. Cellular distribution of lipid droplets during appressorium morphogenesis by *M. grisea*.

A. Conidia of Guy 11 and Δ pth2-1 mutant were incubated in water droplets on glass coverslips and allowed to form appressoria. Samples were removed at 4 h, 8 h, 12 h and 24 h intervals and stained with Nile Red for the presence of triacylglycerol. Coverslips were viewed under DIC and epifluorescence microscopy. Lipid bodies in Guy 11 were mobilized to the tips of germ tubes and coalesced within appressoria before disappearing during the onset of turgor generation. The Δ pth2-1 mutant showed a delay in lipid mobilization with lipid bodies being retained within the germ tubes during the onset of turgor generation. Bar = 10 μ m for all panels.

B. Quantitative analysis of lipid distribution during appressorium morphogenesis. The percentage of fungal structures of Guy 11 and Δ pth2-1 mutant that contained lipid bodies at a given time was recorded from a sample of 300 germinated conidia in three replications of the experiment.

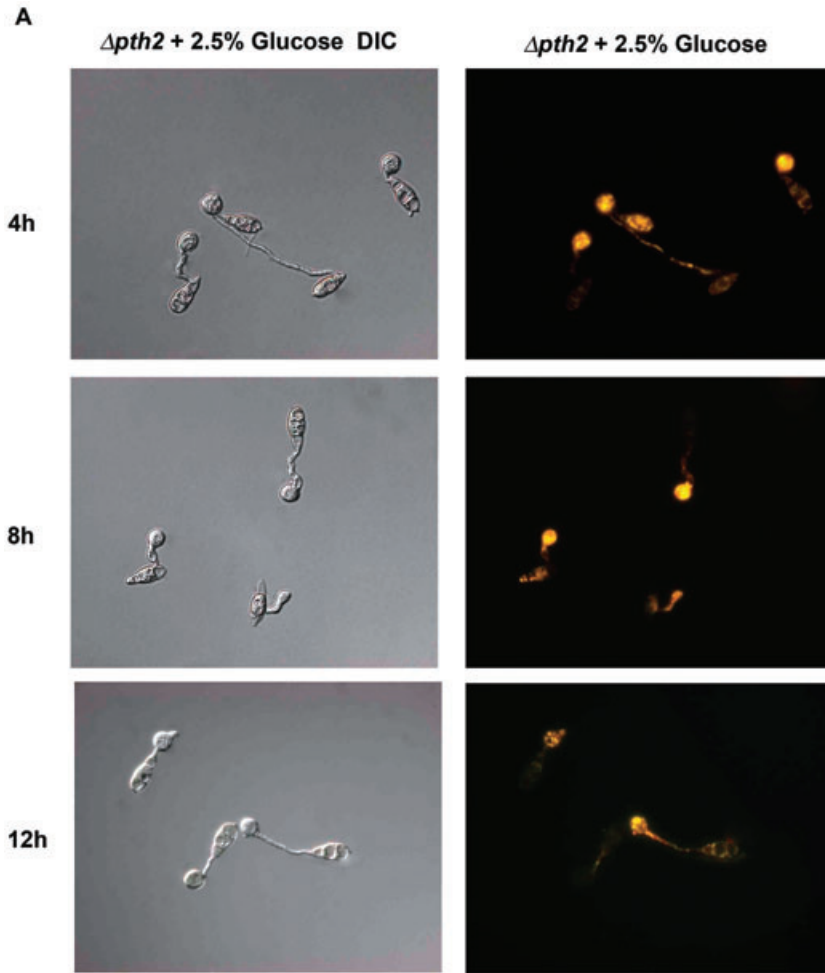
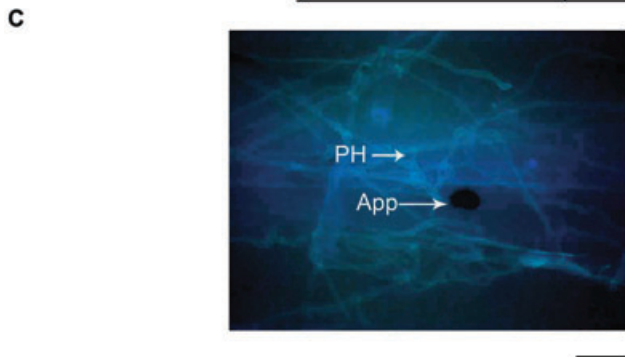
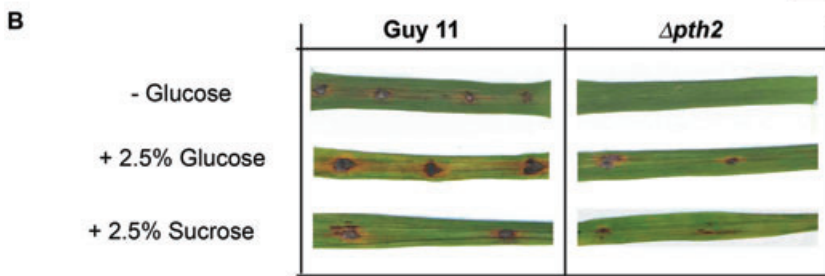


Fig. 7. Addition of glucose partly restores the ability of $\Delta pth2$ mutants to cause rice blast disease.

A. Conidia of $\Delta pth2-1$ mutant were incubated in water droplets on glass coverslips with the addition of a 2.5% glucose solution and allowed to form appressoria. Samples were removed at 4 h, 8 h and 12 h intervals and stained with Nile Red for the presence of triacylglycerol. Each sample was viewed under DIC and epifluorescence microscopy (right). Lipid bodies migrated to appressoria before disappearing during the onset of turgor generation as observed in Guy 11. Bar = 10 μ m.

B. Conidial suspensions of Guy 11 and $\Delta pth2-1$ mutant were inoculated onto intact rice leaves in the presence and absence of 2.5% glucose or sucrose solution. In Guy 11, disease lesions were produced in the absence and presence of glucose or sucrose. The addition of glucose or sucrose partially restores lesion formation in the $\Delta pth2-1$ mutant.

C. Aniline blue staining of invasive hyphae within rice epidermal cell layers of $\Delta pth2-1$ in the presence of 2.5% glucose solution. App, appressorium; PH, penetration hyphae. Bar = 10 μ m.



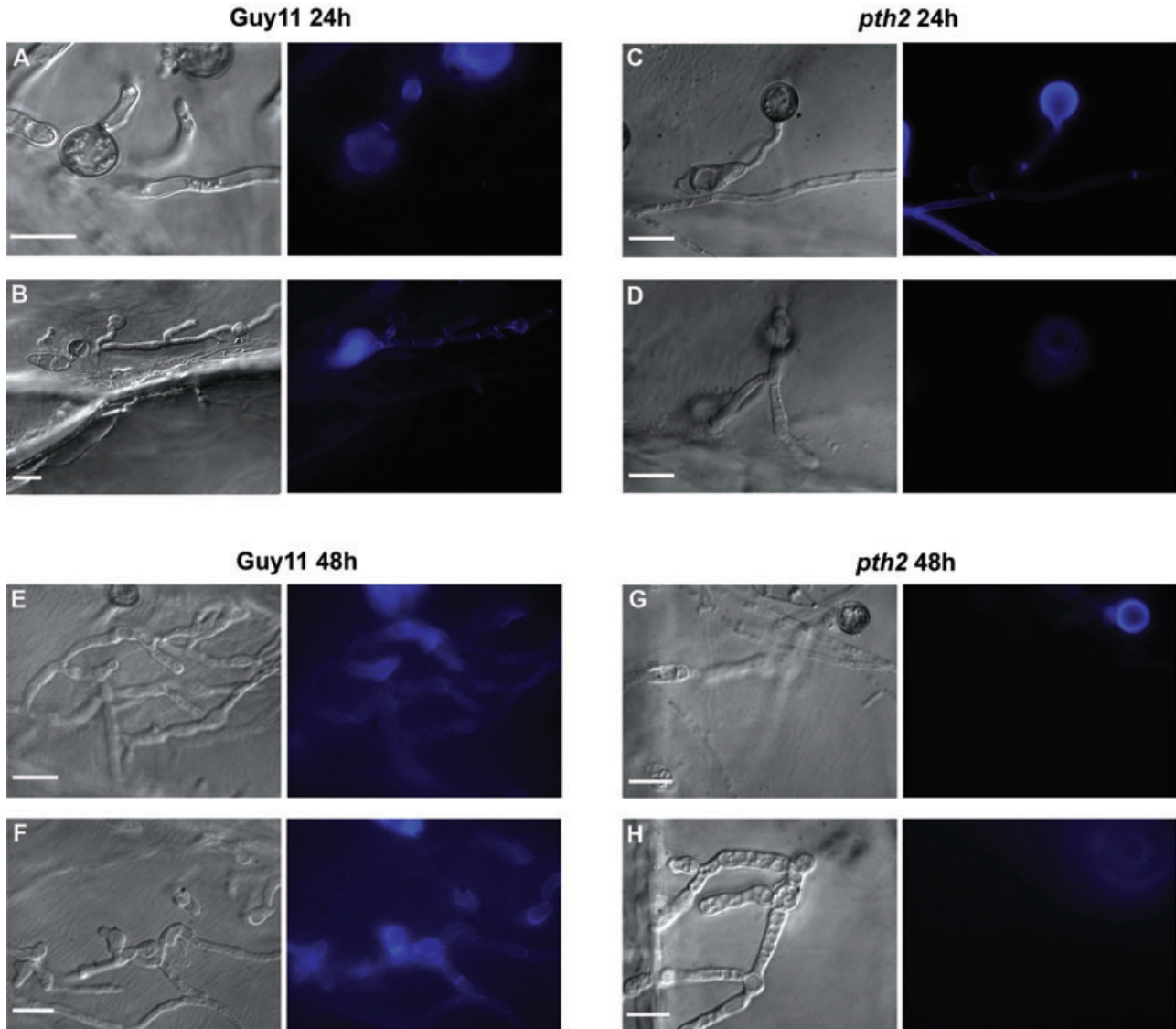


Fig. 8. Cell wall staining of *M. grisea* invasive hyphae with calcofluor white to show chitin deposition. Guy 11 and $\Delta pth2$ -1 mutant appressoria were allowed to form on onion epidermis and penetration hyphae allowed to form for 48 h. After 24 h appressoria could be clearly stained with calcofluor white (A and C). Guy 11 invasive hyphae elaborated from these appressoria also stained well with calcofluor white after 24 h or 48 h (B, E, F). $\Delta pth2$ -1 mutant penetration hyphae failed to stain with calcofluor white at 24 h (D) or 48 h (G and H). Bar = 10 μ m.

mutants to utilize acetate suggests that there may be a separate cytosolic pool of Pth2, although we have demonstrated that the protein is predominantly peroxisomal. Alternatively, it is possible that some, or all, of the enzymes of the glyoxylate shunt are located in glyoxysomes, as suggested for *N. crassa* (Gainey *et al.*, 1992), requiring Pth2 activity for entry of acetyl CoA in this separate compartment.

The function of PTH2 in rice blast disease

Generation of a targeted $\Delta pth2$ deletion mutant highlighted the significance of CAT to fungal pathogenicity.

Cytological analysis showed that $\Delta pth2$ mutants are impaired in rice cuticle penetration and we therefore set out to explore the likely roles of Pth2 in plant infection by *M. grisea*. We distinguished three possible virulence-associated functions for the enzyme and designed experiments to test the likelihood of each. The first possibility was that Pth2 is required for turgor generation necessary for appressorium-mediated penetration (de Jong *et al.*, 1997). Acetyl CoA from fatty acid β -oxidation could, for example, be processed via the glyoxylate cycle and gluconeogenesis to yield precursors to glycerol biosynthesis, which is necessary for turgor generation (Thines *et al.*, 2000). Evidence gained from incipient cytorrhysis assays

however, showed, no observable difference in appressorium turgor in $\Delta pth2$ mutants, which is inconsistent with such a role.

The second possibility we considered was that *PTH2* is involved in the supply of acetate units for melanin biosynthesis via the dihydroxynaphthalene pathway (Howard and Valent, 1996). A large body of experimental evidence has shown that appressorium melanization is vital for plant infection by *M. grisea* (Howard *et al.*, 1991; Money and Howard, 1996; Howard and Valent, 1996; de Jong *et al.*, 1997) and $\Delta pth2$ mutants clearly showed a reduction in melanin production in appressoria when observed by electron microscopy (see also Ramos-Pamplona and Naqvi, 2006). Three distinct lines of evidence, however, argue that although there is a reduction in melanization, this is not the primary reason for loss of pathogenicity in $\Delta pth2$. First of all, both light and electron microscopy confirmed that appressorial melanin biosynthesis does occur in $\Delta pth2$ mutants. Secondly, appressorium turgor generation was normal in $\Delta pth2$ mutants, and finally addition of a melanin biosynthesis intermediate, scytalone, failed to restore lesion formation in $\Delta pth2$ mutants. Therefore, we conclude that although Pth2 activity very likely contributes to melanin biosynthesis, CAT is not essential for turgor generation in appressoria.

The third model that we considered is that peroxisomal fatty acid β -oxidation is vital for facilitating appressorium-mediated infection in *M. grisea* and that Pth2 acts to make acetyl CoA available to the glyoxylate cycle which feeds through reactions of the gluconeogenesis pathway to support glucan and chitin biosynthesis, which are required for cell wall generation and growth of penetration hyphae. Evidence to support such a role for Pth2 arises from the observation that addition of exogenous glucose or sucrose to appressoria of $\Delta pth2$ partially restores their ability to cause disease. Furthermore, calcofluor white staining revealed that $\Delta pth2$ mutants have reduced chitin within infectious hyphae when compared with Guy 11 and that such hyphae do not form on hard surfaces such as rice leaves. It is possible therefore that Pth2 is necessary for efficient cell wall biosynthesis during penetration peg development. The mutant phenotype of $\Delta pth2$ mutants is, however, completely non-pathogenic in contrast with *M. grisea* $\Delta icl1$ mutants, which lack isocitrate lyase, but only show a delay in disease symptom expression (Wang *et al.*, 2003). Furthermore, $\Delta icl1$ mutants produce normal chitin-rich penetration hyphae (data not shown). CAT is therefore of considerably more importance to appressorium physiology than simply providing substrate for the glyoxylate cycle. It is more likely that perturbation of the pool of cytosolic acetyl CoA, which results from loss of Pth2, is a catastrophic event for the appressorium due to the importance of acetyl CoA as a substrate for several metabolic and biosynthetic pathways. The accompanying

manuscript (Ramos-Pamplona and Naqvi, 2006) provides independent corroboration of the significance of Pth2 for appressorium-mediated infection in *M. grisea* and provides further evidence that cell wall biosynthesis is compromised in $\Delta pth2$ mutants.

The importance of CAT activity to the plant infection process by *M. grisea* therefore highlights the dependence of foliar plant pathogens on lipid metabolism for supporting initial growth and development on the leaf surface. The absence of external nutrients during spore germination, which is essential to promote appressorium development by *M. grisea* (Bourett and Howard, 1990), means that lipid mobilization, peroxisomal fatty acid β -oxidation and generation of a pool of acetyl CoA are likely to be vital to allow this fungal pathogen to enter its host. This conclusion is consistent with a study of the anthracnose fungus *Colletotrichum lagenarium*, in which peroxisomal biogenesis was shown to be necessary for appressorium function (Kimura *et al.*, 2001). Peroxisome formation has now also been shown to be important for *M. grisea* (Ramos-Pamplona and Naqvi, 2006; Z.Y. Wang and N.J. Talbot, manuscript in preparation). Deletion of the *PEX6* peroxin-encoding prevents peroxisome biogenesis and renders *M. grisea* completely non-pathogenic, demonstrating the significance of peroxisomal activities in appressorium function. Interestingly, $\Delta pex6$ mutants also lack Woronin bodies – the peroxisomal bodies required for septal occlusion in fungi (Ramos-Pamplona and Naqvi, 2006). Woronin bodies have previously been shown to be essential for appressorium function in *M. grisea* (Soundararajan *et al.*, 2004), highlighting the diverse functions fulfilled by the peroxisomal compartment in fungi.

In the wheat pathogen *Stagonospora nodorum* (Solomon *et al.*, 2004), the brassica pathogen *Leptosphaeria maculans* (Idnurm and Howlett, 2002), and the human pathogen *Candida albicans* (Lorenz and Fink, 2001), the glyoxylate cycle has also been shown to be necessary for pathogenesis. Recent transcriptional profiling experiments from the barley powdery mildew fungus *Blumeria graminis* have also shown that coordinated expression of lipid metabolic genes during appressorium-mediated plant infection (Both *et al.*, 2005). The characterization of Pth2 is therefore likely to be important in defining the spatial regulation and diverse functions of fatty acid β -oxidation during appressorium-mediated plant infection. Experiments to determine the interplay between Pth2 and other components of peroxisome function in *M. grisea* are currently underway.

Experimental procedures

Fungal strains, culture conditions and DNA analysis

Wild-type and mutant strains of *M. grisea* are stored in the laboratory of N.J. Talbot, University of Exeter. Standard pro-

cedures of *M. grisea* growth, maintenance, nucleic acid extraction and transformation were performed as described previously (Talbot *et al.*, 1993). Gel electrophoresis, restriction enzyme digestion and DNA gel blot hybridization were performed using standard procedures (Sambrook *et al.*, 1989).

Identification and targeted gene disruption of *PTH2*

Two, independent, non-pathogenic mutants were identified in which the *PTH2* gene had been mutated by restriction enzyme-mediated integration, as described previously (Sweigard *et al.*, 1998). A HindIII fragment spanning the *PTH2* gene locus was selected and an 80 bp EagI–NcoI fragment was removed and replaced with a 1.4 kb hygromycin phosphotransferase gene cassette (Carroll *et al.*, 1994), bestowing resistance to hygromycin B. The resulting construct, pCB965 (kindly donated by Dr J.A. Sweigard, DuPont Company, Wilmington, DE) was used to transform protoplasts of Guy 11 (Talbot *et al.*, 1993). For complementation of *pth2* mutants, a 4.7 kb ApaI–NotI fragment was cloned into pCB1532, which carries a sulphonylurea resistance selectable marker gene (Carroll *et al.*, 1994).

Rice and barley infections

Plant infection assays were performed by spraying seedlings of rice (*Oryza sativa*) cultivar CO-39 and barley (*Hordeum vulgare*) cultivar Golden Promise with suspensions of *M. grisea* conidia at a concentration of 10^5 conidia ml⁻¹, using an artist's airbrush (Dixon *et al.*, 1999).

Assays for infection-related morphogenesis

Magnaporthe grisea appressorium development was observed on plastic coverslips, as described previously (Dixon *et al.*, 1999). Appressorium-mediated penetration of sterile onion epidermis (*Allium cepa*) and detached rice leaves was assayed by placing individual drops of 1×10^4 conidia ml⁻¹ inoculum onto 1 cm² sections of onion epidermis and/or rice leaf epidermal strips. The frequency of cuticle penetration was recorded at time intervals of 24 h and 48 h and penetration peg formation was viewed using an Axiophot 2 compound microscope (Zeiss, UK) under phase-contrast illumination. Wounding assays of rice leaves were performed by inoculating 1×10^5 conidia ml⁻¹ suspensions onto abraded rice leaves with their cuticles removed and observed 4 days post inoculation for lesion formation. Rice leaf assays were also performed with 1×10^4 conidia ml⁻¹ suspensions in the presence or absence of a 2.5% glucose or sucrose concentration and lesion formation was observed 4 days post inoculation.

Carnitine acetyltransferase enzyme assay

Protein extracts were prepared by grinding 0.2 g of mycelium (wet weight) with glass beads in 1 ml of 50 mM Tris-HCl (pH 7.5) and centrifuging the resulting slurry at maximum speed in a microcentrifuge for 1 min as described by Stemple *et al.* (1998). CAT assays were performed as described by

Kawamoto *et al.* (1978). The reaction was monitored spectrophotometrically at 30°C by monitoring the release of CoA-SH from acetyl-CoA using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). The reaction mixture contained 40 mM KH₂PO₄ (pH 8.0), 0.05 mM acetyl-CoA, 0.12 mM DTNB and 0.1 ml of crude extract. The reaction was initiated by the addition of 2.2 mM DL-carnitine (β -hydroxy- γ -trimethylammonium butyrate) chloride, and the final reaction volume was 1 ml. A blank was used to which no carnitine was added, as a slight background rate of carnitine-independent release of CoA-SH from acetyl-CoA was observed. Protein concentrations were determined by using the Bio-Rad Protein Assay reagent, and CAT activities are expressed as nanomoles of CoA-SH produced per minute per microgram of protein, assuming an extinction coefficient of 13,600/M/cm for the chromophore formed from DTNB.

Cytological assays

Lipid droplets within germinating conidia and appressoria were visualized by staining with Nile Red solution (Greenspan *et al.*, 1985; Weber *et al.*, 2001) consisting of 50 mM Tris/maleate buffer, pH 7.5, with 20 mg ml⁻¹ polyvinylpyrrolidone and 2.5 μ g ml⁻¹ Nile Red Oxazone (9-diethylamino-5H-benzo[α]phenoxazine-5-one; Sigma). Cytological analysis was then performed on harvested conidia in the presence or absence of a 2.5% glucose solution incubated on glass cover slips. Material was directly mounted in the Nile Red stain. Lipid droplets begin to fluoresce within a few seconds when viewed with a Nikon Optiphot-2 microscope with the B-2 A filter (excitation at 450–490 nm, 505 nm dichroic mirror, 520 nm barrier filter). Percentage of conidia, germ tubes and appressoria exhibiting fluorescence were counted after 0 h, 2 h, 4 h, 6 h, 8 h, 12 h and 24 h time intervals and recorded. Penetration peg formation within rice leaf tissue was visualized by staining with Aniline Blue consisting of 0.05% w/v Aniline Blue stain in 0.067 M K₂HPO₄ pH 9.0. Cytological analysis was then performed on harvested conidia inoculated onto detached rice leaves at standard conidial concentrations. Four days post inoculation, rice leaves were incubated in 50 ml of 1 M KOH in a 70°C water-bath for 30 min. Rice leaves were washed 3 times with sterile distilled water and then transferred to a sterile glass universal containing 20 ml of 0.067 M K₂HPO₄ pH 9.0. The sample was then mounted onto a glass slide and the 0.05% aniline blue stain was added. Samples were viewed immediately with an Axiophot-2 microscope (Zeiss, UK) with the DAPI filter (excitation at 365 nm and emission at 420 nm).

Pth2 subcellular localization

For construction of the Pth2–GFP gene fusion vector, the sGFP coding sequence was removed from pMJK80 (Kershaw *et al.*, 1998) and inserted before the penultimate codon of *PTH2* ensuring the integrity of the SKL peroxisomal targeting sequence. The resulting fragment was cloned into pCB1532, which contains a sulphonylurea resistance selectable marker (Sweigard *et al.*, 1998) and confirmed by DNA sequencing. For construction of the RFP expression vector, pLMH21 was used (kindly donated by Lori Maggio-Hall and Nancy Keller) comprising a *gpdA* promoter-red fluorescent

protein (RFP) fused in-frame to 164 amino acids of the C-terminus of the β -oxidation enzyme, *FoxA*, containing a type-1 peroxisomal-targeting signal (Maggio-Hall and Keller, 2004). Plasmid pLMH21 was digested with EcoRI to excise the *argB* cassette and replace this with a hygromycin phosphotransferase gene cassette. The resulting construct was introduced into *M. grisea* strains expressing Pth2-GFP.

Transmission electron microscopy

Ultrastructural investigations were performed by material prepared by freeze-substitution fixation. Conidia of Guy 11, $\Delta pth2$ and $\Delta buf1$ were inoculated onto onion epidermis and allowed to produce appressoria. After 24 h, small squares of epidermis (2–3 mm²) were frozen in Freon-23, cooled to –180°C with liquid nitrogen, and were then transferred to substitution fluid consisting of 1% (w/v) OsO₄ in anhydrous acetone (Howard and O'Donnell, 1987) for 48 h at –80°C and then for 1 h each at –20°C and 0°C. This was followed by three washes in anhydrous acetone at room temperature and infiltration in Spurr's resin (Spurr, 1969) for 24 h each in four- and twofold dilutions in acetone and in pure resin. Samples were polymerized by heating to 70°C for 8 h. Thin sections were then contrasted for 10 min in 1% (w/v) uranyl acetate in ethanol and for 3 min in lead citrate (Reynolds, 1963) and viewed at 80 kV with transmission electron microscope (model JFM 100-S, Tokyo, Japan).

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