An ATP-driven efflux pump is a novel pathogenicity factor in rice blast disease

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Cells tolerate exposure to cytotoxic compounds through the action of ATP-driven efflux pumps belonging to the ATP-binding cassette (ABC) superfamily of membrane transporters. Phytopathogenic fungi encounter toxic environments during plant invasion as a result of the plant defense response. Here we demonstrate the requirement for an ABC transporter during host infection by the fungal plant pathogen *Magnaporthe grisea*. The *ABC1* gene was identified in an insertional mutagenesis screen for pathogenicity mutants. The *ABC1* insertional mutant and a gene-replacement mutant arrest growth and die shortly after penetrating either rice or barley epidermal cells. The *ABC1*-encoded protein is similar to yeast ABC transporters implicated in multidrug resistance, and *ABC1* gene transcripts are inducible by toxic drugs and a rice phytoalexin. However, *abc1* mutants are not hypersensitive to antifungal compounds. The non-pathogenic, insertional mutation in *ABC1* occurs in the promoter region and dramatically reduces transcript induction by metabolic poisons. These data strongly suggest that *M.grisea* requires the up-regulation of specific ABC transporters for pathogenesis; most likely to protect itself against plant defense mechanisms.

Keywords: ABC transporter/insertional mutagenesis/*Magnaporthe grisea*/multidrug resistance/phytopathogenicity

Introduction

The ability to withstand toxic compounds in the natural environment is an important adaptation for all microorganisms. In particular, microorganisms often encounter an array of pre-formed and inducible chemical barriers during their attempts to infect plants. To defend themselves against fungal pathogens, plants produce an array of antifungal proteins (Dixon and Lamb, 1990; Dixon *et al.*, 1994) as well as specialized antibiotics called phytoalexins (for a review see Osbourn, 1996). Only pathogens which can evade these plant defense responses during early infection stages are able to survive and cause disease. Although some fungi are capable of producing phytoalexin-detoxifying enzymes, mutants defective in these enzymes show only modest reductions in virulence (VanEtten *et al.*, 1989, 1994). Thus, other fungal

mechanisms must exist to ensure survival in plant cells. An understanding of these pathogenic mechanisms can lead to improved and informed design of antifungal compounds and genetically engineered crops.

The rice blast fungus *Magnaporthe grisea* causes the most economically devastating disease of cultivated rice (Ou, 1985). Genetic and physiological studies have provided an ever increasing understanding of the mechanisms of fungal attachment and pathogenicity (Talbot, 1995; Hamer *et al.*, 1997). Penetration is mediated by the differentiation of a specialized cell called the appressorium. Once inside the plant cell, the fungus forms bulbous infection hyphae that rapidly spread to adjacent cells. Eventually, lesions develop at the infection site which yield mycelia that sporulate and release more conidia to reinitiate the disease cycle. Few genes are known to be required for invasive growth, although numerous fungal metabolites have been proposed to act as toxins (Umetsu *et al.*, 1972; Nukina, 1988) and many rice metabolites have been proposed as phytoalexins (Akatsuka *et al.*, 1985; Kodama *et al.*, 1992).

To identify genes required for invasive growth and survival in plant cells, we performed an insertional mutagenesis screen using DNA-mediated transformation and screened transformants for defects in pathogenicity by leaf sheath injection (Xu and Hamer, 1996; Lau and Hamer, 1998). Insertional mutagenesis has been used to identify genes involved in fungal morphogenesis and pathogenesis (Lu *et al.*, 1994; Bölker *et al.*, 1995; Dufresne *et al.*, 1998; Sweigard *et al.*, 1998). Leaf sheath injection obviates the need for *M.grisea* to infect from appressoria, and thus would be predicted to identify genes involved in plant cell invasion.

Here we describe the characterization of a novel insertional mutant of *M.grisea* dramatically reduced in pathogenicity. The affected gene, *ABC1*, encodes a protein with a high degree of relatedness to proteins of the ATP-binding cassette (ABC) transporter family. We show that mutations in *ABC1* arrest fungal growth early in pathogenicity. *ABC1* mutants do not display drug sensitivity phenotypes characteristic of other mutated fungal ABC transporter genes. An analysis of the regulation of *ABC1* mRNA levels in wild-type and mutant strains shows that up-regulation of *ABC1* is essential for pathogenicity in *M.grisea*. Our findings provide the first evidence that specific ABC transporters may play a role in fungal pathogenicity, most likely as a defense against antimicrobial compounds produced by the host.

Results

Isolation of *ABC1*

In an insertional mutagenesis screen for non-pathogenic mutants of *M.grisea* wild-type strain Guy11 (Lau and

Hamer, 1998), the mutant strain TF7-3131 was isolated as a strain with dramatically reduced pathogenicity on rice cultivar CO-39. Random ascospore analysis indicated that the reduced pathogenicity phenotype co-segregated with the hygromycin resistance marker conferred by the integrating plasmid (data not shown). The plasmid insertion in strain TF7-3131 was rescued and transformed in *Escherichia coli*. The recovered plasmid, pRP27, contained a 3.9 kb *Bgl*II DNA fragment, which detected a restriction polymorphism between Guy11 DNA (wild-type) and the TF7-3131 genomic DNA digested with the enzymes *Bgl*II, *Kpn*I, *Mlu*I, *Eco*RI and *Xho*I (Figure 1A). A subcloned fragment contained in pRP27 revealed high similarity to ATP-binding cassettes (ABCs) from genes of the ABC transporter superfamily. This fragment was used to isolate clones from a genomic λ library and a cDNA library. DNA sequence analysis from the genomic λ clones and cDNA clones revealed a large open reading frame (ORF) for an ABC transporter in *M.grisea*, hereafter designated *ABC1*. Low stringency Southern blot hybridization demon-

strated that *ABC1* is a single-copy gene (data not shown). The plasmid insertion of strain TF7-3131 is located in the putative promoter region 718 bp upstream of the start codon of *ABC1* (Figure 1B). The insertional mutation in TF7-3131 was designated *abc1-1*.

Features of the *ABC1* gene

An ORF for a protein of 1619 amino acids (Figure 2) was deduced by sequence analysis. This required proposing the existence of five introns of 231, 73, 68, 72 and 85 bp in length, respectively. The presence of these five introns was verified by sequencing three independent cDNA clones. The *ABC1* genomic sequence is available in the DDBJ/EMBL/GenBank database under the accession No. AF032443. The ORF is preceded by putative promoter elements, including CAAT consensus sequences at positions -524 and -477, and a TATA-like consensus (TATAAC) at position -114.

Comparison of the deduced protein with sequences in the SWISS-PROT protein sequence database revealed significant similarity with members of the ABC transporter superfamily. The highest degree of identity was obtained with Cdr1 from *Candida albicans* (Prasad *et al.*, 1995) and Pdr5 from *Saccharomyces cerevisiae* (Balzi *et al.*, 1994) using the FASTA program (Pearson and Lipman, 1988). Both proteins show 47% identity to Abc1. Hydrophathy analysis (Kyte and Doolittle, 1982) and protein alignments (Figure 2A) show that Abc1 displays a structure and domain organization typical of membrane proteins of the ABC transporter superfamily. It is composed of two homologous halves, each comprising one N-terminal hydrophilic domain followed by a C-terminal hydrophobic domain (Figure 2B). Each hydrophilic domain contains an ABC typical of ABC transporters: the N-terminal hydrophilic domain consists of a Walker A motif (GPPGSGCST), an ABC signature sequence (VSGG-ERKRVIA) and a Walker B motif (QCWDNSTRGLD) (Walker *et al.*, 1982). The C-terminal ABC shows a Walker A motif (GVSGAGTTL), a Walker B motif (LFVDEPTSGLD) and a modified ABC signature sequence (NVEQRKRLTIGV) identified in fungal ABC transporters from *S.cerevisiae* and *C.albicans* (Decottignies and Goffeau, 1997). For each of the two hydrophobic domains, six transmembrane-spanning domains were predicted using the program PREDICT PROTEIN (Rost *et al.*, 1995) (Figure 2B).

Disruption of *ABC1*

The insertional mutation in strain TF7-3131 (*abc1-1*) results in a dramatic reduction in disease symptoms (see below). However, the insertion may not create a null phenotype because it occurred 718 bp upstream of the ORF. Therefore, we created an *ABC1* deletion allele (*abc1-2 Δ*) by one-step gene replacement (Figure 3A). We replaced 1.9 kb of the ORF of *ABC1* with a gene encoding hygromycin phosphotransferase (*HPH*) (Carroll *et al.*, 1994) in gene replacement vector p Δ ABC1. The deleted region contains almost all of the N-terminal half of the ABC transporter (Figure 2B) including the N-terminal ABC and the N-terminal six transmembrane domains. p Δ ABC1 was linearized with *Not*I and transformed into strain Guy11. Transformants were selected on hygromycin-containing medium. Ten out of 48 hygromycin-resistant

Fig. 1. The *ABC1* locus in *M.grisea*. (A) Five micrograms of genomic DNAs isolated from strains Guy11 (lanes 1, 3, 5 and 7) and TF7-3131 (lanes 2, 4, 6 and 8) were digested with *Bgl*II (lanes 1 and 2), *Kpn*I (lanes 3 and 4), *Mlu*I (lanes 5 and 6) or *Eco*RI (lanes 7 and 8), separated on a 1% agarose gel and blotted. The blot was hybridized with probe SC1.0, a 1 kb *Sac*I DNA fragment of the rescue plasmid pRP27, indicated in (B). (B) Restriction map of a 10 kb DNA region containing the *ABC1* gene indicating the position and orientation of the insertion in TF7-3131. The large arrow indicates the direction of transcription and the length of the ORF. Introns are indicated by hooks. The site of plasmid insertion is shown. The *HPH* gene is indicated as a white box. Restriction enzymes are: N, *Not*I; K, *Kpn*I; E, *Eco*RI; M, *Mlu*I; X, *Xba*I; S, *Sal*I; Bg, *Bgl*II.

A

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ABC1 1 MSQPVEDPSHDOARNDAQTETDTQNASMPKTHCHDQESSATCISSSPADTLMDEKQAAFTPEPSTTEKARVPDPRIASVASDTEAEFFDDNDSEQRNRN
PDR5 1 ..MPKAKNNVNDVSYSSASSSENADLHN..WNOGDER.....FEAR.....TOKLARTLAQSMQNSQSAFNRSD.....
CDR1 1 .....MSDSKSSQDESLEKKAISQDSSETHSYNE..WNOGDA.....TSN.....IQNLARTTHDSFKDDSSAGLLKYL.....

ABC1 101 EMVQQARVYTSRSNASAAADEYGNANFELIASDPF..DSPFNPSQNFERYAWARALACMVAAEGGSRF..TTCGCFQNNMVGFQAAADQKTVSNVW
PDR5 67 ..AQSISSSGVEG.....VNPIFSDPAPGYPFLDPNSENENSSAAMVKMMAHLSAADPDPYKPYSLGCWKNLSASGASADVAYQSTVVMNIP
CDR1 67 .....TMSSEVPG.....VNPIKSHHEINN.....DQLNEDSENFAKFWVKNLRLRFESDPEYKPSRLGIQYRNLRAAGVANDSDYQPTVNAL

ABC1 197 LEANLMDRTAVGMGKTRIDILKGFNGVVRNGELVVLVLPSSGCCSTFLKTIAGEFTNGLNVDSQAYFNVQGLSAEEMHKHNRGEATYAEVDVHFQQLS
PDR5 154 VRLKSLGRKFQKSKENTFQILKPMDCGKMPGELLVVLGRPGSGCCTLLKSHSSNTHGFDLGADTKISVSGYSGDDIKKHNRGEVYVNAEADVHLPLHL
CDR1 148 KKLATEGFRRFQKDDERYFDILKSMDAKMRPGELTVVLGRPGAGCSTLLKTIATVNTYGFHIGKESQITVDGLSPDIERHVRGDDVIYSAEVDVHFPHLS

ABC1 296 VGDILTFAANARAPRRAPPGVSKTLFANHRDVMNATIGSHTNTNVGNLYRGVSGGERKRVIAEAALSGAPLQCWDNSTRGDLSANALEECKTDRV
PDR5 254 VFTFLVTVARLTPQNRIKGVDRSEYANHCALVAMATYGLSHTRNTNVGNDIVRGVSGGERKRVIAEVSICGSKFQCWDNATRGDLSATALEFIRALKT
CDR1 248 VGDITLFAARLRTFQNRGEGADREYARHMASVYMATYGLSHTRNTNVGNDIVRGVSGGERKRVIAEASLSGANQCWDNATRGDLSATALEFIRALKT

ABC1 396 CTRIFOTTACVSIYQAPASAYD.FDKRAVVLYEGYQIYFGPADEAKQYFVNLCGECPAR..TTPDPLTSTAPHERIVRPGDEGR...APRTEPEFAIAWEN
PDR5 354 QADISMTSATVAIYQCSQDAYDLEKQVLDDEGYQIYFGPADKAKKYFEDMGVPCSRQTTADPLTSVTSPPSERTENKMDLKKGIHMPQTPKEMNDYVWK
CDR1 348 SAVILDTPTLTAIYQCSQDAYDLEKQVVLVLYEGYQIFGRATRAKKEFEREMGMKCPORQTTADPLTSTNPAEREPLPGVEDK...VPRTAQREETVWKN

ABC1 492 SAELYTAQADIIEYKSSHPINGPDAAEARKSRNAQQCGRGORPNSPYTTSEFYQQTKLCLWRGWKRLLGDPALTVCALFANTLMALVSSIFFNLQMT..TS
PDR5 454 SPVYKELMKKEVDQRLND..DEASREATENHNAKQSRARPSPYTVSMMQVKYLLIRNMWRLNNIGFTFEMILGNCSMALILGSMFFKMKKGDT
CDR1 445 SPEYAEITKEIDEYFVECE.RSNRREYRRESHVAKQSNNTRPASPYTVSFMQVRYGVARNLRLMGDPSTPTESVFGQLVMGLILSSVFNNLSQT..TG

ABC1 590 SFQORGALFFACILNCFPALEILFLAQRPIVEKEHDYALYHPSADAAVASMCDMPYKVFNAIVFNLVLYFMANLRRPFGAFFYLLHSFATVLAMSH
PDR5 552 AFYVRCGAMFFAFLFNAFSSLLLEIFSLFEARPTIEKEKHTVSLYHPSADAFASVLSSEPSKLITAVCFNLIFVFIVDFRRNGGVFFYLLINIVAFESMSH
CDR1 542 SFYVRCGAMFFAFLFNAFSSLLLEIFSLFEARPIVEKEHDYALYHPSADAFASVLSSEPSKLAMSMSENFVFYFVMVFNRRNPGRFFFYWLCICWCTFVMSH

ABC1 690 MFRFIASMSRSLSCAMVPAALNLLILNITGFIPLDYMFLPWRWNYIILILAYSFESLLINEFAGQRYTCTEMVPRAEFPGYDLSGTRNVCAVGSVA
PDR5 652 LFRCVGSHITKTLSCAMVPAALNLLILNITGFIPLDYMFLPWRWNYIINPLAYFESLLINEFHGKFFCAEYVPRG..PAYANISSTESVCTVVGAVP
CDR1 642 LFRSIGAVSSTSCAMVPAVLLLAIVLYTGFIPTPSMLGWSRWINYINPVGYVFESLVNVEFHGREFCQAQYVPSG..PGVENISRSNQVCTAVGSVP

ABC1 790 GQPVVKGMDYLYSSFRFESANKWRNFGILIAFMFFCSRTWLRPRMCKRKSKEGLVFRF.....GQPPAAIKDAKTDPBAGPPK..VGGAVVBAANMT
PDR5 750 GQDYVLGDDFTRGAYQYVHKDKWRGFGIGMAVVFVFFVVFVFCENEGAKQKGEILVFPRSIVKRMKNRGVLTERRNANDPENVGERSDLSDDRMKLGES
CDR1 740 GNEVMSCTNYLASAYQYVNSHKWRNLGITGFAVFLAIYALTEENKGAQKGEIVFLKGSLEKKEKRTAASNKGDIAGVAVGLDYQDEAEVWNE

ABC1 882 GBNAG.....FXQRTSTFGWRDVCYEVQIKKETRRILDHVDGWVKPGTLTALMGVSGAGKTLLDCLADRTSMGVIT..GEMLVGHORDASFORKTGY
PDR5 850 SEESDSTYGEHGLSKSEATFHWRLCYEVQIKKETRRILNVDGWVKPGTLTALMGASGAGKTLLDCLAEVTVMGVIT..GDLVNGIIPDKSFPRSIGY
CDR1 840 KFTKGTGCTGVDFENREIEFWRLDTYQVKIKKEDRVILDHVDGWVKPGTLTALMGASGAGKTLLNCLSERVTGTGHTDGERLVNGHADDSFQRSIGY

ABC1 975 VQOQDLHLQATVREALFSAFLLROPAEVPRAEKLAAYDEVITLLDQYADAVVGVGEGLNVEQKRRTIGVELAAKPPLLLVDEPTSGLDSQTSWA
PDR5 949 QOQDLHLKATVRESLRFSAYLROPAEVSLEENRNVDEVITLLEMEKYADAVVGVGEGLNVEQKRRTIGVELAAKPKLLVFLDEPTSGLDSQTAWS
CDR1 940 VQOQDVHLPTSTVREALFSAYLROSNKISLKERDDYVDVVIDLELTDYADAVVGVGEGLNVEQKRRTIGVELAAKPKLLVFLDEPTSGLDSQTAWS

ABC1 1075 ILDTGKLTGSGQAILCTIHOPSAALFQRFDRLLFLAKGGKTVYFGDLGNSKIMTDYFERNGCFPCPDANPAENWMLVVGASPGTSDIDWHQAWRES
PDR5 1049 ICQLMVKLANHGQAILCTIHOPSAALMOEDRLLFLQGGKTVYFGDLGEGCKTMDYFESHGAKCPADANPAENWMLVVGGAAPGSHANQDYMEVWRNS
CDR1 1040 ICKLMVKLANHGQAILCTIHOPSAALMAEDRLLFLQKGGRATVYFGDLGNCQPMIYFERYGADPCPKANPAENWMLVVGGAAPGSHANQDYMEVWRNS

ABC1 1175 PECADVHAELDRKKEQVNTPEPTEDKASYREFAAFTHOQIYAVTRRVFQOYWRTPSYIYKAADCAVTALFIGFVFDAPNTQOGLQONQMAFHNILT
PDR5 1149 EBYRAVQSELDWMERELPKKGSIAAEDKH.EFSQSIYQTKLVSRIRFQOYWRSDYLVSKFILTITFNQLFIGFPPFRKACTSLOGLQONQMLAVFMETVI
CDR1 1140 SEYQAVREIEFMRMEABLSKLE.RDNDPEALLKYAAPWKQYLVLSWRTIVQDWRSPGYYSKIFLVVSAALFNGSFFKAKNNMQGLQONQMFVFMETIP

ABC1 1275 FGOVLVOQTMPEFVHORDLYEVRERPSSVYSKWKVLSQIIVLVEIPWNSLNAVIMFFCWYYPVGLERNAILADQVTERGATAMLYWGLIFTSFTDLMIA
PDR5 1248 FNPVVOQYLPSEVQORDLYEAREPSTRFSWISIFIAQIVVEVPWNLAGTIAMFIYYPGTFYNSASAAGOLBERGALFVLSCAFVYVVGSMGLLVIS
CDR1 1239 FNPVVOQMLPYFVHORDLYEVRENPSTRFSWFAIAQIATSEIPIYQAVGTIAFFCWYYPGLYNNATPTDSVNPGRGLVMMVLTAFYVVTATMGQCMIS

ABC1 1375 GFETAEAGGNHANFESLCLNFCGVLANPDMPRFWIFMYRVSPFTYVSGLLSVVANSEVRCASNETLHEDPLN.GTCAEFMRNYINGTTTIPGLGRIP
PDR5 1348 ENQVABSAANLASLFTMSLSEFCGVMTTPSAMPRFWIFMYRVSPFTYVQALLAVGVANVDVKADVELLEETPPSGMTCCQYVMSPYLQAKT.....
CDR1 1339 ESEADNANLANLFLFTMCLNFCGVLAGPDVLPSEFWIFMYRCNPFTYVQAMLSLGLANFVKCAEREYVSVKPPNGESSTVEDPYIKFAGG.....

ABC1 1474 GAGGYLRPDESSRSNCAFCEPKDNTNIFLQGAHANVNDWRNFCGLFVYIENITIALEFVYVAVRVPKKLGKGDAAAGVGAGAGAARASASNEKGKMQR
PDR5 1440 ...GYLTDN...ATDTCFCQISTNDYLANVNSFYSERWRNFGIFCYIAFNITACVFFYWLARVPKK..NGKLSKK.....
CDR1 1431 ...YFETRN...DGCAPFCQMSSTNIFLKSVNSLYSERWRNFGIFIAFLAINIILTVIFYWLARVPKGN.REKKNKK.....

ABC1 1574 EKGEVEGLTAVLGTSVAGSDAPMTTTEGEGERAKRRTSGDEVVR
PDR5 .....
CDR1 .....

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B

transformants that had the *ABC1* gene replaced with the *abc1-2Δ* allele (data not shown) were identified in a preliminary screen. *ABC1* deletion transformants were confirmed by Southern blot analysis. Hybridization analysis with probe BA1.6 (Figure 3A) showed that the strain Guy11 transformants AM30 and AM36 contained the *ABC1* 3.0 kb *EcoRI* fragment (Figure 3B). In addition, transformants AM30 and AM36 contained a high molecular weight fragment of >14 kb, indicating an integration of the gene replacement vector elsewhere in the genome. Transformants AM25, AM27, AM31 and AM35 show the 1.9 kb *EcoRI* fragment as expected for a correct gene replacement event.

Infection assays on rice cultivar Sariceltic were performed to test the pathogenicity of *ABC1* deletion mutants. Mutants TF7-3131 (*abc1-1*) and AM25 (*abc1-2Δ*) are reduced in pathogenicity to the same extent when compared with control inoculations of strain Guy11 or transformed strains with an intact *ABC1* gene (AM30) (Figure 4 and Table I). Pathogenicity assays of additional independently derived *abc1-2Δ* strains gave identical results (data not shown).

Magnaporthe grisea strain Guy11 can cause lesions on other rice cultivars and on barley. Thus we inoculated two other host plants, rice cultivar CO-39 and barley cultivar Golden Promise, with strains AM25 (*abc1-2Δ*) and TF7-3131 (*abc1-1*). The pathogenicity defect of *abc1* mutants was similar on all tested host plants (data not shown).

Resistance to rice blast is correlated with visible necrotic pinpoint lesions, called hypersensitive (Type 1) lesions (Valent *et al.*, 1991). Microscopic examination of infected leaves did not show a high induction of Type 1 lesions (data not shown), suggesting that *abc1* mutants arrest growth and die early in the pathogenicity process. However, rare large lesions were observed following inoculation with the insertional mutant (*abc1-1*) and the deletion mutant (*abc1-2Δ*) (see Figure 4, leaves D and E). The formation of rare large lesions is not due to rare suppressor mutations because strains obtained from these rare lesions produced phenotypes identical to the original mutants when re-inoculated onto susceptible hosts (data not shown).

A detached leaf assay (see Materials and methods, and Figure 5) was used to see if *abc1* mutants were weakly pathogenic. In this assay, more concentrated inoculations can be used to test if strains induce a hypersensitive response, or if they are weakly pathogenic. Drops of conidial spore suspensions were placed on 4×12 mm pieces of rice leaves from cultivars CO-39 and Sariceltic. After 6 days incubation, only rice leaves inoculated with strains Guy11 and AM30 showed visible fungal growth and sporulation. Strain nn78, which is defective in appressorium formation, and mutants AM25 (*abc1-2Δ*) and TF7-3131 (*abc1-1*) did not show detectable fungal growth.

Furthermore, viable *abc1* cultures could not be recovered from these assays (data not shown). These results demonstrate that the block in pathogenicity in *abc1* mutants is caused during early steps in pathogenesis.

Table I and Figure 6 show the results of a quantitative penetration assay on detached barley leaves (see Materials and methods). The isogenic wild-type strain penetrated barley epidermal cells and differentiated dense spreading infection hyphae within 60 h (Figure 6A and B). In contrast, the majority of *abc1* mutants formed appressoria that failed to elaborate extensive infection hyphae (Figure 6C). Only in rare instances were small infection hyphae observed (Figure 6D).

The ability of *M.grisea* to infect its host and cause disease symptoms is a complex phenotype. Fungal conidia have to germinate, form appressoria and produce lesions. We compared growth rates and conidiation on oatmeal plates, spore attachment and the rate of appressorium formation on plastic coverslips. However, we could not detect any significant difference between Guy11 and *abc1*

Fig. 3. Replacement of the *M.grisea* *ABC1* gene. (A) *ABC1* locus and gene-replacement vector. (B) Southern analysis of *abc1-2Δ* transformants. The genomic DNAs prepared from wild-type strain Guy11 (lane 1), *ABC1* deletion mutants AM25, AM27, AM31 and AM35 (lanes 2, 4, 5 and 6) and ectopic integration transformants AM30 and AM36 (lanes 3 and 7) were digested with *EcoRI* and separated in a 1% agarose gel. The blot was hybridized with probe BA1.6, a 1.6 kb *Bam*HI fragment from pBA1.6, indicated in (A).

Fig. 2. Predicted structure of the Abc1 transporter of *M.grisea*. (A) Amino acid sequence alignment of Abc1 with *S.cerevisiae* Pdr5 and *C.albicans* Cdr1. Sequences are aligned using the CLUSTAL W program (Thompson *et al.*, 1994). Identical amino acids are highlighted on a black background, while similar amino acids are shown on a gray background. Gaps in the alignment are indicated by dots. (B) Topological model of the Abc1 protein. Secondary structure was predicted using the program PredictProtein (Rost *et al.*, 1995). The predicted Abc1 protein consists of two halves. Each half comprises one hydrophilic domain followed by one hydrophobic domain. The two hydrophilic domains are predicted to be cytoplasmic (IN), and contain one Walker A and one Walker B ATP-binding motif each (boxed). The conserved ABC signature sequence (black box) in the N-terminal and the modified ABC signature sequence (white box) in the C-terminal hydrophilic domain are indicated as well. The hydrophobic domains are predicted to be integrated into the plasma membrane through six transmembrane helices (shaded boxes). The deleted region in *abc1-2Δ* mutant strains is indicated.

Table I. Phenotypic characterization of *abc1* mutant strains

	Guy11	AM25	TF7-3131
Pathogenicity ^a (lesions/leaf)	10.3 ± 5.8	0.2 ± 0.7	0.8 ± 1.3
Infection hyphae ^b (%)	65 ± 12	4 ± 1	4 ± 3
Growth ^c (cm/day)	0.28 ± 0.02	0.27 ± 0.04	0.27 ± 0.03
Conidiation ^d (×10 ⁴ conidia/ml)	32 ± 10	70 ± 19	47 ± 15
Spore attachment ^e (%)	90 ± 14	92 ± 7	82 ± 8
Appressorium formation ^f (%)	>95	>95	>95

^aPathogenicity was quantified as disease lesions produced per leaf after spray inoculation of rice cultivar Sariceltic. Lesions of 56 second rice leaves were counted; mean ± SD.

^bFormation of infection hyphae was determined microscopically using detached barley leaves of cultivar Golden Promise 60 h after infection. At least 800 appressoria were evaluated per strain.

^cColonies were grown on oatmeal agar under fluorescence light. Growth rates were recorded after 5, 7 and 10 days. The experiment was repeated twice with four replicates.

^dConidia were collected from 10-day-old oatmeal agar plates and counted. The experiment was repeated twice with four replicates.

^eConidial suspensions were placed on plastic microscope coverslips (Fisher) and allowed to attach for 2 h. More than 100 conidia in marked areas were counted and coverslips were washed three times with water from a squeeze bottle. Marked areas were then recounted. Results are combined data from two experiments, each with three replicates.

^fConidia that formed appressoria on plastic coverslips (Fisher) were counted after 24 h. Results are combined data from two experiments, each with six replicates; 100 germinated conidia were examined per replicate.

Fig. 4. *abc1* mutants are highly reduced in pathogenicity. Seedlings from rice cultivar Sariceltic were spray-inoculated with gelatin solution (A), or conidial suspensions (2×10⁴ conidia/ml) of (B) wild-type strain Guy11, (C) ectopic integration transformant AM30, (D) deletion mutant AM25 (*abc1-2Δ*) or (E) insertional mutant TF7-3131 (*abc1-1*). Leaves A, B and C represent typical leaves from inoculated plants. Leaves D and E show leaves containing rare lesions (see text). Typical leaves lacked signs of infection.

mutants (Table I). We conclude that the majority of *abc1* mutant conidia fail early in pathogenesis, most likely just after penetration.

***ABC1* and drug sensitivity**

Mutations in the yeast multidrug resistance (MDR) transporter proteins Pdr5 and Cdr1 result in acute sensitivity

to a range of metabolic poisons and antifungal drugs (Balzi *et al.*, 1994; Hirata *et al.*, 1994; Sanglard *et al.*, 1996). To determine whether *ABC1* plays a role in multidrug resistance, we tested the effects of metabolic poisons and antifungal drugs on wild-type strains and mutant strains AM25 and TF7-3131. In Table II, sensitivity concentrations for each drug were determined using the wild-type strain Guy11, so as to reduce appressorium formation to 50% in spore germination assays. In growth assays on agar plates, minimal inhibitory drug concentrations (MICs), sufficient to suppress fungal mycelial growth, were determined. The tested drugs included protein synthesis inhibitors (cycloheximide and chloramphenicol), sterol biosynthesis inhibitors (fenarimol, fenpropimorph, metconazole, miconazol, propiconazole, tebuconazole and terbinafine), an inhibitor of protein secretion (brefeldin A), a microtubule poison (benomyl), the mutagenic agent 4-nitroquinoline-1-oxide (4-NQO) and the rice phytoalexin sakuranetin (Kodama *et al.*, 1992). Our assays did not detect any difference in drug sensitivities between *abc1* mutants and Guy11 (Table II).

Transcriptional regulation of *ABC1*

MDR-type ABC transporters, such as Pdr5 or the homologous transporter Snq2 in *S.cerevisiae* which are involved in the efflux of xenobiotic compounds, are transcriptionally up-regulated by exposure to metabolic poisons and by heat shock (Miyahara *et al.*, 1996). To investigate whether *ABC1* is subject to MDR regulation, we examined the effects of a variety of antifungal compounds and metabolic poisons on *ABC1* mRNA levels by RNA blotting.

For these experiments, a genomic 1.8 kb *SalI*–*KpnI* DNA fragment containing the N-terminal hydrophilic ABC domain and parts of the N-terminal transmembrane-spanning domains was used as a probe. The specificity of this probe was tested in a blot analysis of RNA prepared from strain AM25 to exclude the possibility of cross-hybridization with other ABC transporters. In AM25, the corresponding 1.8 kb *SalI*–*KpnI* DNA fragment is deleted and no signals were detected in RNA prepared from this strain (data not shown).

Figure 7A shows that treatment of wild-type cultures with the antifungal compounds miconazole and metconazole, as well as the rice phytoalexin sakuranetin and the protein synthesis inhibitor hygromycin dramatically induced *ABC1* transcript levels. Stationary phase growth, dimethylsulfoxide (DMSO) treatment, heat shock or nitrogen starvation had no effect on *ABC1* transcript levels (data not shown).

Interestingly, the insertion of the transforming plasmid into the 5' region of *ABC1* results in the same pathogenicity phenotype as the deletion allele. Thus loss of pathogenicity in *abc1-1* strains may be due to an inability to express or up-regulate *ABC1*. To investigate this possibility, we monitored *ABC1* transcript levels in TF7-3131 and Guy11 before and following exposure to cycloheximide.

Figure 7B shows that *ABC1* is transcribed at a very low level in strains Guy11 and TF7-3131. The addition of cycloheximide results in a 200-fold induction of *ABC1* transcript levels in the wild-type strain but only a slight (<4-fold induction) in strain TF7-3131. We conclude that the plasmid insertion in the *abc1-1* allele does not abolish *ABC1* expression but significantly reduces *ABC1* induction

Fig. 5. *abc1* mutants do not cause symptoms on detached leaves. Short pieces of rice leaves from cultivars CO-39 (upper row) and Sariceltic (bottom row) were drop-inoculated, floated on water for 6 days in the dark, dried and photographed. (A) Water, (B) wild-type strain Guy11, (C) ectopic integration transformant AM30, (D) deletion mutant AM25 (*abc1-2Δ*), (E) insertional mutant TF7-3131 (*abc1-1*) and (F) strain nn78 ($\Delta pmk1$) defective in appressorium formation. The assay was repeated three times, and typical results from one assay are shown.

by metabolic poisons. Because strain TF7-3131 has a pathogenicity phenotype indistinguishable from that of an *abc1* deletion allele, we conclude that up-regulation of *ABC1* mRNA is required for pathogenicity.

Discussion

Molecular analysis of an insertional mutant of the rice blast pathogen *M. grisea* identified the ABC transporter-encoding gene, *ABC1*, as an important pathogenicity determinant. Fungal ABC transporters play important roles as efflux pumps, providing resistance to a variety of metabolic poisons (Balzi *et al.*, 1994; Hirata *et al.*, 1994; Sanglard *et al.*, 1996). Studies of an insertional mutation and a deletion mutation show that *ABC1* is required as an efflux pump during early stages of *M. grisea* pathogenesis of rice.

Abc1 has high levels of similarity to the yeast ABC transporters Pdr5 and Cdr1. Together with Abc1, these proteins and Pdr5 homologs in the filamentous fungus *Aspergillus nidulans* (Del Sorbo *et al.*, 1997) share an N-terminal modified ABC signature sequence found in fungal ABC transporters which act as efflux pumps (Decottignies and Goffeau, 1997). Like *PDR5*, *ABC1* transcript levels are dramatically elevated in response to metabolic poisons. However, unlike the yeast transporter genes *PDR5* and *CDR1*, promoter or deletion mutations

in *ABC1* do not result in acute sensitivity to metabolic poisons or antifungal compounds. This finding suggests that *ABC1* may not play a general role in MDR but may have a more specialized role in pathogenicity.

Both *C. albicans* and *S. cerevisiae* contain other ABC transporters involved in MDR. The *S. cerevisiae* transporter, Snq2, has distinct but overlapping drug specificities with Pdr5 (Hirata *et al.*, 1994). Mutations in the *C. albicans* transporter Cdr2 do not result in acute drug sensitivity; however, $\Delta cdr1\Delta cdr2$ strains appear to be more sensitive than $\Delta cdr1$ mutants (Sanglard *et al.*, 1997). By analogy with *C. albicans*, *M. grisea* may contain a second ABC transporter. A partial overlap in function between this transporter and Abc1 may account for the rare lesion phenotype observed in whole plant pathogenicity assays. Experiments to test this hypothesis are in progress.

A role for *ABC1* in pathogenicity

A surprising result from this study is that an insertional mutation 718 bp upstream of the start codon of *ABC1* dramatically reduces the up-regulation of *ABC1* transcription after exposure to metabolic poisons and results in a pathogenicity phenotype indistinguishable from a deletion mutation. This finding argues strongly for the up-regulation of *ABC1* as an important consequence and requirement for *M. grisea* pathogenicity of rice.

At least two hypotheses can be proposed to account for

Fig. 6. Penetration assays on detached barley leaves after 60 h. (A) Wild-type strain Guy11 forms appressoria (white arrow) visible in a bright field image. (B) The same sample as in (A) using aniline blue epifluorescence. (C) AM25 conidia form appressoria without extensive infection hyphae at the site of penetration. (D) Rare small infection hyphae formed by strain AM25. Bar = 10 µm.

Table II. Sensitivity of wild-type strain Guy11, and *abc1* mutant strains AM25 and TF7-3131 to various antifungal drugs

Compound	EC ₅₀ ^a (µg/ml)	MIC ^b (µg/ml)
Benomyl	15.0	nt
Brefeldin A	40.0	40.0
Chloramphenicol	nt	150.0
Cycloheximide	0.3	2.5
Fenarimol	20.0	2.0
Fenpropimorph	160.0	7.5
Metconazole	20.0	0.25
Miconazole	nt	7.5
Sakuranetin	0.8	nt
Tebuconazole	30.0	0.25
Terbinafine	nt	1.0
Propiconazole	70.0	1.0
4-NQO	0.3	nt

^aEffective concentration of the tested drug, which inhibits 50% of appressorium formation.

^bMinimal inhibitory drug concentration sufficient to inhibit fungal mycelic growth on minimal agar plates.

the role of *ABC1* in pathogenesis. First, *Abc1* may be required to export a fungal toxin during an early stage of pathogenesis. This hypothesis seems unlikely for several reasons. Despite a variety of studies identifying low molecular weight compounds produced by *M.grisea*, there is no definitive evidence for a toxin in rice blast disease (Ou, 1985), and early stages of blast fungus pathogenesis

are characterized by a lack of plant cell damage or necrosis. Furthermore, *abc1* mutants show an abrupt and dramatic growth arrest immediately after penetration. *Tox⁻* strains of other fungal pathogens do not demonstrate this type of growth arrest. Finally, *abc1* mutants are fully viable whereas transporter mutants of the toxigenic maize pathogen *Cochliobolus carbonum* are predicted to be lethal due to toxin accumulation (Pitkin *et al.*, 1996). We recognize that despite these and other arguments, until the substrate for *ABC1* is identified, we cannot rule out this hypothesis.

The alternative hypothesis is that *Abc1* provides a defense function during early stages of pathogenesis, by acting as an efflux pump to provide resistance to antimicrobial compounds present in rice and/or barley cells. Support for this role is found in the extensive similarity of *Abc1* to other fungal ABC transporters which act as MDR-type efflux pumps, and the observation that *ABC1* transcripts are up-regulated in the presence of metabolic poisons, antifungal drugs and the rice phytoalexin, sakuranetin. Interestingly this up-regulation is required for pathogenicity because an insertional mutation which disrupts this regulation is non-pathogenic. Although we have yet to identify the substrates of *Abc1*, numerous molecules isolated from rice cells have been proposed to act as phytoalexins during rice blast disease (Akatsuka *et al.*, 1985; Sekido *et al.*, 1987; Kodama *et al.*, 1992). The availability of isogenic *M.grisea* strains containing

Fig. 7. Transcriptional regulation of *ABC1*. (A) Induction of *ABC1* transcript level by various drugs. RNA was prepared from untreated strain Guy11 (lane 1) or strain Guy11 treated with 80 µg/ml miconazole (lane 2), 440 µg/ml metconazole (lane 3), 40 µg/ml sakuranetin (lane 4) or 1 mg/ml hygromycin (lane 5). (B) The up-regulation of the *ABC1* transcript level is affected in insertional mutant TF7-3131. The expression of the *ABC1* gene was analyzed in strains Guy11 and TF7-3131 (*abc1-2Δ*) untreated or treated with 1 mg/ml cycloheximide. Equal loading of lanes with RNA was checked by ethidium bromide staining. Blots were hybridized with probe SK1.8 as indicated in Figure 1B.

or lacking *ABC1* may facilitate the identification and characterization of biologically relevant molecules.

ABC-type efflux pumps have been shown to play important roles as genetic determinants of MDR in humans (Decottignies and Goffeau, 1997) and fungi (Nishi *et al.*, 1992; Balzi *et al.*, 1994; Sanglard *et al.*, 1997). It has long been speculated that fungi possess efflux pumps to resist metabolic poisons present in the environment. We propose that a natural role for these fungal transporters is to permit intimate associations between plant and fungal cells. If this hypothesis is correct, then the role we have proposed here for the Abc1 transporter of *M. grisea* is likely to be generalized for other invasive fungal phytopathogens.

Materials and methods

Growth and manipulation of *M. grisea*

Strains of *M. grisea* used in this study are listed in Table III. Methods for propagating, transforming and manipulating *M. grisea* have been described previously (Crawford *et al.*, 1986; Talbot *et al.*, 1993; Xu and Hamer, 1996). Minimal agar was prepared as described (Harris *et al.*, 1994).

Table III. *Magnaporthe grisea* strains used in this study

Strains	Genotype	Description and source
Guy11	Wild-type	Leung <i>et al.</i> (1988)
TF7-3131	Guy11 <i>abc1-1</i> , Hyg ^R	insertional mutant; this study
AM25	Guy11 <i>abc1-2Δ</i> , Hyg ^R	deletion allele; this study
AM27	Guy11 <i>abc1-2Δ</i> , Hyg ^R	deletion allele; this study
AM31	Guy11 <i>abc1-2Δ</i> , Hyg ^R	deletion allele; this study
AM35	Guy11 <i>abc1-2Δ</i> , Hyg ^R	deletion allele; this study
AM30	Guy11, Hyg ^R	ectopic transformant; this study
AM 36	Guy11, Hyg ^R	ectopic transformant; this study
nn78	Guy11Δ <i>pmk1</i> , Hyg ^R	Xu and Hamer (1996)

Low stringency Southern blots were performed at 55°C. For RNA expression experiments, rapidly growing liquid cultures in CM medium were filtered through Miracloth (Calbiochem), washed with sterile water and resuspended in half the volume of fresh medium containing the respective compound. The cultures were then incubated for another 5 h, harvested again by filtration and immediately frozen in liquid nitrogen. Heat shock experiments were performed by shifting rapidly growing cultures to 37°C. Aliquots were taken after 0, 10 and 30 min and 1 h. Northern analyses were done as described by Virca *et al.* (1990).

Assays

Infection assays were performed as previously described (Xu and Hamer, 1996). The insertional mutagenesis screen is described in Lau and Hamer (1998).

For the detached leaf assay, second leaves from rice or barley were cut into 4×12 mm pieces and floated on sterile water in a 24-well tissue culture plate (Sarstedt). Conidial suspensions were prepared in water and filtered through two layers of Miracloth. Small droplets of conidial suspensions (1×10⁵ conidia/ml) were applied onto floated leaf pieces using a 26G1/2 needle attached to a 1 ml tuberculin syringe. Samples were stored in the dark at 25°C. After 6 days, rice leaf pieces were dried and photographed. For the penetration assay on cut barley leaves, leaf samples were incubated for 60 h with fungal conidia (5×10⁴ conidia/ml) as described above. Samples were then fixed with 1 M KOH, heated to 70°C for 30 min, stained with aniline blue, observed and photographed by epifluorescence microscopy (Hood and Shew, 1996).

Vegetative growth and conidiation rates were measured as described (Liu and Dean, 1997) using oatmeal agar plates. Spore attachment and appressorium formation rates were determined using plastic microscope coverslips (Liu and Dean, 1997; Xu *et al.*, 1997).

Cloning and sequence analysis of *ABC1*

Plasmid rescue was performed as described earlier by Kuspa and Loomis (1992). The recovered plasmid, pRP27, contained a 3.9 kb genomic DNA fragment, which was used to screen genomic clones from a λGEM-11 genomic Guy11 library and cDNA clones from a λZAPII (Stratagene) cDNA library constructed with RNA isolated from nitrogen-starved Guy11 mycelial culture (Xu and Hamer, 1996). Because the genomic clones did not include 2.4 kb of the 3' end of the ORF, two sets of primers AB3 (GGAATTCCATCT TGGCCGACCAGGTCA) and AB4 (GGAATTCCACTTTTCAGGAAACATACG); AB5 (CCGGTTTCAT-CCAGAGGC) and AB6 (GTTCGCAATGTTGCCACC), derived from the cDNA sequence, were used to amplify genomic DNA of the corresponding region. The sequence is available in the DDBJ/EMBL/GenBank database under accession No. AF032443.

Plasmids and plasmid constructions

pBA1.6 contains a 1.6 kb fragment of *ABC1* (nucleotides 3026–4691 in AF032443) amplified by primers AB5 and AB6 in pGEM-T (Stratagene). pBL10K contains a 10 kb *KpnI* fragment of genomic DNA comprising the upstream region and 2.5 kb of the 5' end of the *ABC1* gene in pBluescript KS+ (Stratagene). pSK1.8 contains a 5.7 kb *BamHI* fragment of pBL10K containing the 5' end of the *ABC1* gene in pBC KS-.

Disruption of the *ABC1* gene

To generate pBC1, the 1.6 kb *BamHI* in pBA1.6 was cloned into pBC KS- (Stratagene). The 5' region of *ABC1* was introduced into pBC1 as a 5.5 kb *SalI* fragment from pBL10K to generate pBC2. The modified *HPH* gene (Carroll *et al.*, 1994) was inserted between both *ABC1* fragments in pBC2 as a 1.5 kb *EcoRI* fragment to generate the replacement vector pΔABC1. For *M. grisea* transformation, pΔABC1

was linearized with *NorI* and transformed into Guy11. Putative transformants were pre-screened by PCR analysis of crude mini DNA extracts (Wu *et al.*, 1997) with *ABC1* primers AB1 (GCAGAAGTTGGTAAGTA-GTTGCAT) and AB2 (AAGATCTGGATGGACCGAGTTAGC) for the absence of a 1.1 kb *ABC1* fragment.

Drug assays

Sterol biosynthesis inhibitors were a gift from Dr Mark Bossie (American Cyanamid Company). The rice phytoalexin, sakuranetin, was a gift from Drs Shigeyuki Mayama (Kobe University) and Osamu Kodama (Ibaraki University). Brefeldin A was dissolved in ethanol. All other drugs were dissolved in DMSO.

Spore germination tests were performed as published (Akatsuka *et al.*, 1985) with slight modifications. Briefly, conidial suspensions of 2×10^4 conidia/ml were prepared in water and filtered through Miracloth. Then, 99 μ l drops of spore suspensions were placed on plastic coverslips, and different amounts of drugs were added in a 1 μ l volume. Drug concentrations were determined which suppress appressorium formation rates of fungal conidia down to 50% (EC₅₀) (Table II). For tests on agar plates, increasing amounts of drug, eventually sufficient to stop growth, were incorporated into minimal agar. Plugs (6 mm) of 10-day-old mycelium were placed on the agar, and radial growth of colonies was evaluated after 8–12 days. MICs of the tested drugs are given (Table II). Experiments were done in duplicate.

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