

A P-type ATPase required for rice blast disease and induction of host resistance

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To cause diseases in plants, pathogenic microorganisms have evolved mechanisms to deliver proteins directly into plant cells, where they suppress plant defences and facilitate tissue invasion^{1–3}. How plant pathogenic fungi, which cause many of the world's most serious plant diseases, deliver proteins during plant infection is currently unknown. Here we report the characterization of a P-type ATPase-encoding gene, *MgAPT2*, in the economically important rice blast pathogen *Magnaporthe grisea*, which is required for exocytosis during plant infection. Targeted gene replacement showed that *MgAPT2* is required for both foliar and root infection by the fungus, and for the rapid induction of host defence responses in an incompatible reaction. Δ *Mgapt2* mutants are impaired in the secretion of a range of extracellular enzymes and accumulate abnormal Golgi-like cisternae. However, the loss of *MgAPT2* does not significantly affect hyphal growth or sporulation, indicating that the establishment of rice blast disease involves the use of *MgApt2*-dependent exocytotic processes that operate during plant infection.

Plant pathogenic fungi are able to breach the external surfaces of plants and proliferate rapidly within plant tissue⁴. The rice blast fungus *M. grisea* produces specialized infection structures, called appressoria, to gain entry to the rice leaf, and subsequently develops a network of invasive hyphae to colonize host tissue^{5,6}. The fungus grows extensively in host cells before causing disease symptoms within 3–4 days of infection⁵. *M. grisea* can also infect roots, and elaborates alternative infection structures that allow hyphae to invade root tissue and the vascular system to cause systemic plant infection⁷.

The rapid internalization and spread of *M. grisea* hyphae through plant tissue occurs before disease symptoms become apparent, indicating that the fungus might have a capacity for the evasion or suppression of plant defences⁵. Sequencing of the *M. grisea* genome also revealed that the fungus produces many secreted proteins, indicating that the deployment of a large armoury of secreted peptides might be an important aspect of its pathogenicity⁸. To investigate the biology of plant tissue colonization by *M. grisea*, we decided to focus attention on the operation of the Golgi apparatus and protein trafficking in the fungus. Here we describe the characterization of a gene encoding an integral membrane P-type ATPase from *M. grisea*, belonging to the type IV, Drs2 family of aminophospholipid translocases (APTs). These enzymes are required in *Saccharomyces cerevisiae* for efficient Golgi function and are involved in both endocytosis and exocytosis^{9–11}. APTs maintain the asymmetrical distribution of aminophospholipids in cellular membranes, in which the aminophospholipids phosphatidylserine and phosphatidylethanolamine are enriched within the inner leaflet of the membrane bilayer, and phospholipids such as sphingomyelin and glycosphingolipids are found predominantly in the outer leaflet^{10,11}. Phospholipid asymmetry is important in membrane fusion events, such as vesicle budding and docking, which take place at both the

plasma membrane and in the *trans*-Golgi network¹⁰. *M. grisea* possesses four putative APT-encoding genes, *MgPDE1*, *MgAPT2*, *MgAPT3* and *MgAPT4* (Fig. 1a). Phylogenetic analysis revealed the *MgAPT2* gene product (*M. grisea* genome database accession number MG02767, Broad Institute, USA) to be closely related to P-type ATPases of the Drs2 family of APTs (Fig. 1a and Supplementary Table 1). The *MgAPT2*-encoded protein consists of 1,524 amino acids with a predicted molecular mass of 171.3 kDa, has ten predicted membrane-spanning domains, and all conserved features expected of a P-type ATPase¹² (Fig. 1b). To determine whether *MgAPT2* is functionally related to the Drs2 family of APTs, complementation experiments were performed. In yeast, a Δ *dnf1* Δ *dnf2* Δ *dnf3* yeast mutant exhibits hypersensitivity to calcofluor white (CW)¹⁰. An *MgAPT2* complementary DNA was expressed in the Δ *dnf1* Δ *dnf2* Δ *dnf3* triple mutant PFY3273A under the control of the *GAL1* promoter. Expression of *MgAPT2* in two independent yeast transformants restored the ability of PFY3273A to grow in the presence of 2.5 μ g ml⁻¹ CW (Fig. 1c). *MgAPT2* therefore encodes a P-type ATPase that is functionally related to the Drs2 family of APTs. However, *MgAPT2* failed to complement the temperature-sensitive growth phenotype of a *drs2* Δ mutant (Supplementary Fig. 1) and did not show plasma membrane aminophospholipid translocase activity on expression in yeast (Supplementary Fig. 2), indicating that in yeast *MgApt2* is able to fulfil some, but not all, of the functions of the Drs2 family of APTs^{10,11} and is unlikely to be localized in the plasma membrane.

To test the role of *MgAPT2* in rice blast disease, a one-step gene replacement was performed in which the 4.7-kilobase (kb) coding sequence of *MgAPT2* was removed and replaced with a 1.4-kb gene cassette conferring hygromycin resistance¹³ (Supplementary Fig. 3). Growth of six independent Δ *Mgapt2* mutants revealed no significant effect on either hyphal development or sporulation (data not shown). However, the ability of Δ *Mgapt2* mutants to cause rice blast disease in the susceptible rice cultivar CO-39 was significantly affected (Fig. 2a and Supplementary Fig. 3). The mean density of disease lesions on leaves inoculated with the wild-type *M. grisea* strain Guy11 was 34.33 ± 3.6 lesions per 5 cm leaf tip compared with 2.23 ± 1.5 in seedlings inoculated with the Δ *Mgapt2* mutant TM2105.6 ($P < 0.0005$). All the Δ *Mgapt2* mutants showed less epidermal invasion than seedlings inoculated with Guy11 and TM2105.1 ($P < 0.0001$) (Supplementary Fig. 3d). To test whether the pathogenicity defect was associated with appressorium function, rice leaves were abraded to remove the cuticle, and spore suspensions were applied. Under these conditions, *M. grisea* invades plant tissue without the need for an appressorium⁵. In rice seedlings inoculated with Guy11 and TM2105.1, disease symptoms appeared within 48 h in wounded leaves. In contrast, leaves inoculated with Δ *Mgapt2* mutant TM2105.6 did not exhibit symptoms until 72–96 h after inoculation (Fig. 2c). Plant tissue colonization was therefore delayed

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in leaves infected with $\Delta Mgapt2$, even in the absence of appressorium formation. Reduced fungal biomass and sporulation was observed from wounded leaves (not shown). The ability of $\Delta Mgapt2$ to infect roots, which does not require appressorium formation, was also completely impaired (Fig. 2b), although the root surface was colonized normally (data not shown). Ultrastructural analysis of appressoria revealed that $\Delta Mgapt2$ mutants formed abnormal penetration hyphae compared with Guy11 and did not completely penetrate plant epidermal cells (Fig. 2d–g), often arresting growth before invasion of the epidermal cell.

In yeast, the Drs2p APT and the Drs2p-related P-type ATPases Dnf1p and Dnf2p have been implicated in endocytosis. Drs2p is a Golgi-localized APT that has a role in late Golgi function¹¹; *drs2Δ* mutants are synthetically lethal with clathrin heavy chain (*chc1*) mutants and *arf1Δ* ADP-ribosylation factor mutants, and show

endocytotic defects, particularly in endosome–vacuole docking^{10,11}. Dnf1p and Dnf2p are both plasma membrane APTs, and null mutants show a cold-sensitive defect in receptor-mediated and bulk-phase endocytosis¹⁴. Furthermore, Drs2p is required for the formation of a specific subset of clathrin-coated secretory vesicles, indicating a role in exocytosis¹⁰. To determine the role of MgApt2p, we investigated the subcellular location of the protein. A V5 viral epitope tag sequence was incorporated into the 3' end of the *MgAPT2* gene. Expression of the resulting MgApt2–V5 fusion protein, after transformation of an *M. grisea* $\Delta Mgapt2$ mutant, complemented all mutant phenotypes and restored the ability to cause rice blast disease (Fig. 2a). For localization studies we generated an *M. grisea* strain expressing both the MgApt2–V5 fusion protein and a fusion protein of Ktr1 with green fluorescent protein (GFP). *MgKTR1* encodes a 1,2- α -mannosyltransferase (*M. grisea* genome database accession number MG08692, Broad Institute, USA), which is homologous to the Golgi-localized *S. cerevisiae* Ktr1 protein¹⁵. Immunofluorescence showed a punctate intracellular distribution pattern of the V5 epitope¹⁶ (Fig. 3a, b) co-localizing with MgKtr1–GFP fluorescence, which is consistent with localization to the Golgi. In filamentous fungi, discrete stacks of Golgi cisternae are rarely observed; instead, a disperse, vesicular Golgi apparatus is normally seen¹⁶. Immunogold electron microscopy confirmed the localization of MgApt2p to an internal vesicular compartment (Fig. 3c, d). We also performed an

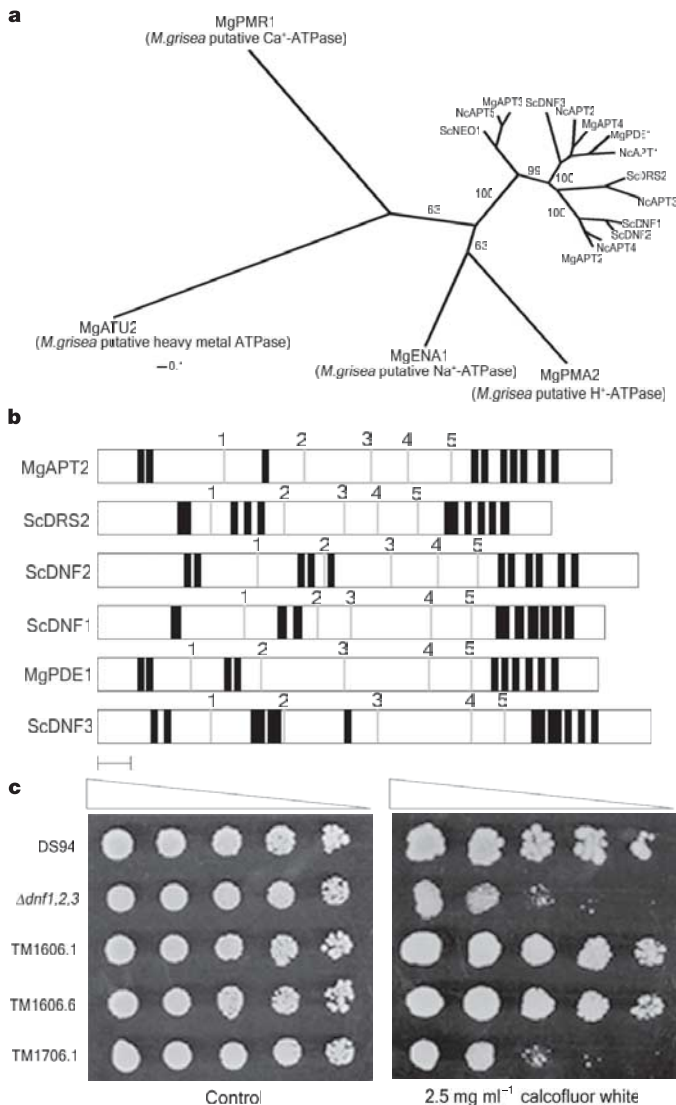


Figure 1 | Characteristics of *M. grisea* APT2. **a**, Phylogram of aminophospholipid translocases (APTs) from *M. grisea*, *S. cerevisiae* and *Neurospora crassa* (see Supplementary Information). **b**, Predicted topology of MgApt2, MgPde1 and yeast APTs. Thick lines indicate transmembrane helices. Thin lines are P-type ATPase motifs (1, LDGET; 2, DKTGTLT phosphorylation site; 3, KGA nucleotide-binding site; 4, LTGD.ATP binding site; 5, GDGXND hinge motif). Scale bar, 100 amino acids. **c**, *MgAPT2* was expressed in the *S. cerevisiae* CW-sensitive $\Delta dnf1,2,3$ mutant (TM1606.1 and TM1606.6). TM1706.1 is $\Delta dnf1,2,3$ transformed with the empty vector. DS94 was used as wild type. The triangle indicates reduced concentration of the inoculum.

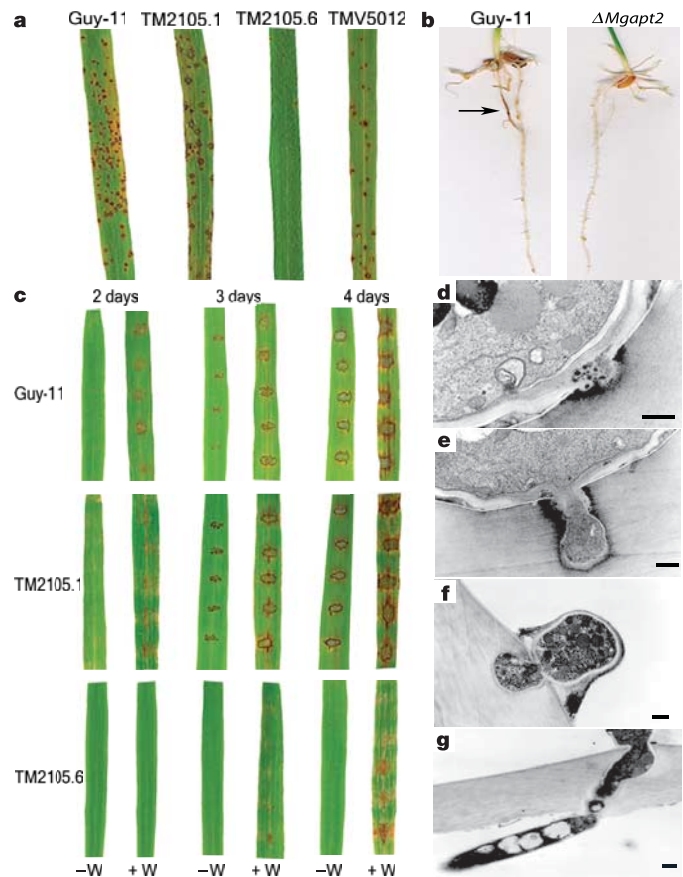


Figure 2 | Characterization of $\Delta Mgapt2$ mutants. **a**, Rice seedlings (cv. CO-39) were inoculated with Guy11, TM2105.1, TM2105.6 ($\Delta Mgapt2$) and TMV5012 ($\Delta Mgapt2$ expressing V5-epitope-tagged allele of *MgAPT2*). **b**, Blast symptoms on rice roots. Arrow shows necrotic lesion. **c**, Disease symptoms on intact (–W) and wounded leaf tissue (+W) inoculated with spore droplets. **d–g**, Transmission electron micrographs showing attempted penetration hypha development (**d**) and cuticle penetration (**e**) by TM2105.6 ($\Delta Mgapt2$) after 24 hours on onion epidermis compared with the same developmental stages in Guy11 (**f** and **g**). Scale bars, 500 nm.

ultrastructural examination of conidia, hyphae and appressoria of $\Delta Mgapt2$ mutants after cryofixation, which revealed the presence of abnormal membrane-bound ring structures within the cytoplasm after cold shock (Fig. 3e, f). Direct comparison with a *drs2Δ* yeast mutant, previously subjected to cold shock, highlighted the similarity of these structures to Berkeley bodies¹¹ (Supplementary Fig. 4). To determine whether endocytosis was affected by the loss of MgApt2p, uptake of the lipophilic styryl dye FM4-64 was studied in Guy11 and $\Delta Mgapt2$, but showed no difference between the strains (Supplementary Fig. 5).

However, we observed that $\Delta Mgapt2$ mutants were unable to grow on a range of substrates when these were provided as sole carbon sources. $\Delta Mgapt2$ mutants did not grow, for example, on starch, amylopectin or glycogen (Fig. 4a and Supplementary Table 2), and grew poorly on casein or lipids. We reasoned that these growth phenotypes could be a consequence of an exocytotic defect, preventing the secretion of extracellular depolymerizing enzymes. To test this idea we investigated the export of the starch-degrading enzyme α -amylase. Fungal mycelium was grown for 10 days in either glucose-containing medium or starch-containing medium, and α -amylase was detected in the culture filtrate with an enzyme-linked

immunosorbent assay (ELISA). The enzyme is induced only in the presence of starch and was readily detected at high concentration in culture filtrates of Guy11. In contrast, only trace amounts of α -amylase were detected in culture filtrates of the $\Delta Mgapt2$ mutant (Fig. 4b, c). Immunogold electron microscopy with an anti- α -amylase antibody confirmed the secretion defect of $\Delta Mgapt2$ mutants, and showed a predominantly cytoplasmic distribution of gold particles, which accumulated around the inside of the plasma membrane (Supplementary Fig. 6). However, only a subset of extracellular enzymes seem to require MgApt2p for secretion. An acidic trehalase, for instance, was secreted normally and the overall

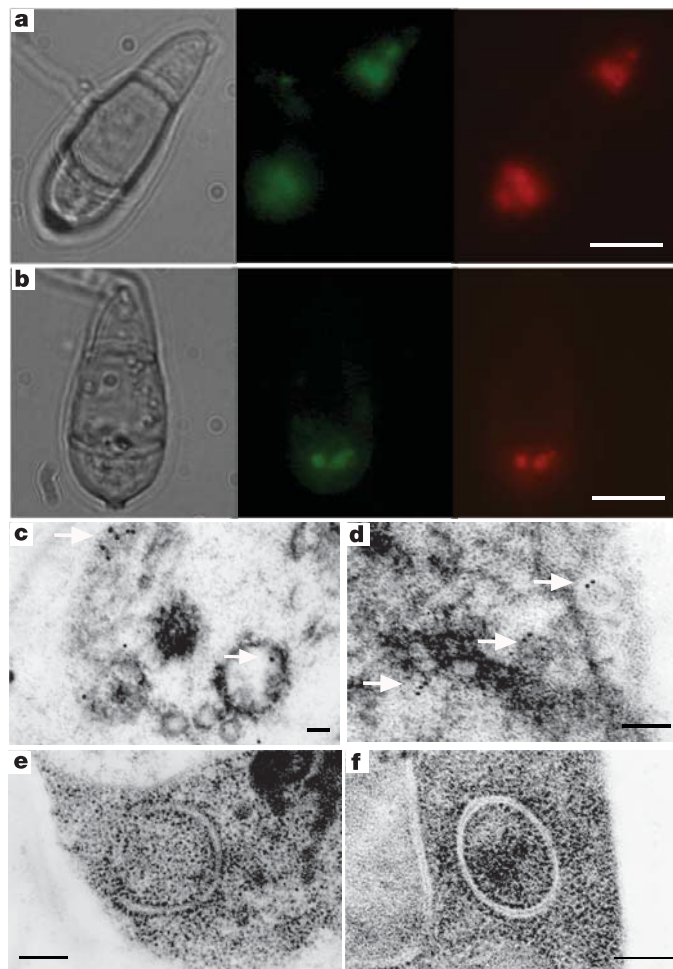


Figure 3 | Subcellular co-localization of MgApt2 and MgKtr1 Golgi protein. **a, b**, Micrographs of *M. grisea* conidium (left) expressing MgKtr1-GFP (middle) and MgApt2-V5 epitope-tagged allele (right) detected with Texas red (see Methods). Scale bars, 10 μ m. **c, d**, Representative immunogold electron micrographs of conidia from TMV5012 with the use of anti-V5 antibody. Scale bar, 100 nm (**c**); 250 nm (**d**). **e, f**, Transmission electron micrographs of TM2105.6 ($\Delta Mgapt2$) hyphae, incubated at 4 °C for 4 h before fixation, showing typical accumulation of circular intracellular membrane structures. Scale bars, 150 nm.

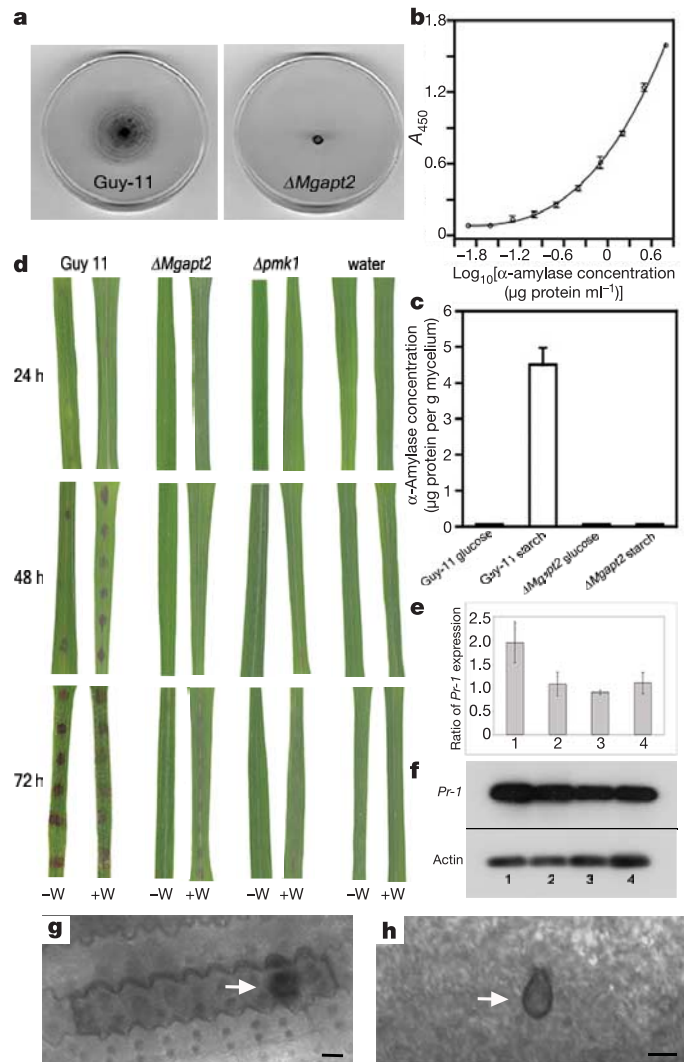


Figure 4 | $\Delta Mgapt2$ mutants are impaired in secretion and induction of rice defence responses. **a**, Impaired growth of $\Delta Mgapt2$ on starch-containing medium. **b, c**, Immunodetection of α -amylase in culture filtrates of Guy11 and $\Delta Mgapt2$ grown in glucose or starch. Enzyme concentrations in **c** were determined by converting absorbances from ELISA with anti-fungal α -amylase serum to equivalents of protein concentration by using a calibration curve (**b**) of purified fungal α -amylase. **d**, Elicitation of hypersensitive reaction in rice cultivar IR-68 in non-wounded (-W) and wounded (+W) tissues. **e**, Bar chart showing the expression of rice *Pr-1* relative to actin gene expression 48 h after challenge by Guy11 (1), $\Delta Mgapt2$ (2), $\Delta pmk1$ (3) and water (4), based on RT-PCR analysis shown in (**f**). Total RNA and resulting cDNA were quantified with an Agilent 2100 Bioanalyser. Values in **b, c** and **e** are means \pm s.d. for three repetitions of the experiment. **g, h**, Hypersensitive cell death in rice detected with Trypan blue, after challenge with Guy11 (**g**) or $\Delta Mgapt2$ (**h**). Appressoria indicated by arrows. Scale bars, 10 μ m.

amount of protein secreted from growing hyphae was not affected by the loss of MgApt2p (Supplementary Fig. 7). To determine whether other virulence-associated proteins require MgApt2 for exocytosis, we characterized the behaviour of Δ Mgapt2 in an incompatible interaction with its host. Rice cultivars resistant to *M. grisea* often exhibit a hypersensitive reaction in response to challenge with the pathogen. Rice and *M. grisea* show a gene-for-gene interaction in which single major genes for resistance in the host are required for the recognition of pathogen-derived molecules encoded by fungal avirulence genes^{2,5}. In *M. grisea* several avirulence gene products are now known⁸ and in AvrPi-ta and its cognate resistance protein Pi-ta a direct interaction within rice cells has been shown¹⁷. We inoculated rice cultivar IR-68, which is resistant to *M. grisea* Guy11, with a Δ Mgapt2 mutant and found an inability to elicit the hypersensitive reaction after 48 h (Fig. 4d). We reasoned that this might be due to the inefficiency of appressorium-mediated cuticle penetration of Δ Mgapt2 mutants. We therefore removed the cuticle by abrasion and inoculated IR-68 leaves with Guy11, a Δ Mgapt2 mutant and a non-appressorium-forming Δ pmk1 mutant¹⁸. Even after abrasion, hypersensitivity did not become apparent until 72 h after inoculation with a Δ Mgapt2 mutant (Fig. 4d). To investigate the plant defence response, the expression of the rice defence gene *Pr-1* (ref. 19) was analysed in IR-68 tissue by semiquantitative polymerase chain reaction with reverse transcription (RT-PCR) and found to be significantly decreased ($P < 0.05$) on challenge with the Δ Mgapt2 mutant compared with Guy11 (Fig. 4e, f). At the cellular level, hypersensitive cell death in IR68 as a response to Guy11 was observed by Trypan blue staining and occurred at a much lower frequency in response to Δ Mgapt2 infections (Fig. 4g, h). When considered together, these results indicate that the secretion of fungal proteins perceived by the rice plant host during a resistance response might also require MgApt2.

Characterization of the MgApt2 P-type ATPase has consequences for our understanding of fungal pathogenicity and the development of rice blast disease. It is clear from the genome sequence of *M. grisea* that a family of APTs exists in the fungus. Previous work has established that Pde1p (a functional homologue of yeast Dnf3p) acts as a virulence determinant in *M. grisea* by affecting appressorium function²⁰. The current report has shown that MgApt2, an APT with a role in exocytosis, is required for plant tissue colonization. Taken together, the studies provide evidence that APTs regulate membrane characteristics important to fungal pathogens, both for infection-related morphogenesis and for the efficient delivery of virulence-associated proteins. The identification of MgApt2p will allow the dissection of the processes required for secretion by pathogenic fungi during plant infection and will provide a method of identifying effector proteins essential for rice blast disease.

METHODS

Full details are given in Supplementary Methods.

Fungal growth conditions. All *M. grisea* strains used in this study are derived from Guy11 and were maintained as described previously. Yeast strains were manipulated in accordance with standard methods²¹.

Expression of MgApt2 in *S. cerevisiae*. A full-length MgAPT2 cDNA was obtained by rapid amplification of cDNA ends (RACE)²² with a Clontech SMART kit. Individual 3' and 5' RACE products were cloned into the pGEM-T vector (Promega), and the full-length cDNA was sequenced. DNA sequencing used a Global IR2 NEN automated DNA sequencer (LI-COR) and fluorescent infrared-labelled primers. The 4.5-kb MgAPT2 cDNA was cloned into pYES-2 (Invitrogen) and introduced into the *S. cerevisiae* *dnf1,2,3Δ* mutant²¹. Transformants were confirmed by PCR. Sensitivity to CW¹¹ was assessed by plating a dilution series of yeast cells (10^5 – 10^4 cells ml⁻¹) on yeast extract/peptone (YP) plates⁹ containing 2.5 μg ml⁻¹ CW in the presence of galactose for 17 h at 29 °C.

Targeted gene replacement of MgAPT2. An MgAPT2 gene replacement vector was constructed by amplifying 1.3-kb and 1.8-kb flanking sequences of the MgAPT2 coding sequence by PCR from Guy11 genomic DNA with 35 cycles. The products were cloned into pGEM-T and a BamHI-linked hygromycin

phosphotransferase selectable marker gene¹³ ligated between the two flanking sequences⁸. The linear 4.5-kb gene replacement construct was excised with *ApaI* and *SpeI* and introduced into *M. grisea*²³. Putative transformants were confirmed by Southern blots, as described previously.

Rice infections. Rice infections were performed with a susceptible Indica rice (*Oryza sativa*) cultivar CO-39 (ref. 24) or the resistant cultivar IR-68 (ref. 25). Uniform conidial suspensions were prepared and applied to rice leaves as described previously²³. Appressorium-mediated penetration was assessed on onion (*Allium cepa*) epidermal strips²⁶. Infection of wounded rice leaf tissue was performed by gently abrading the leaf surface with glasspaper before placing leaf sections on water agar plates (4% w/v) and inoculating with 10 μl droplets containing 10⁴ conidia ml⁻¹.

Subcellular co-localization of MgDRS2p and MgKTR-GFP. A 6.6-kb genomic DNA fragment spanning the MgAPT2 protein-coding region and 1.6-kb upstream sequence was amplified and cloned into pTOPO 2.1 (Invitrogen). A V5 epitope was incorporated by performing inverse PCR and ligation of the resulting amplicon¹⁶. The successful incorporation of the V5 epitope was confirmed by DNA sequencing. A sulphonylurea resistance gene²⁷ was cloned into the BamHI site of pMJGgAPT5 and the plasmid was transformed into the Δ Mgapt2 mutant. Sulphonylurea-resistant transformants were selected and analysed by Southern blotting. Co-localization studies were performed by generating a MgKtr1–GFP gene fusion. A 3.5-kb fragment of the MgKTR1 gene was isolated and an *XhoI* site was engineered at the 3' end of the gene to allow an in-frame fusion to the sGFP gene (see Supplementary Methods for details). The resulting construct was transformed into an *M. grisea* strain expressing pMJGgAPT5.

Immunological procedures. Immunofluorescence microscopy was performed as described²⁸. Conidia (10⁴ ml⁻¹) were allowed to germinate and form appressoria on multi-test ten-well glass slides (ICN) for 2–18 h. To allow antibodies access to internal structures, enzymatic digestion of the cell wall with Glucanex (50 mg ml⁻¹) was performed for 90 min at 20 °C. Anti-V5 antibody (Invitrogen) and Texas red-conjugated anti-mouse secondary antibody were added as described in Supplementary Methods. Samples were viewed on a Zeiss Axioskop 2 microscope with the Axiovision 3.0 software. For immunogold electron microscopy, procedures were as described previously²⁸.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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