Genomics 101 (2013) 354–361

Contents lists available at SciVerse ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

Genome-scale analysis of ABC transporter genes and characterization of the ABCC type transporter genes in *Magnaporthe oryzae*

Yongnam Kim [a,1], Sook-Young Park [d,1], Dongyoung Kim [a], Jaeyoung Choi [d], Yong-Hwan Lee [d], Jong-Hwan Lee [a,b,c], Woobong Choi [a,b,c,*]

[a] *Department of Biomaterial Control, Dong-Eui University, Busan 614-714, Korea*
[b] *Department of Biotechnology and Bioengineering, Dong-Eui University, Busan, 614-714, Korea*
[c] *Blue-Bio Industry RIC, Dong-Eui University, Busan 614-714, Korea*
[d] *Department of Agricultural Biotechnology, Fungal Bioinformatics Laboratory, Center for Fungal Genetic Resources, and Center for Fungal Pathogenesis, Seoul National University, Seoul 151-921, Korea*

ARTICLE INFO

Article history:
Received 27 November 2012
Accepted 3 April 2013
Available online 11 April 2013

Keywords:
ABC transporters
Magnaporthe oryzae
Phylogenetics
Pathogenicity
Abiotic stress

ABSTRACT

Rapid adaptation to various environmental stresses is a prerequisite for successful infection in fungal pathogens. ABC transporters are responsible for regulating intracellular levels of cytotoxic or xenobiotic compounds, suggesting a crucial role in pathogenesis. Here, we report genome-scale identification of putative ABC transporter genes in *Magnaporthe oryzae*. A total of 50 ABC transporter genes were predicted and phylogenetic analysis divided them into 11 subfamily groups: ABCA, ABCB, ABCC-1, ABCC-2, ABCD, ABCE, ABCF, ABCG-1, ABCG-2, ABCI, and YDR061W-like. In the 11 ABCC subfamily genes, the transcript levels were elevated during infection stages and after exposure to various abiotic stresses. Based on expression pattern, three representative genes, *MoABC5*, *MoABC6* and *MoABC7*, were selected. Functional analysis of *MoABC5*, *MoABC6* and *MoABC7* revealed that the genes may be responsible for virulence, abiotic stress tolerance, and conidiation, respectively. Our data will be providing valuable information to examine the role of ABC transporter genes in *M. oryzae*.

1. Introduction

For successful infection of the host plant, phytopathogenic fungi need to adapt to a specific host environment and subsequently overcome the cytotoxic and antifungal compounds such as phytoalexins produced by the host plant [1]. The ATP-binding cassette (ABC) transporter protein family is one of the largest gene families in most organisms. They are key players in tolerance and resistance of toxic substances, either sequestering the toxic hydrophobic compounds into specialized designated organelles, or by directing them for secretion [2]. In humans, it is important for maintaining the blood–brain barrier or for mediating cellular resistance to chemotherapeutic drugs [3,4]. In plants, ABC proteins are involved in stomatal movement in response to various stresses and are required for normal seed germination and lateral root development [5,6]. In phytopathogenic fungi, the transporter proteins are involved in resistance mechanisms against cytotoxic compounds or fungicides for successful disease development [7].

In eukaryotes, the ABC transporters are integral membrane proteins transporting a wide range of substrates such as lipids, drugs, and heavy metals. Nine different subfamilies have been defined by their structure and the location of the nucleotide-binding domain (NBD), N-terminal extension (NTE), and the transmembrane segment (TMD) [8]. The general structure of ABC transporters includes four core domains, two NBDs and two TMDs. Most recently, fungal ABC transporters were analyzed at the genome scale using a set of 27 fungal species [9]. They classified highly conserved subfamilies of ABC proteins and group-specific, diversified ABC protein subfamilies. However, ABC transporter genes in *Magnaporthe oryzae* have not been confirmed in detail. Furthermore, the genes have not been systematically explored at the genome-level.

Unlike other subfamilies, most of the ABCC subfamily proteins were found to be full-length transporters with an N-terminal hydrophobic region present in most eukaryotes. In *Arabidopsis*, AtABCC1 and AtABCC2 are responsible for detoxification of toxic compounds by expulsion from the cell or by sequestration in the vacuole [10]. In animals, some of ABCC transporters act either as ATP-gated channels or as potassium channel regulators although they are not primarily active. However, a few studies have been carried out on this subfamily in phytopathogenic fungi.

Only a few ABC transporter genes have been functionally analyzed in fungal species other than human pathogens such as *Candida albicans*

* Corresponding author at: Department of Biomaterial Control, Dong-Eui University, Busan 614-714, Korea. Fax: +82 51 890 2632.
E-mail address: wbchoi@deu.ac.kr (W. Choi).
[1] These authors contributed equally to this work.

0888-7543/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ygeno.2013.04.003

[11], *Aspergillus fumigatus* [12], and *Cryptococcus neoformans* [13–15]. In *M. oryzae*, four ABC transporter genes, *ABC1* to *ABC4*, have been studied [16–19]. The *M. oryzae* ABC1 [16] and ABC4 [19] are required for pathogenicity [16], helping the fungus to cope with the cytotoxic environment during infection. In addition, ABC2 [17] and ABC3 are required for multidrug resistance. ABC3 specifically helps to overcome cytotoxicity and oxidative stress within the appressoria during early stages of infection-related morphogenesis and likely imparts defense against certain antagonistic and xenobiotic conditions encountered during pathogenic development [18].

In this study, we report genome-scale identification of *M. oryzae* ABC transporter genes in detail. We found that 50 ABC transporter genes are present in this fungus. Gene expression analysis revealed that they are induced during early and late infection stages and under various abiotic stresses. Functional analysis of three genes, *MoABC5*, *MoABC6* and *MoABC7*, from ABCC subfamily revealed that *MoABC5* and *MoABC6* are involved in pathogenicity and hyphal growth, respectively. Drawing together the results from the expression and the functional analyses, evidence supports the role of the ABCC subfamily proteins in tolerance of stress conditions encountered during colonization of the host, directly linked to pathogen fitness.

2. Results

2.1. ABC transporter genes in M. oryzae

In a previous study, 50 putative ABC transporter genes were identified in *M. oryzae* genome by Kovalchuk and Driessen [9]. The 50 genes were divided into nine different subfamilies, ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, ABCG, ABCI, and YDR061w-like. However, no comparisons were made between the 50 genes. Based on this previous work, we collected the 50 genes from *M. oryzae* genome database (Table S1). Subfamilies of ABCB (19 genes, 38%), ABCC (11 genes, 22%), and ABCG (8 genes, 16%) occupied higher proportions than the other subfamilies in *M. oryzae*. We mapped the loci of the 50 genes to putative *M. oryzae* chromosomes by chromosome location analysis (http://cfgp.riceblast.snu.ac.kr). The genes were mapped onto all seven chromosomes and a dispensable chromosome (Fig. 1A). We found that the members of a single subfamily can be scattered around different chromosomes instead of linkage and the protein sizes are variable, ranging from 206 (MGG_13339) to 1683 (MGG_04855) residues (Table S1). All the ABC transporter genes were conserved in other 32 organisms including chromista, fungi, metazoa, and vidiriplantae, except MGG_13339 (Table S2).

Phylogenetic analysis was carried out to determine the evolutionary relationship of the 50 genes. The resulting tree showed that they are classified into 11 subgroups (Fig. 1B). ABCB and ABCC subfamilies were more closely related to each other than to the other subfamilies. Interestingly, one gene (MGG_06024) from ABCC subfamily was separated as a distinct branch on its own. Moreover, ABCG subfamily was also divided into two branches.

All the identified *M. oryzae* ABC transporter proteins have more than two AAA ATPase domains (IPR003593) and two to four ABC transporter-like domains (IPR003439). The locations of the ATPase and ABC transporter-like domains are variable. Unlike other subfamilies, ABCB and ABCC transporters contain integral membrane type 1 ABC transporters (IPR017940) and transmembrane domain type 1 ABC transporters (IPR011527), resulting in close position of the two groups in the topology of the phylogenetic tree (Fig. 1B). ABCG transporters in *M. oryzae* contain ABC2 type transporter domain (IPR013525) (Fig. 2).

2.2. ABCC subfamily genes are expressed during infection and various abiotic stresses

Previous reports on ABC1 to 4 transporter encoding genes in *M. oryzae* have shown that they regulate cytotoxicity and mediate tolerance against antifungal agents as well as oxidative stress, allowing the pathogen to perform successful infection. Therefore it was suggested that these genes are required for pathogenicity [16–19]. As these four genes belong to ABCA (*ABC4*), ABCB (*ABC3*), and ABCG (*ABC1* and *ABC2*) subfamilies in *M. oryzae*, we directed our attention to the ABCC subfamily, which was the largest subfamily whose members have not been studied previously.

To obtain insights into the physiological roles of ABCC transporters, we performed expression analysis of 11 ABCC transporters in various conditions including cell developmental stages, infection stages, and abiotic stress conditions (Table S3 and Fig. 3). The results showed that the 11 transporter genes are classified into three major groups based on their expression patterns (Fig. S1). All the 11 genes were up-regulated under LiCl, sorbitol, and Iprobenfos treatments, implying ABCC transporter genes are generally required for those abiotic stress conditions in *M. oryzae*, whereas we did not find conditions where all 11 genes are down-regulated.

Group I contains three transporter genes, MGG_04855, MGG_03736, and MGG_08309, which are activated mainly by hygromycin. Group II-1 contains five transporter genes, MGG_13880, MGG_06024, MGG_01674, MGG_11025, and MGG_05044, which are up-regulated mainly by carbon starvation, heat shock, Triflumizol, Isoprothiolane and nitrogen starvation. Group II-2 contains three transporter genes, MGG_07567, MGG_05746, and MGG_05009, which are activated when grown on minimal medium, or at 72 h post inoculation on rice, or under nitrogen starvation, heat shock stress, NaCl, KCl, Triflumizol, and Isoprothiolane-treated conditions.

2.3. Targeted gene disruption of three ABCC transporter genes

Building on the information from the expression analysis, we examined the function of the three genes, MGG_04855, MGG_05044, and MGG_05009, from each Group I, II-1, and II-2 (Table S4). We designated gene names; *MoABC5* for MGG_05009, *MoABC6* for MGG_05044, and *MoABC7* for MGG_04855. We performed targeted gene disruption for each of the three genes. Gene disruption constructs with hygromycin gene cassettes were created as in Fig. S2 and were introduced into the wild-type strain to generate KO mutants. The transformants were primarily screened for hygromycin resistance. Southern blot analysis with appropriate probes confirmed successful gene disruption with a single integration event (Fig. S2). To confirm that the phenotypes shown by Δ*Moabc5* or Δ*Moabc6* or Δ*Moabc7* mutants are the result of gene inactivation, we carried out transcription analysis by qRT-PCR. This clarified that all Δ*Moabc5* or Δ*Moabc6* or Δ*Moabc7* mutants were down-regulated compared with that of the wild-type. The transcript abundance from complementation strains was identical to that of wild-type (Fig. S2).

2.4. Phenotype of ΔMoabc5, ΔMoabc6, and ΔMoabc7

The effects of deletion of *MoABC5*, *MoABC6*, and *MoABC7* genes on *M. oryzae* development and pathogenicity are summarized in Table 1. Deletion mutants of *MoABC6* and *MoABC7* genes exhibited significant reduction of conidiation compared to the wild-type. However, we observed no significant difference in germination and appressorium formation between the mutants and the wild-type (Table 1).

In spray-inoculation tests, the Δ*Moabc5* mutant showed reduced virulence on a susceptible rice cultivar, Nakdongbyeo, whereas the wild-type KJ201, Δ*Moabc6*, Δ*Moabc7* and the complemented transformants of each deletion mutant caused typical susceptible-type spreading lesions (Fig. 4 and Table 1). Dramatic differences in disease severity were observed, suggesting that *MoABC5* gene may be involved in pathogenesis (Table 1).

There was also no apparent difference between the wild-type and the mutants for mycelial growth on CM, MM, nitrogen starvation, carbon starvation, 1 M sorbitol, 1 M KCl, and 1 M NaCl. However,

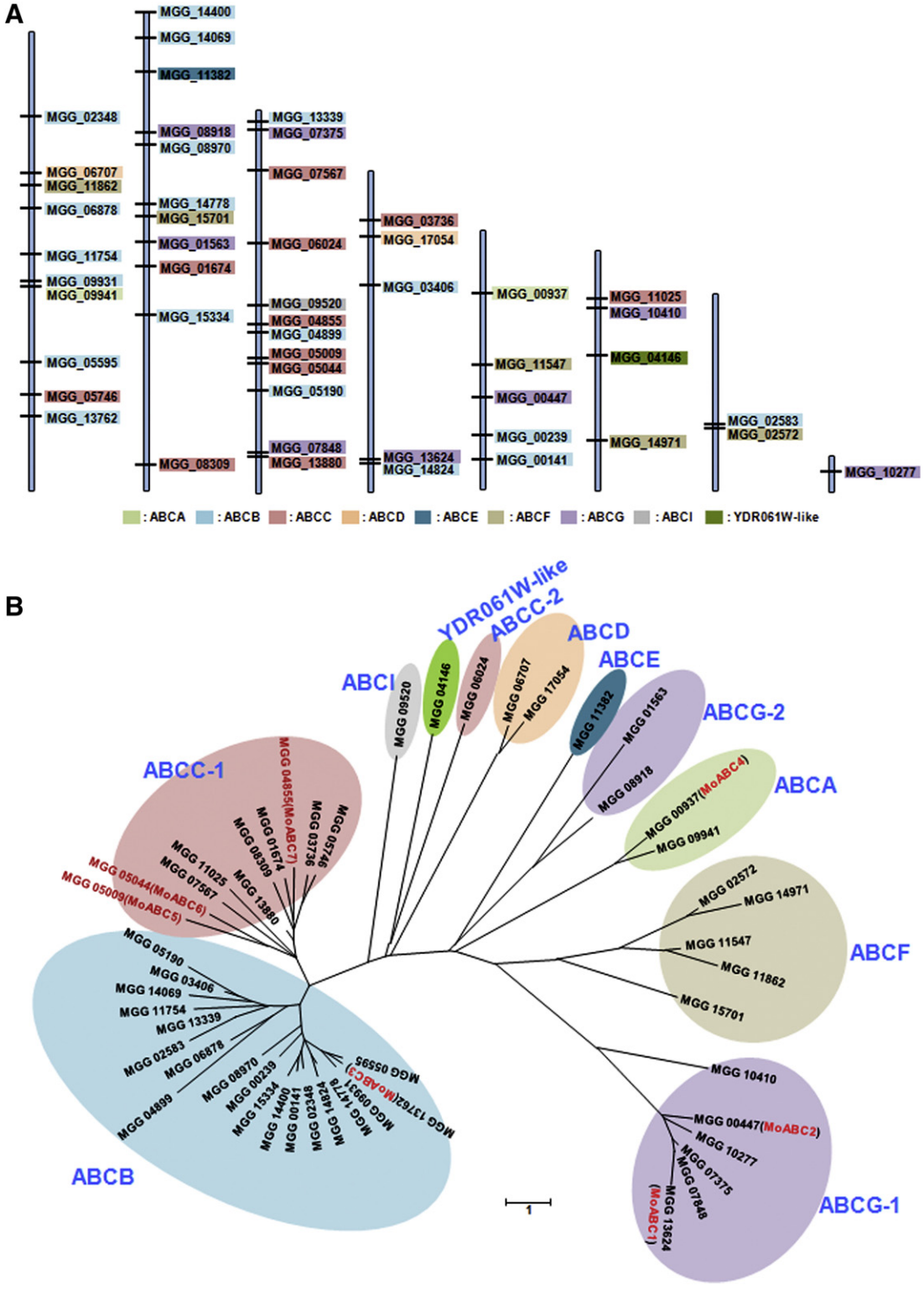

Fig. 1. Chromosomal distribution and phylogenetic analysis of ABC transporters in *M. oryzae*. A. Genome wide distribution of putative 50 ATP-binding cassette (ABC) transporter genes in *Magnaporthe oryzae*. B. Phylogenetic analysis of the 50 ABC proteins in *M. oryzae*. The protein sequences were aligned with ClustalW, and the MEGA software version 4.0 was used to perform a 2000 bootstrap phylogenetic analysis using the neighbor joining method. The terminology of ABC subfamily, ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, ABCG, ABCI, and YDR061W-like, were adopted from Kovalchuk and Driessen [9].

the Δ*Moabc6* mutant showed a significant reduction in mycelial growth on minimal medium, 1 M sorbitol, 1 M KCl and 1 M NaCl, implying that the *MoABC6* gene may be involved in abiotic stress responses (Table 2). Similarly, the Δ*Moabc5* mutant showed a significant reduction in mycelial growth on minimal medium.

It has been reported that two ABC transporter genes mediate the efflux of various compounds, including toxicants in *M. oryzae* [17,19]. We examined whether the products of *MoABC5*, *MoABC6*, and *MoABC7* genes can mediate the removal of toxicants. No differences were seen in mycelial growth on potato dextrose agar medium

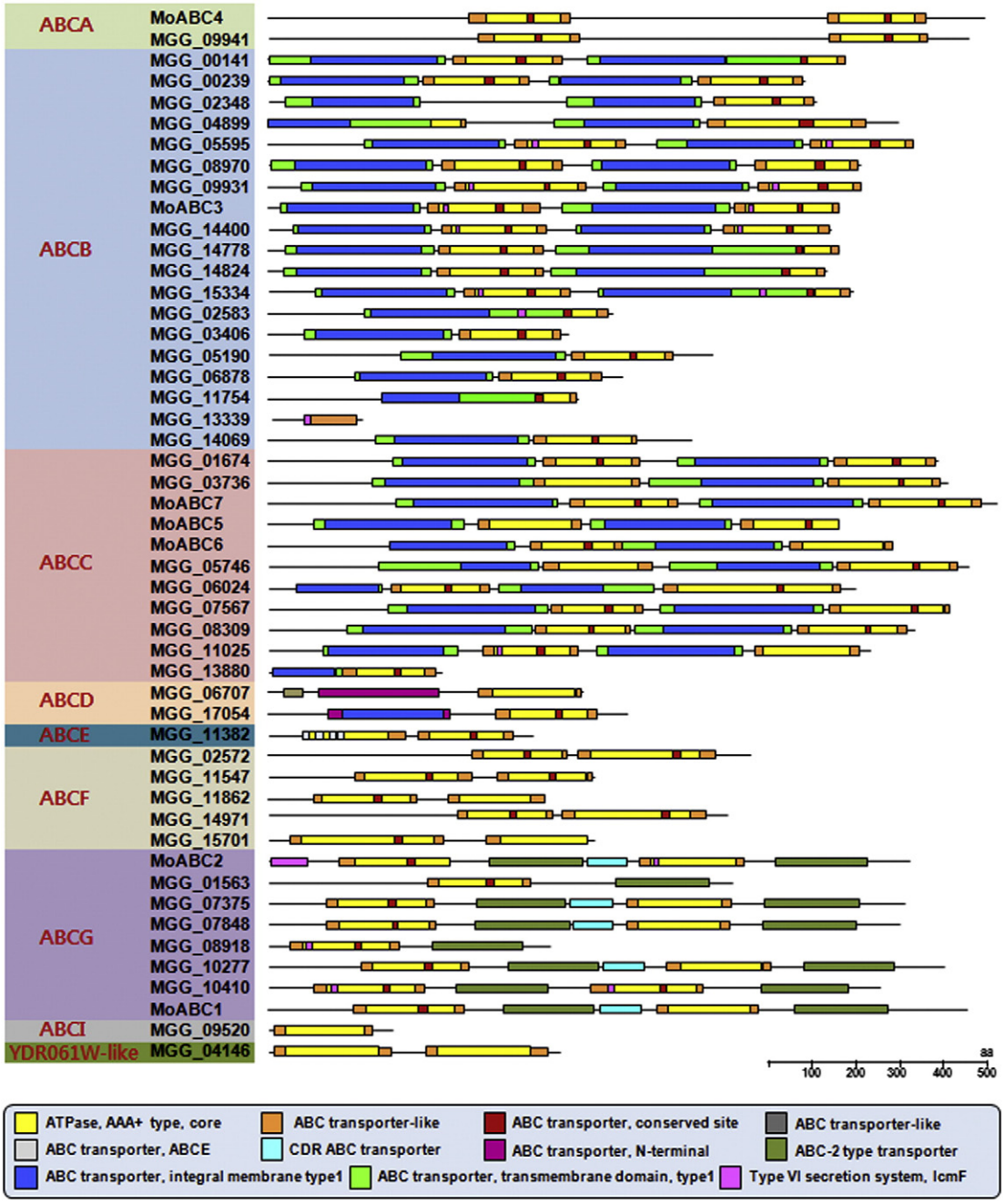

Fig. 2. Protein domain structures of the 50 putative *M. oryzae* ABC transporters were obtained from the Interpro protein database (http://www.ebi.ac.kr/interpro).

(PDA) supplemented with appropriate quantities of benomyl (binds to microtubules, interfering meiosis and intracellular transport), iprobenfos (inhibitor of fungal choline biosynthesis), isoprothiolane (inhibitor of fungal choline biosynthesis), kresoxim-methyl (inhibitor of electron transport in mitochondria and disrupting metabolism), thiophanate-methyl (cholinesterase inhibitor), tricyclazole (a polyketide melanin pathway inhibitor), and triflumizol (cholinesterase inhibitor).

To confirm that the phenotypes exhibited by the mutants was due to the deletion of *MoABC5*, *MoABC6*, and *MoABC7* genes, we complemented the mutation by introducing fragments containing the ORFs of the corresponding WT alleles with 1.5 kb 5′ flanking regions that correspond to the promoter regions of the genes. RT-PCR using mycelial RNA confirmed recovery of each *MoABC5*, *MoABC6* and *MoABC7* gene transcripts to a level comparable to that of wild-type (Fig. S2). The defective phenotypes from the deletion of *MoABC5*, *MoABC6*, and *MoABC7* genes were also rescued (Tables 1, 2, and Fig. 4).

3. Discussion

We have carried out a genome scale analysis of ABC transporters in *M. oryzae* and elucidated the phylogenetic relationship between these genes. The ABCC subfamily was explored in greater depth by expression analysis of three representative genes to deduce the general biological significance of the group. We found that two of ABC transporter genes, *MoABC5* and *MoABC6*, are associated with pathogenicity and response to abiotic stress, respectively.

We found that 50 putative ABC transporter proteins are present in the *M. oryzae* genome. This is in accordance with the prediction from

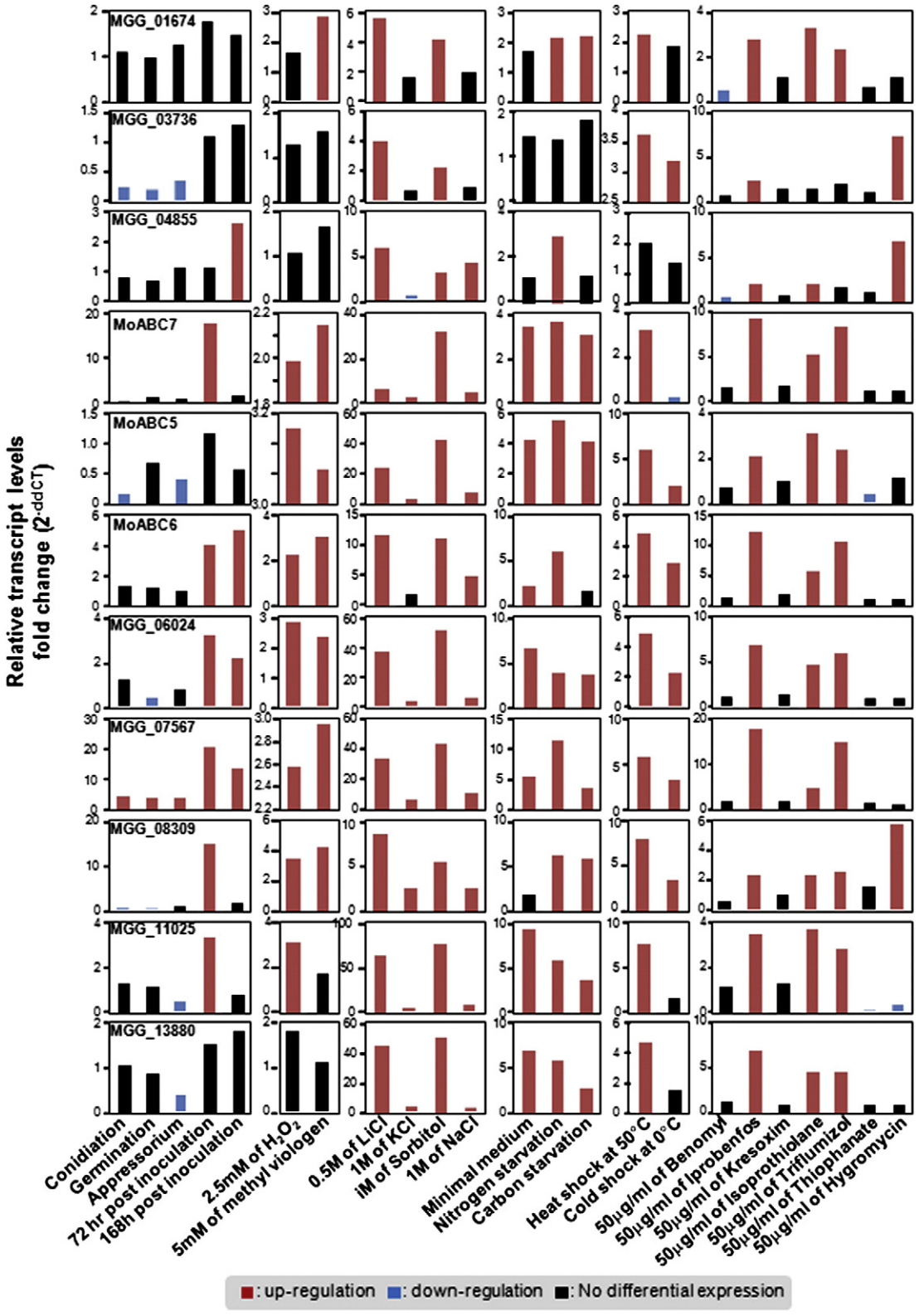

Fig. 3. Expression of 11 MoABCC type transporter genes during infection-related conditions, under various abiotic stresses, and chemical stresses. Total RNA samples were extracted from appropriate materials. All the conditions are listed in Table S3. Transcript levels were determined by quantitative real-time PCR (qRT-PCR). Relative abundance of transcript was compared with untreated sample. Up-regulated genes (32 fold) were indicated in red, down-regulated genes (≤0.5 fold) were indicated in blue, and no differential expression compared with untreated samples was indicated in black.

a previous study [9]. Most of these genes presented high protein sequence similarity to ABC transporters in other fungi, plants and the human, showing that the ABC transporter proteins are evolutionarily conserved in *M. oryzae* also. However, one transporter gene, MGG_13339, which belongs to the ABCB subfamily showed low similarity compared with ABC transporters in 29 fungi, one chromist, three

Table 1
Characterization of transformants including deletion mutants for *MoABC5*, *MoABC6*, and *MoABC7* genes in *M. oryzae* development and pathogenicity.

Strain	Conidiation (10^4/ml)[a]	Germination (%)[b]	App. Formation (%)[c]	Pathogenicity[d]
Wild-type	41.9 ± 7.2[Ae]	98.3 ± 1.5[A]	99.0 ± 1.7[A]	4.2 ± 0.4[A]
Δ*Moabc5-2*	39.5 ± 6.2[A]	98.4 ± 1.5[A]	97.4 ± 0.6[A]	2.3 ± 0.5[B]
Moabc5c[f]	40.7 ± 7.2[A]	98.9 ± 0.3[A]	98.6 ± 0.3[A]	4.0 ± 0.7[A]
Δ*Moabc6-2*	30.1 ± 3.1[B]	99.7 ± 0.5[A]	97.8 ± 1.5[A]	4.3 ± 0.6[A]
Moabc6c	39.5 ± 5.4[A]	99.6 ± 0.3[A]	98.9 ± 0.4[A]	4.4 ± 0.2[A]
Δ*Moabc7-1*	29.4 ± 3.7[B]	98.7 ± 1.5[A]	98.7 ± 1.5[A]	4.5 ± 0.6[A]
Moabc7c	44.3 ± 1.2[A]	99.0 ± 1.0[A]	99.1 ± 0.8[A]	4.3 ± 0.0[A]

[a] Conidiation was measured by counting the number of conidia collected with 5 ml of sterilized distilled water from 7-day-old V8 Juice agar plates. Data were presented as means ± SD from three independent experiments.
[b] Percentage of conidial germination on hydrophobic surfaces was measured under a light microscope using conidia harvested from 7-day-old V8 juice agar plates.
[c] Percentage of appressorium formation on hydrophobic surfaces was measured using conidia harvested from 7-day-old V8 juice agar plates.
[d] Scores of disease severity were measured 7 days after inoculation, as previously described Valent et al. (Genetics, 1991) [34].
[e] Tukey's test was used to determine significance at the 95% probability level. The same letters in a column showed no significant difference.
[f] Complement strain.

metazoans, and two viridiplantae (Table S2), suggesting the gene is a *M. oryzae* specific transporter gene. Further examination is needed to reveal whether this gene plays a critical role for *M. oryzae*.

Although Kovalchuk and Driessen [9] have performed massive phylogenetic analysis with 27 fungal species including *M. oryzae*, the resolution of the tree structure was relatively low for the proteins in a given fungal species. Our data revealed that the 50 ABC transporter proteins were divided into 11 subfamilies (Fig. 1B and Table S1). These ABC protein subfamilies are generally divided by their overall conserved architectures, which consist of a membrane-spanning domain (MSD) with multiple transmembrane spans and a nucleotide-binding domain (NBD) [20–22]. In this context, we can expect the members of the same subgroup family to be clustered together. In the previous studies, phylogenetic analysis and domain architecture analysis using 30 Yeast ABC transporter proteins have shown that all the proteins in the same subfamily clustered together [21]. However, the ABCC and ABCG subfamilies in *M. oryzae* were both divided into two separate branches (Fig. 1B). This suggests that the two subfamilies may need to be subdivided further. The divergence might be caused by non-synonymous substitutions. Although our current results do not support cause of subdivision of ABCC and ABCG subfamilies, we anticipate a more comprehensive resolution of the phylogenetic relationships between the ABC transporters in *M. oryzae* in the future.

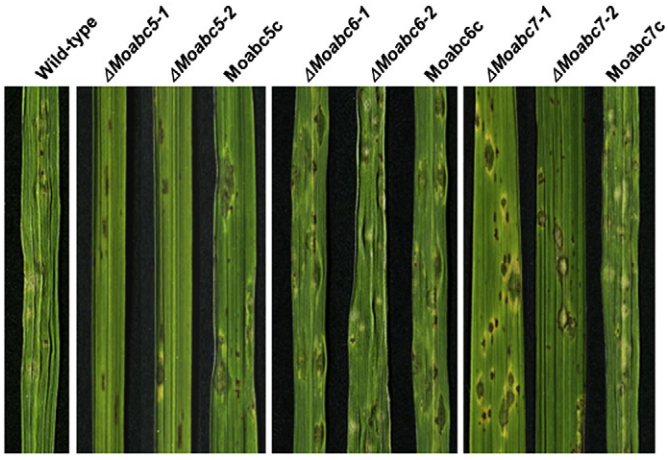

Fig. 4. Assay for pathogenicity. Conidial suspensions (1×10^5 conidia/ml) of the indicated isolates were sprayed on 3–4 leaf-stage rice leaves. Photographs were taken 7 days after inoculation.

Quantitative expression analysis has proven to be a powerful and highly sensitive method for generating a large amount of data for gene expression analysis. Our qRT-PCR analysis revealed that the 11 ABCC subfamily genes were differentially expressed under the given 24 conditions, suggesting the 11 genes may be involved in response to different conditions. Although the majority of the 11 ABC transporter genes studied here showed variable levels of expression in response to a given condition, we could group the genes based on their gene expression patterns under the tested conditions using heat map analysis (Fig. S1). The result implies that each group of genes may perform similar functions or act in response to the given conditions [23].

All the 11 genes were activated by LiCl and sorbitol salt stresses, and antifungal agent, Iprobenfos (Fig. 3). This suggested a role of the ABCC transporters in tolerating these abiotic stress conditions. Moreover, we found that the six genes that were up-regulated under H_2O_2 stress (MGG_08309, MGG_06024, MGG_11025, MGG_07567, MGG_05746, and MGG_05009) were also induced at 72 h post inoculation. This suggests involvement of the transporter genes in countering host defense responses during primary pathogenesis. This speculation is strongly supported by previous studies [18,24]. ABC3 of the ABCB subfamily was required for host penetration, and the ABC3 mutants showed sensitivity to oxidative stress [18]. Transcriptional activity assays of *M. oryzae ABC1* gene demonstrated that the gene is activated under oxidative stress [16].

More specifically, we found that *MoABC5*, *MoABC6*, and *MoABC7* genes were all up-regulated when treated with iprobenfos and isoprothiolane, which inhibit fungal choline biosynthesis. This result demonstrated that all the genes may be necessary for efflux of choline biosynthesis-related toxicants. However, this is inconclusive because the deletion of *MoABC5*, *MoABC6*, and *MoABC7* genes did not show any sensitivity to the chemicals during mycelial growth compare to that of wild-type.

Gene deletion mutants of three ABCC transporter genes, *MoABC5* (MGG_05009), *MoABC6* (MGG_05044), and *MoABC7* (MGG_04855) were used for functional analysis. Our results indicated that the MoABC5 transporter activity is most probably required for overcoming host defense during the host colonization step. We observed that Δ*Moabc5* mutant was able to form mature appressorium (Table 1) and successfully penetrate rice sheath (data not shown). However, Δ*Moabc5* mutant showed a striking reduction in pathogenicity on 3-week old rice seedlings (Fig. 4), thus suggesting that the *Moabc5* is responsible for efflux of phytotoxic metabolites produced by the fungus upon induction by interactions on the rice leaf.

The greater than 10-fold up-regulation of *MoABC5* gene at 72 hpi supports this speculation. There was no significant induction of *MoABC5* transcription at 168 hpi (1.5 fold), which is a relatively late stage of infection. This highlights the importance of MoABC5 in early infection. However, since we did not include earlier infection stages in our study, the significance of MoABC5 before 72 hpi has yet to be determined. Nevertheless, up-regulation of *MoABC5* gene under H_2O_2 stress condition further supports our speculation because the pathogen must overcome plant defenses during the early stages of pathogenesis for successful infection [24].

Our expression analysis showed that *MoABC6* transcript levels were up-regulated in response to salt stresses and nutrient deficient conditions including carbon starvation, nitrogen starvation and minimal nutrient conditions for *M. oryzae*, but not during infection-related development, clearly indicating the *MoABC6* gene may be responsible for salt stress tolerance and adaptation to poor nutrient conditions. Functional analysis of *MoABC6* using the deletion mutant supports this. Δ*Moabc6* mutant showed statistically significant growth reduction on minimal media and under sorbitol, KCl, and NaCl salt stressed conditions (Table 2). This indicates that the *MoABC6* is required for various abiotic stresses and its function is most likely critical in starvation and osmotic stresses.

Table 2
Characterization of transformants including deletion mutants for *MoABC5*, *MoABC6*, and *MoABC7* genes in *M. oryzae* growth on stress conditions.

Strain	CM (mm)[a]	MM (mm)[b]	−N (mm)[c]	−C (mm)[d]	Sorbitol (mm)[e]	KCl (mm)[f]	NaCl (mm)[g]
Wild-type	29.3 ± 0.6^{A} [h]	30.0 ± 1.0^{A}	27.5 ± 0.5^{AB}	27.8 ± 0.3^{A}	21.0 ± 1.0^{BC}	36.5 ± 1.0^{CD}	33.5 ± 0.5^{D}
Moabc5-2	25.2 ± 3.4^{A}	27.2 ± 1.0^{B}	28.2 ± 0.3^{AB}	28.2 ± 0.6^{A}	21.8 ± 0.3^{BC}	36.3 ± 0.3^{CD}	31.3 ± 1.5^{BCD}
Moabc5c[i]	29.5 ± 0.2^{A}	30.0 ± 0.2^{A}	27.9 ± 0.5^{AB}	28.2 ± 0.3^{AB}	21.9 ± 0.4^{C}	36.5 ± 0.3^{CD}	32.5 ± 0.3^{CD}
Moabc6-2	25.3 ± 1.8^{A}	23.2 ± 0.3^{C}	26.0 ± 0.5^{A}	27.5 ± 0.5^{A}	18.5 ± 0.5^{A}	31.5 ± 1.0^{A}	27.3 ± 0.8^{A}
Moabc6c	28.3 ± 1.0^{A}	29.5 ± 1.2^{A}	27.0 ± 1.0^{AB}	28.3 ± 0.6^{A}	21.7 ± 0.3^{C}	35.8 ± 0.7^{BC}	33.2 ± 0.4^{BC}
Moabc7-1	27.8 ± 0.3^{A}	28.8 ± 1.3^{AB}	29.5 ± 0.9^{B}	28.8 ± 1.0^{AB}	22.3 ± 0.6^{C}	34.7 ± 0.8^{BC}	29.5 ± 1.3^{AB}
Moabc7c	28.5 ± 0.5^{A}	28.0 ± 1.0^{AB}	28.6 ± 0.3^{AB}	27.6 ± 0.4^{A}	21.5 ± 0.7^{BC}	36.5 ± 1.0^{CD}	32.5 ± 1.0^{D}

Data were presented as means ± SD from three independent experiments.
Vegetative growth was measured at 5 days post-inoculation: [a]on complete agar medium (CM); [b]on minimal agar medium (MM); [c]on nitrogen-starvation agar medium (−N); [d]on carbon-starvation agar medium (−C); [e]on 1 M sorbitol amended with MM; [f]on 1 M KCl amended with MM; and [g]on 1 M NaCl amended with MM. [h]Tukey's test was used to determine significance at the 95% probability level. The same letters in a column showed no significant difference. [i]Complement strain.

Expression analysis showed that most of the 11 ABCC subfamily genes were up-regulated when the mycelia were treated with triflumizol, iprobenfos, and isoprothiolane (Fig. 3). One of the well-known resistance mechanisms in fungi is the exclusion or expulsion of the fungicide mediated by ABC transporter proteins. However, susceptibility of the gene deletion mutants in the *in vitro* fungicide assay was not greater than that of the wild-type. At least we may hypothesize that the 11 ABCC subfamily genes are up-regulated (Fig. 3) because they play a role in detoxification and oxidative resistance in *M. oryzae*. It is difficult to conclusively test this hypothesis since we cannot produce a multiple deletion mutant of all 11 ABCC genes in *M. oryzae*.

In conclusion, this is the first report of genome-scale analysis of ABC transporter genes in *M. oryzae*. Chromosomal distribution of all 50 ABC transporter genes and their phylogenetic relationship may provide valuable information to understand the regulation of ABC transporter genes at the whole genome scale in *M. oryzae*. Moreover, the expression analysis of ABCC subfamily genes may be helpful in elucidating the functionality of these genes.

4. Materials and methods

4.1. Identification of ABC transporter genes in M. oryzae

To collect ABC transporter genes in *M. oryzae*, we mostly adopted the method from Kovalchuk and Driessen [9] with slight modification. In brief, multiple blastP and tblastn searches against *M. oryzae* genome (http://www.broadinstitute.org/annotation/genome/magnaporthe_comparative) were performed. All known fungal ABC subfamily proteins were used as queries. Cutoff e-values of less than 10^{-4} were applied for protein similarity in all hits.

Using obtained protein sequences, phylogenetic analysis was performed. The protein sequences were aligned with ClustalW using the MEGA4.1 software with default parameters [25]. A phylogenetic tree was constructed using the neighbor-joining method [26] in the MEGA4.1 software with the following parameters with 2000 bootstrap replicates. Protein structure of the ABC transporter genes was obtained from the InterPro database (http://www.ebi.ac.uk/interpro).

4.2. Fungal strains and culture conditions

M. oryzae isolates KJ201 was obtained from the Center for Fungal Genetic Resources (CFGR) at Seoul National University, Seoul, Korea. All fungal cultures were routinely grown at 25 °C under continuous fluorescent light on oatmeal agar medium (OMA) [27] or V8 (4% V8 juice, pH 7.0) agar medium. DNA and RNA were isolated from mycelia, which were grown in liquid complete medium (CM) [27] for 3–4 days.

4.3. Analysis of transcript levels

Quantitative real-time RT-PCR (qRT-PCR) was employed to measure transcript levels. Total RNA samples and first-strand cDNA were prepared as described previously [24]. qRT-PCR was carried out in MicroAmp Optical 96-Well Reaction Plate (PE Biosystems, Foster City, CA, USA) and an Applied Biosystems 7500 Real-Time PCR System. Each well contained 5 μl of Power 2× SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK), 2 μl of cDNA (12.5 ng/μl), and 15 pmol of each primer (Table S5). All the reactions were performed in more than two biological replicates using combined three RNA samples extracted from independent fungal materials. A *β-tubulin* gene was included in the assays as internal control for normalization. All amplification curves were analyzed with a normalized reporter threshold of 0.1 to obtain the threshold cycle (Ct) values. The comparative ΔΔCt method was used to evaluate the relative quantities of each amplified product in samples. Fold changes were calculated as $2^{-\Delta\Delta Ct}$ [28].

Pearson's correlation coefficient and Spearman's rank were used to measure the similarity between gene expression profiles and the similarity between samples, respectively. These 24 samples demonstrated acceptable internal consistency (>0.7), with a Cronbach's alpha coefficient of 0.84, but not in 11 genes (Cronbach's alpha coefficient = 0.64). A heat map of the clustered genes and samples was generated by complete linkage. We applied the following cut-off values; ≥2-fold changes were defined as up-regulation and ≤0.5 changes were defined as down-regulated.

4.4. Developmental and stress conditions

Wild-type KJ201 was used to collect fungal materials for developmental and stress samples. To collect developmental samples, conidia were obtained from 14 day old cultures on OMA media by rubbing the mycelia with water followed by filtration through Miracloth (Calbiochem, San Diego, USA). Conidiation sample and germinated conidia were harvested as described previously [29], and appressoria were collected 6 h after dropping the conidia on a hydrophobic surface. For infected plant samples, after inoculating 3 week old rice seedlings (3–4 leaf stage) with 10 ml of KJ201 conidia (1×10^5 conidia/ml), leaves were collected at 72 and 168 h post inoculation.

For collection of abiotic stress samples, cultures of 100 ml liquid CM inoculated with 1 ml of a conidial suspension (5×10^4 conidia/ml) were incubated at 25 °C for 3 days in an orbital shaker (100 rpm). The cultured mycelia were harvested, washed twice with 1 liter of sterilized distilled water, then transferred to fresh liquid CM or minimal medium [30] containing each treatment, and then cultured for 4 or 16 h (Table S3). All the samples were harvested from three replicates of three biological repeats, immediately frozen using liquid nitrogen, and stored at −80 °C until processed.

4.5. Generation of deletion mutants in three ABCC subfamily genes

To generate gene deletion mutants, constructs were prepared by Double Joint-PCR method [31]. Gene disruption (Fig. S2) and fungal transformation were prepared by homologous recombination strategy

as described previously [32]. Putative mutants were confirmed by Southern blot analysis [27].

4.6. In vitro growth assay, monitoring of infectious growth, and pathogenicity assays

Vegetative growth, pigmentation, conidiation, conidial size, conidial germination, appressorium formation, and infection assays on onion epidermis, rice sheath cells, and rice seedlings were conducted as described previously [32,33].

For stress compound test, mycelial agar plugs (4-mm diameter) were taken from the margins of mycelia on MM and placed upside down on CM medium supplemented with toxicants at different concentrations. After incubation at 25 °C, the colony diameters were measured.

Concentration of stress compounds was determined in effective concentration inhibiting 50% mycelial growth. The concentration of the fungicide was as follows: Benomyl (0.4 mg/ml), Iprobenfos (8 mg/ml), Isoprothiolane (3 mg/ml), Kresoxim-methyl (0.1 mg/ml), Thiophanate-methyl (6.5 mg/ml), Tricyclazole (80 mg/ml), and Triflumizole (0.3 mg/ml). The compounds were added in dimethyl sulfoxide (DMSO). The concentration of the other abiotic stresses was as follows: 1 M of sorbitol, KCl, and NaCl. Statistical analysis was performed using SPSS software v.18.0 (SPSS Inc., Chicago, IL, USA).

Acknowledgments

This research was supported by Dongeui University Research Grant 2010AA196. Blue-Bio industry RIC (RIC 08-06-07) under the Ministry of Knowledge Economy contributed for experimental equipments. Coauthors of Y. Kim and D. Kim were the recipients of graduate fellowships from the Ministry of Education through the Brain Korea 21 Project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygeno.2013.04.003.

References

[1] G.N. Agrios, Plant Pathology, Fifth edition Elsevier Academic Press, San Diego, CA, 2005.
[2] D.C. Rees, E. Johnson, O. Lewinson, ABC transporters: the power to change, Nat. Rev. Mol. Cell Biol. 10 (2009) 218–227.
[3] M.M. Gottesman, V. Ling, The molecular basis of multidrug resistance in cancer: the early years of P-glycoprotein research, FEBS Lett. 580 (2006) 998–1009.
[4] A. Haimeur, G. Conseil, R.G. Deeley, S.P. Cole, The MRP-related and BCRP/ABCG2 multidrug resistance proteins: biology, substrate specificity and regulation, Curr. Drug Metab. 5 (2004) 21–53.
[5] S.J. Suh, Y.F. Wang, A. Frelet, N. Leonhardt, M. Klein, C. Forestier, B. Mueller-Roeber, M.H. Cho, E. Martinoia, J.I. Schroeder, The ATP binding cassette transporter AtMRP5 modulates anion and calcium channel activities in *Arabidopsis* guard cells, J. Biol. Chem. 282 (2007) 1916–1924.
[6] J. Kang, J.U. Hwang, M. Lee, Y.Y. Kim, S.M. Assmann, E. Martinoia, Y. Lee, PDR-type ABC transporter mediates cellular uptake of the phytohormone abscisic acid, Proc. Natl. Acad. Sci. U.S.A. 107 (2010) 2355–2360.
[7] F.L. Stefanato, E. Abou-Mansour, A. Buchala, M. Kretschmer, A. Mosbach, M. Hahn, C.G. Bochet, J.P. Metraux, H.J. Schoonbeek, The ABC transporter BcatrB from *Botrytis cinerea* exports camalexin and is a virulence factor on *Arabidopsis thaliana*, Plant J. 58 (2009) 499–510.
[8] P.J. Verrier, D. Bird, B. Burla, E. Dassa, C. Forestier, M. Geisler, M. Klein, U. Kolukisaoglu, Y. Lee, E. Martinoia, A. Murphy, P.A. Rea, L. Samuels, B. Schulz, E.J. Spalding, K. Yazaki, F.L. Theodoulou, Plant ABC proteins—a unified nomenclature and updated inventory, Trends Plant Sci. 13 (2008) 151–159.
[9] A. Kovalchuk, A.J. Driessen, Phylogenetic analysis of fungal ABC transporters, BMC Genomics 11 (2010) 177.
[10] W.Y. Song, J. Park, D.G. Mendoza-Cozatl, M. Suter-Grotemeyer, D. Shim, S. Hortensteiner, M. Geisler, B. Weder, P.A. Rea, D. Rentsch, J.I. Schroeder, Y. Lee, E. Martinoia, Arsenic tolerance in *Arabidopsis* is mediated by two ABCC-type phytochelatin transporters, Proc. Natl. Acad. Sci. U.S.A. 107 (2010) 21187–21192.
[11] H. Wolfger, Y.M. Mamnun, K. Kuchler, Fungal ABC proteins: pleiotropic drug resistance, stress response and cellular detoxification, Res. Microbiol. 152 (2001) 375–389.
[12] J.W. Slaven, M.J. Anderson, D. Sanglard, G.K. Dixon, J. Bille, I.S. Roberts, D.W. Denning, Increased expression of a novel *Aspergillus fumigatus* ABC transporter gene, atrF, in the presence of itraconazole in an itraconazole resistant clinical isolate, Fungal Genet. Biol. 36 (2002) 199–206.
[13] M. Sanguinetti, B. Posteraro, M. La Sorda, R. Torelli, B. Fiori, R. Santangelo, G. Delogu, G. Fadda, Role of AFR1, an ABC transporter-encoding gene, in the in vivo response to fluconazole and virulence of *Cryptococcus neoformans*, Infect. Immun. 74 (2006) 1352–1359.
[14] S.J. Thornewell, R.B. Peery, P.L. Skatrud, Cloning and characterization of CneMDR1: a *Cryptococcus neoformans* gene encoding a protein related to multidrug resistance proteins, Gene 201 (1997) 21–29.
[15] B. Posteraro, M. Sanguinetti, D. Sanglard, M. La Sorda, S. Boccia, L. Romano, G. Morace, G. Fadda, Identification and characterization of a *Cryptococcus neoformans* ATP binding cassette (ABC) transporter-encoding gene, CnAFR1, involved in the resistance to fluconazole, Mol. Microbiol. 47 (2003) 357–371.
[16] M. Urban, T. Bhargava, J.E. Hamer, An ATP-driven efflux pump is a novel pathogenicity factor in rice blast disease, EMBO J. 18 (1999) 512–521.
[17] Y.J. Lee, K. Yamamoto, H. Hamamoto, R. Nakaune, T. Hibi, A novel ABC transporter gene *ABC2* involved in multidrug susceptibility but not pathogenicity in rice blast fungus, *Magnaporthe grisea*, Pesticide Biochemistry and Physiology 81 (2005) 13–23.
[18] C.B. Sun, A. Suresh, Y.Z. Deng, N.I. Naqvi, A multidrug resistance transporter in *Magnaporthe* is required for host penetration and for survival during oxidative stress, Plant Cell 18 (2006) 3686–3705.
[19] A. Gupta, B.B. Chattoo, Functional analysis of a novel ABC transporter ABC4 from *Magnaporthe grisea*, FEMS Microbiol. Lett. 278 (2008) 22–28.
[20] M. Dean, A. Rzhetsky, R. Allikmets, The human ATP-binding cassette (ABC) transporter superfamily, Genome Res. 11 (2001) 1156–1166.
[21] C.M. Paumi, M. Chuk, J. Snider, I. Stagljar, S. Michaelis, ABC transporters in *Saccharomyces cerevisiae* and their interactors: new technology advances the biology of the ABCC (MRP) subfamily, Microbiol. Mol. Biol. Rev. 73 (2009) 577–593.
[22] K.J. Linton, Structure and function of ABC transporters, Physiology (Bethesda) 22 (2007) 122–130.
[23] M.B. Eisen, P.T. Spellman, P.O. Brown, D. Botstein, Cluster analysis and display of genome-wide expression patterns, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 14863–14868.
[24] M.H. Chi, S.Y. Park, S. Kim, Y.H. Lee, A novel pathogenicity gene is required in the rice blast fungus to suppress the basal defenses of the host, PLoS Pathog. 5 (2009) e1000401.
[25] K. Tamura, J. Dudley, M. Nei, S. Kumar, MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0, Mol. Biol. Evol. 24 (2007) 1596–1599.
[26] N. Saitou, M. Nei, The neighbor-joining method: a new method for reconstructing phylogenetic trees, Mol. Biol. Evol. 4 (1987) 406–425.
[27] S.Y. Park, M.G. Milgroom, S.S. Han, S. Kang, Y.H. Lee, Diversity of pathotypes and DNA fingerprint haplotypes in populations of *Magnaporthe grisea* in Korea over two decades, Phytopathology 93 (2003) 1378–1385.
[28] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2001) 402–408.
[29] J. Jeon, J. Goh, S. Yoo, M.H. Chi, J. Choi, H.S. Rho, J. Park, S.S. Han, B.R. Kim, S.Y. Park, S. Kim, Y.H. Lee, A putative MAP kinase kinase kinase, MCK1, is required for cell wall integrity and pathogenicity of the rice blast fungus, *Magnaporthe oryzae*, Mol. Plant Microbe Interact. 21 (2008) 525–534.
[30] N.J. Talbot, H.R.K. McCafferty, M. Ma, K. Koore, J.E. Hamer, Nitrogen starvation of the rice blast fungus *Magnaporthe grisea* may act as an environmental cue for disease symptom expression, Physiological and Molecular Plant Pathology 50 (1997) 179–195.
[31] J.H. Yu, Z. Hamari, K.H. Han, J.A. Seo, Y. Reyes-Dominguez, C. Scazzocchio, Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi, Fungal Genet. Biol. 41 (2004) 973–981.
[32] S. Kim, S.Y. Park, K.S. Kim, H.S. Rho, M.H. Chi, J. Choi, J. Park, S. Kong, J. Goh, Y.H. Lee, Homeobox transcription factors are required for conidiation and appressorium development in the rice blast fungus *Magnaporthe oryzae*, PLoS Genet. 5 (2009) e1000757.
[33] H. Koga, K. Dohi, O. Nakayachi, M. Mori, A novel inoculation methods of *Magnaporthe grisea* for cytological observation of the infection process using intact leaf sheaths of rice plants, Physiol. Mol. Plant. Pathol. 64 (2004) 67–72.
[34] B. Valent, L. Farrall, F.G. Chumley, *Magnaporthe grisea* genes for pathogenicity and virulence identified through a series of backcrosses, Genetics 127 (1991) 87–101.