*MOLECULAR PLANT PATHOLOGY* (2006) **7**(1), 17–29 DOI: 10.1111/J.1364-3703.2005.00313.X

# Investigating the role of ABC transporters in multifungicide insensitivity in *Phytophthora infestans*

HOWARD S. JUDELSON* AND GEETHA SENTHIL

*Department of Plant Pathology, University of California, Riverside, CA 92521, USA*

## SUMMARY

Isolates of the oomycete *Phytophthora infestans* exhibit a wide range of intrinsic sensitivities to fungicides, which potentially influences the application rates of chemicals needed to control potato late blight. To help understand what determines such levels of sensitivity, a genetic approach was employed which followed the segregation of sensitivities to structurally diverse fungicides such as metalaxyl and trifloxystrobin. Progeny exhibited broad distributions of sensitivity phenotypes, consistent with the behaviour of a quantitative trait. Measurements of the inhibition of strains by seven fungicides revealed that basal sensitivities to metalaxyl and trifloxystrobin, and to cymoxanil and dimethomorph, correlated at the 95% confidence level. These compounds have distinct modes of action, suggesting the involvement of a multifungicide efflux phenomenon mediated by ABC transporters. To determine whether such proteins contribute to variation in sensitivity, 41 full transporters and 13 half transporters were identified from *P. infestans* and their mRNA levels compared in strains exhibiting higher or lower sensitivities to fungicides. No correlation was observed between the expression of any ABC transporter and fungicide sensitivity. Other genes, or variation in the activities of the transporters, may therefore explain the differences between strains. Five ABC transporters were induced by several fungicides in strains with both higher and lower sensitivities to fungicides, which probably reflects the existence of a network for protecting against natural and artificial toxins.

## INTRODUCTION

Much emphasis in studies of the sensitivity of microbes to antibiotics has focused on genes that have major effects. Such 'major genes' include those that inactivate the chemical, such as aminoglycoside phosphotransferases (Davies and Wright, 1997), or alter its target as in the case of benzimidazole-insensitive tubulins (Orbach *et al.*, 1986). However, sensitivities are also determined by genes with less dramatic, quantitative effects. Such 'minor genes' may encode general detoxifying enzymes such as glutathione S-transferases and cytochrome P450s, osmoregulators, or efflux pumps (Bauer *et al.*, 1999; Leroux *et al.*, 2002; Ochiai *et al.*, 2001). The best known examples of the last are ABC (ATP Binding Cassette) transporters, which are ubiquitous and evolutionarily conserved (Anjard and Loomis, 2002; Bauer *et al.*, 1999; Davies and Coleman, 2000; Dean *et al.*, 2001). Most catalyse the ATP-dependent transport of a broad spectrum of organics, ranging from small molecules to polypeptides, thereby reducing intracellular concentrations of antibiotics. ABC transporters are notorious as a cause of multidrug resistance in animal cells, in which a pharmacological agent selects for mutations that alter the specificity or expression of the transporter (Borst *et al.*, 1999). Reductions in sensitivity mediated by ABC transporters in fungal pathogens of humans has also been described (Ben-Yaacov *et al.*, 1994). In plant pathogenic fungi, laboratory studies have demonstrated that ABC transporters have roles in virulence or in toxicant efflux, but their relevance to fungicide insensitivity in the field is less well understood (Hayashi *et al.*, 2001; Lee *et al.*, 2005; Nakaune *et al.*, 1998; Urban *et al.*, 1999).

That factors besides 'major genes' influence the sensitivity of plant pathogens to crop protection chemicals is evident from surveys of field isolates of various ascomycetes, basidiomycetes and oomycetes (Franke *et al.*, 1998; Gisi *et al.*, 1997; Olaya and Koller, 1999). In the potato late blight pathogen *Phytophthora infestans,* for example, isolates varied 10–100-fold in their reaction to fungicides such as chlorothalonil, cymoxanil and mancozeb (Samoucha and Cohen, 1984; Sujkowski *et al.*, 1995). All isolates in those studies were considered 'sensitive' as they were controllable in the field by such compounds. However, their sensitivity variation is a factor in determining the application rate of a chemical, because the dosage must inhibit the least sensitive element of the pathogen population.

Our laboratory had previously proposed that the response of *P. infestans* to metalaxyl had a multigenic basis, akin to a quantitative trait (Fabritius *et al.*, 1997; Judelson and Roberts, 1999). Others drew a similar conclusion (Lee *et al.*, 1999). Until

* *Correspondence*: Tel.: +1 951 827-4199; E-mail: howard.judelson@ucr.edu

 

highly resistant strains appeared in recent decades, metalaxyl was the major chemical used to control many oomycetes (Fry and Goodwin, 1997). Genetic studies indicated that the high levels of insensitivity in such strains was due to a semidominant gene, *MEX* (Shattock, 1988). However, within a given *MEX* genotype, substantial variation existed, which was proposed to result from genes of minor effect.

The main objectives of this study were to address further the role of these minor genes in the sensitivity of *P. infestans* to metalaxyl, to test if they affect other fungicides and to attempt to determine their molecular nature by testing the role of ABC transporters. A correlation was observed in the effects of the minor genes on sensitivities to both metalaxyl and trifloxystrobin. Notably, these have distinct structures and modes of action: metalaxyl reportedly inhibits transcription by RNA polymerase I, while strobilurins such as trifloxystrobin block electron transport (Bartlett *et al*., 2002; Davidse *et al*., 1988). To test whether ABC transporters play roles in the response to these compounds, members of the transporter superfamily were identified and expression-profiled in strains of high and low sensitivity in the presence and absence of fungicides.

## RESULTS

### Sensitivity to metalaxyl is determined by *MEX* and other genes

We previously defined *MEX* as a major determinant of the response of *P. infestans* to metalaxyl, with highly insensitive, intermediately insensitive and sensitive strains being of the *MEX*/*MEX*, *MEX*/*mex* and *mex*/*mex* genotypes, respectively. Other loci also appeared to be involved as quantitative variation in insensitivity was observed within each *MEX* genotype (Fabritius *et al*., 1997; Judelson and Roberts, 1999). To confirm that conclusion and obtain a sufficient number of progeny for this study, a small cross described previously for isolates 1114 (*MEX/mex*) and 510 (*mex*/*mex*) was expanded by obtaining 244 additional progeny. These were scored for growth in the presence of 5 p.p.m. metalaxyl relative to unamended media, and for genotypes at locus *W4* which is 11 cM from *MEX*; *W4* is the closest known marker to *MEX* (Judelson and Roberts, 1999).

As shown in Fig. 1, well-separated 'resistant' and 'sensitive' groups were not observed in the progeny. A dashed line is drawn between the presumed *MEX*/*mex* and *mex*/*mex* classes (offspring numbers 1–127 and 128–244, respectively); this demarcation may appear arbitrary based on the metalaxyl phenotype, but is supported by the segregation of *W4.* The segregation pattern indicates that whereas *MEX* strongly influences the ability of *P. infestans* to grow in the presence of metalaxyl, other genes with significant aggregate effects also play important roles. Such genes have both additive and antagonistic effects, as many offspring have phenotypes that are more extreme than either parent.

### Some loci may influence responses to multiple compounds

Because reports of variation in the reaction of *P. infestans* isolates to fungicides such as chlorothalonil and mancozeb

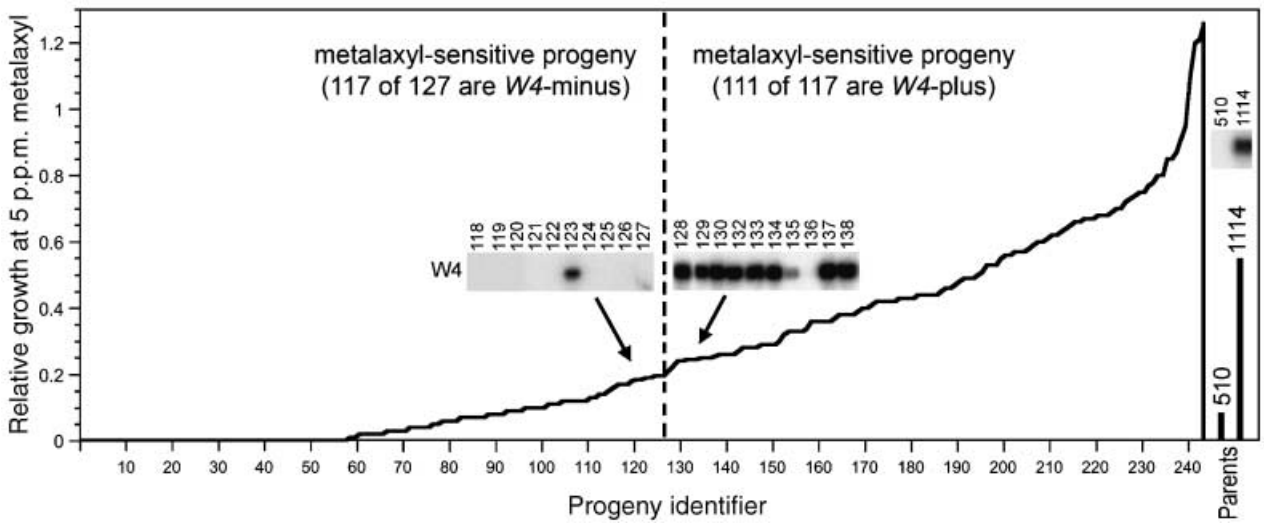


**Fig. 1** Sensitivity of 1114 × 510 offspring to metalaxyl. Indicated is the relative growth of their progeny in 5 p.p.m. metalaxyl relative to unamended media. Values are based on quadruplicate measurements of hyphal dry mass. Progeny to the left and right of the dashed line are presumed to be of *mex*/*mex* and *Mex*/*mex* genotypes, respectively, based on the analysis of locus *W4* (Judelson and Roberts, 1999); as *W4* is not absolutely linked to *MEX*, some recombinants are expected in both classes. The inserts illustrate the genotyping of *W4* for ten progeny to the left and ten to the right of the line. This involved PCR using primers to *W4*, electrophoresis and hybridization with a clone of the *MEX*-linked *W4* band.

(Samoucha and Cohen, 1984; Sujkowski *et al.*, 1995) are reminiscent of the phenomena shown in Fig. 1 for metalaxyl, it was of interest to test for correlations in the responses of isolates to different toxicants. A test of ten diverse isolates against seven structurally unrelated toxicants revealed instances of significant correlation, particularly involving metalaxyl and trifloxystrobin (Table 1). This analysis included *MEX*/*MEX*, *MEX*/*mex* and *mex*/*mex* isolates, to be representative of both older and contemporary isolates. Nevertheless, positive correlations in responses to metalaxyl and trifloxystrobin were also observed within each genotype, such as in *mex*/*mex* strains where $R = 0.98$ ($P = 0.05$).

To understand better the coordinate response of *P. infestans* to metalaxyl and trifloxystrobin, the growth of 33 of the 1114 × 510 progeny in the presence of trifloxystrobin was also examined (Fig. 2A). To eliminate the possible involvement of *MEX*, these 33 were selected from *mex*/*mex* progeny only. Although the 33 were selected randomly, they were checked to ensure that the entire spectrum of insensitivity levels was represented. Compared with all *mex*/*mex* progeny, which averaged 6.6% relative growth (standard deviation = 6.0%) in the presence of metalaxyl, the 33 selected averaged 8.4% (SD = 8.2%). The growth of the other 94 *mex*/*mex* offspring on trifloxystrobin was not measured. Based on measurements of dry mass in 9-day broth cultures containing 5 p.p.m. metalaxyl, 30 p.p.m. trifloxystrobin or unamended media, responses to the compounds by the 33 offspring correlated strongly ($R = 0.428$, $P = 0.0127$). Radial growth assays on agar media yielded similar conclusions (data not shown). Tests of six representative offspring of the 1114 × 510 progeny also indicated a correlation in responses to metalaxyl and another strobilurin, azoxystrobin ($R = 0.882$, $P = 0.021$).

Whether such correlations existed in other genotypes was tested using progeny from 216 × Ca65 and 3029 × 550 crosses, which both represent *MEX*/*mex* × *mex*/*mex* pairings. As before, only *mex*/*mex* progeny were examined, which were selected based on metalaxyl-sensitivity and *W4* phenotypes. A good correlation ($P = 0.073$) was observed in the 216 × Ca65 group, but not in the 3029 × 550 set. The findings from these two crosses should be considered preliminary because it was difficult to produce large numbers of progeny for analysis, as is often the case in *P. infestans* (Whittaker *et al.*, 1991). However, they do indicate that some genes in 216 or Ca65 reduce sensitivity to both compounds, as seen in the 1114 × 510 study. Distinct alleles of those loci may exist in 3029 or 550.

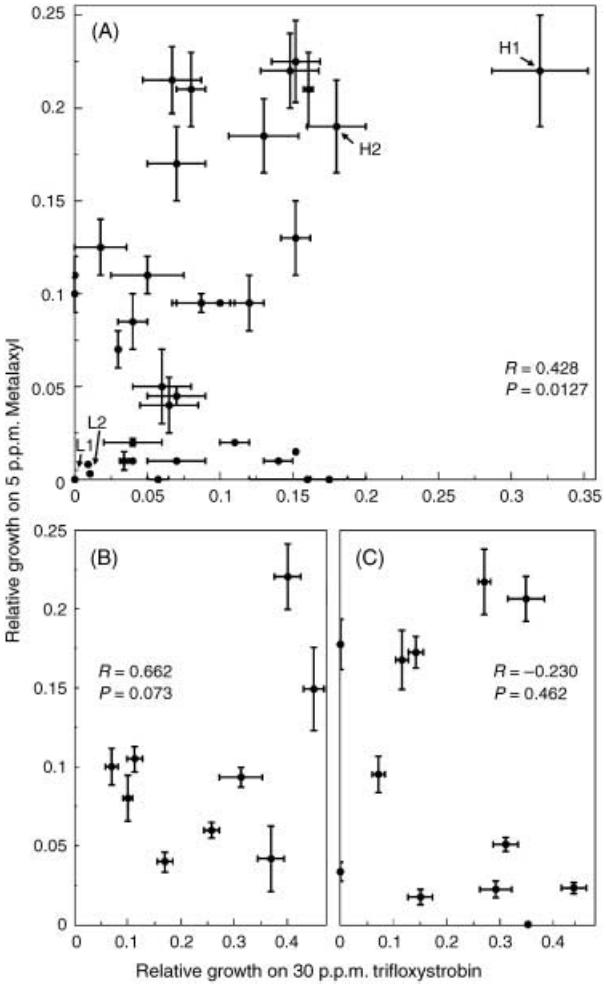


**Fig. 2** Sensitivity of *P. infestans* progeny to trifloxystrobin (30 p.p.m.) and metalaxyl (5 p.p.m.). Representative *mex*/*mex* offspring were selected from crosses 1114 × 510 (A), 216 × Ca65 (B) and 3029 × 550 (C). Growth rates were determined using dry mass assays, with relative growth defined as the mass of hyphae in the presence compared with absence of the chemical. Assays were performed in quadruplicate and error bars represent standard deviations. Indicated in each panel is the correlation coefficient, *R*, and *P*-value. In panel A, offspring H1, H2, L1 and L2 are noted, which are used in later experiments.

## ABC transporters in *P. infestans*

Whether ABC transporters might be involved in the reduced-sensitivity phenomena described above was considered, due to the role of such proteins as determinants of multitoxin insensitivity in other species. To enable this to be tested, it was necessary first to define the nature of the ABC transporter superfamily in *P. infestans.* Whether any genes are expressed differentially in the more-sensitive vs. less-sensitive strains will be addressed later in this paper.

In an 18 000 unigene set recently reported for *P. infestans* (Randall *et al.*, 2005), 73 unigenes had ABC transporters as one or more of their three top BLASTX hits against the SwissProt database ($E < 10^{-15}$). The unigene set was derived from assemblies of 76 000 expressed sequence tags (ESTs) and regions of protein-coding potential extracted from 1× coverage genome sequencing. However, some of the 73 candidates might represent false

matches. This is because the ABC superfamily includes both authentic transporters, which have nucleotide binding fold (NBF) and transmembrane (TMD) domains, and NBF-only proteins that appear derived from ABC transporters but have no transport function. The latter include the RNase L inhibitor and GCN translational regulator groups (eukaryotic ABC families E and F; Dean *et al*., 2001).

Fifty-four of the 73 candidates were deduced to encode authentic ABC transporters (Table 2). This was either because the *P. infestans* sequences contained both NBF and TMD domains or because both features were in their closest relatives, based on BLASTN searches, in the sequenced genomes of *P. ramorum* and *P. sojae* (available online from the Joint Genome Institute of the US Department of Energy). The latter analysis was particularly useful when analysing short EST-derived *P. infestans* unigenes, which would be unlikely to span both an NBF and a TMD. Protein alignments indicated that the 54 *P. infestans* ABC transporters contain NBFs with features seen in authentic transporters from other kingdoms (data not shown). These include the Walker A and Walker B motifs, a glutamate following Walker B that is involved in catalysis, and an ABC transporter signature sequence seen in ABC transporters but not in other ATPase-related NBFs (Gaudet and Wiley, 2001; Orelle *et al*., 2003).

Table 2 also lists, for the 54 *P. infestans* transporters, their closest relative in *P. sojae*. The only exceptions to this involve *PiABCC4* and *PiABCB25* for which no relative was detected, in which cases the closest *P. ramorum* BLASTN matches are shown, and *PiABCA10*, which did not show obvious DNA homology to sequences in either species. In a few cases, one *P. sojae* gene model was the closest relative of multiple *P. infestans* genes, suggesting expansion of the ABC transporter family in the latter species, which is consistent with its larger genome (Voglmayr and Greilhuber, 1998). Also shown in Table 2 is the topology and size of the predicted *P. sojae* or *P. ramorum* homologues. Each of those homologues matched ABC transporters in GenBank (mean and minimum BLASTP *E*-values of $10^{-146}$ and $10^{-55}$, respectively). Fifteen of the *P. sojae* or *P. ramorum* gene models listed in Table 2, which were taken from assembly 1.0 of their respective genome projects, were judged to contain gross defects such as N- or C-terminal truncations or mis-splicing. In such cases, corrected gene models were developed and used for the data in Table 2 and phylogenetic analyses reported below.

**Table 1** Correlation in response to fungicides among ten isolates of *P. infestans*.

| | Correlation coefficients for compounds* | | | | | | |
|---|---|---|---|---|---|---|---|
| | Metalaxyl | Hymexazol | Dimethomorph | Cymoxanil | Chlorothalonil | Propamocarb | Trifloxystrobin |
| Metalaxyl | 1.00 | −0.56 | 0.01 | 0.18 | −0.06 | −0.00 | 0.97† |
| Hymexazol | | 1.00 | 0.23 | 0.22 | 0.38 | −0.17 | −0.54 |
| Dimethomorph | | | 1.00 | 0.71† | 0.40 | 0.60 | 0.06 |
| Cymoxanil | | | | 1.00 | 0.44 | 0.00 | 0.21 |
| Chlorothalonil | | | | | 1.00 | 0.07 | −0.13 |
| Propamocarb | | | | | | 1.00 | −0.04 |
| Trifloxystrobin | | | | | | | 1.00 |

*Compounds were tested at the concentrations listed in the Experimental procedures, using radial growth assays on rye agar media. The isolates examined were of the *MEX*/*MEX* (8811, 115.11, 93H3), *MEX*/*mex* (618, 1114) and *mex*/*mex* (E13a, 1306, 510, Ca65, 550) genotypes.
†Significant at 95% confidence level.

**Table 2** ABC transporters of *P. infestans* characterized in this study, and their relatives.

| | *P. infestans* gene | | | | | Closest *P. sojae* relative | | |
|---|---|---|---|---|---|---|---|---|
| Family | Name | Matching EST* | GenBank accession no. | No. of copies† | Relative mRNA‡ | Name§ | Topology¶ | Size (aa) |
| A | PiABCA1 | pine_30816 | CW812813 | 1 | 0.08 ± 0.01 | estExt_fgenesh1_pg.C_280140(r) | $(NBF\text{-}TMD)_2$ | 1624 |
| | PiABCA2 | PU005B2 | CV943936 | 3 | 0.22 ± 0.04 | estExt_fgenesh1_pg.C_50073 | $(NBF\text{-}TMD)_2$ | 1884 |
| | PiABCA3 | PM049D2 | CV931036 | 1 | — | estExt_Genewise1.C_100204(r) | $(TMD\text{-}NBF)_2$ | 1801 |
| | PiABCA4 | rpvb_3012 | CV949842 | 1 | 0.11 ± 0.02 | estExt_fgenesh1_pg.C_210020 | $(TMD\text{-}NBF)_2$ | 1848 |
| | PiABCA5 | PF039E12 | CV915388 | 1 | 0.11 ± 0.02 | estExt_fgenesh1_pg.C_1060036 | $(TMD\text{-}NBF)_2$ | 2014 |
| | PiABCA6 | PDrpcd_0803 | CV906060 | 2 | 0.25 ± 0.02 | estExt_fgenesh1_pg.C_50065 | $(TMD\text{-}NBF)_2$ | 1949 |
| | PiABCA7 | PYrpcy_8682 | CV964668 | 1 | 0.05 ± 0.02 | estExt_fgenesh1_pg.C_1030070 | $(TMD\text{-}NBF)_2$ | 1959 |
| | PiABCA8 | PVrpvb_4584 | CV950993 | 3 | — | estExt_fgenesh1_pg.C_50073 | $(TMD\text{-}NBF)_2$ | 1884 |
| | PiABCA9 | PJ025E5 | CV924771 | 2 | — | estExt_fgenesh1_pg.C_50065 | $(TMD\text{-}NBF)_2$ | 1949 |
| | PiABCA10 | rpch_7469.y1 | DR783200 | 2 | — | none | — | — |

**Table 2** *Continued.*

| | *P. infestans* gene | | | | | Closest *P. sojae* relative | | |
|---|---|---|---|---|---|---|---|---|
| Family | Name | Matching EST* | GenBank accession no. | No. of copies† | Relative mRNA‡ | Name§ | Topology¶ | Size (aa) |
| B | PiABCB1 | rpxc_1249 | CV954497 | 2 | 1.46 ± 0.08 | estExt_fgenesh1_pg.C_400081 | $(NBF\text{-}TMD)_2$ | 1095 |
| | PiABCB2 | PM045C9 | CV930701 | 1 | 0.07 ± 0.01 | estExt_fgenesh1_pg.C_690041 | $(NBF\text{-}TMD)_2$ | 1320 |
| | PiABCB3 | rpcm_3048 | CV934896 | 1 | 0.02 ± 0.01 | fgenesh1_pg.C_scaffold_24000059 | $(NBF\text{-}TMD)_2$ | 1356 |
| | PiABCB4 | PF034G9 | CV914958 | 2–3 | 0.08 ± 0.02 | estExt_fgenesh1_pg.C_690041 | $(NBF\text{-}TMD)_2$ | 1320 |
| | PiABCB5 | PB050F2 | CV901287 | 1 | 0.07 ± 0.01 | estExt_Genewise1.C_1040010(r) | $(NBF\text{-}TMD)_2$ | 1372 |
| | PiABCB6 | PB040C9 | CV900556 | 3 | 0.04 ± 0.01 | fgenesh1_pg.C_scaffold_425000003 | $(NBF\text{-}TMD)_2$ | 1363 |
| | PiABCB7 | PMrpcm_2904 | CV934850 | 5 | 0.41 ± 0.03 | estExt_fgenesh1_pg.C_130004 | $(NBF\text{-}TMD)_2$ | 1376 |
| | PiABCB8 | pinx_2205.z1 | CW805934 | 5 | 0.05 ± 0.01 | estExt_fgenesh1_pg.C_160121 | $(NBF\text{-}TMD)_2$ | 1321 |
| | PiABCB9 | rpch_0667.y1 | DR783201 | 1 | 0.21 ± 0.03 | estExt_fgenesh1_pg.C_1410015 | $(NBF\text{-}TMD)_2$ | 1369 |
| | PiABCB10 | PN010A10 | CV969707 | 5–10 | 0.08 ± 0.01 | estExt_Genewise1.C_420012(r) | $(NBF\text{-}TMD)_2$ | 1366 |
| | PiABCB11 | MY-24-A-05 | BE777054 | 5–10 | 0.06 ± 0.00 | estExt_fgenesh1_pg.C_30068 | $(NBF\text{-}TMD)_2$ | 1430 |
| | PiABCB12 | PB020F2 | CV899165 | 4 | 0.32 ± 0.02 | estExt_fgenesh1_pg.C_1310045 | $(NBF\text{-}TMD)_2$ | 1354 |
| | PiABCB13 | rpct_2902 | CV935977 | 10 | 0.11 ± 0.01 | estExt_fgenesh1_pg.C_130001(r) | $(TMD\text{-}NBF)_2$ | 1311 |
| | PiABCB14 | PF037D4 | CV915193 | 5 | 0.10 ± 0.02 | estExt_fgenesh1_pg.C_200137(r) | $(TMD\text{-}NBF)_2$ | 1307 |
| | PiABCB15 | PF009D1 | CV912804 | 5 | 0.01 ± 0.00 | fgenesh1_pg.C_200119(r) | $(TMD\text{-}NBF)_2$ | 1307 |
| | PiABCB16 | rpcm_2844 | CV934828 | 1 | 0.03 ± 0.01 | estExt_fgenesh1_pg.C_1310033 | $(TMD\text{-}NBF)_2$ | 1410 |
| | PiABCB17 | PE010A8 | CV910101 | 1 | — | estExt_fgenesh1_pg.C_1310033 | $(TMD\text{-}NBF)_2$ | 1410 |
| | PiABCB18 | PYrpcy_4575 | CV963797 | 5–10 | — | estExt_fgenesh1_pg.C_1550001 | $(NBF\text{-}TMD)_2$ | 1378 |
| | PiABCB19 | PXrpxc_2193 | CV955298 | 3 | 0.16 ± 0.02 | estExt_fgenesh1_pg.C_400093 | NBF-TMD | 713 |
| | PiABCB20 | PU045F05 | CV945664 | 2 | 0.23 ± 0.04 | estExt_Genewise1.C_800012 | NBF-TMD | 456 |
| | PiABCB21 | rpxc_1079 | CV954366 | 5–10 | 0.67 ± 0.08 | estExt_fgenesh1_pg.C_400082 | NBF-TMD | 619 |
| | PiABCB22 | rpct_7932 | CV943423 | 5–10 | 0.27 ± 0.03 | estExt_fgenesh1_pg.C_400082 | NBF-TMD | 712 |
| | PiABCB23 | PHrpch_0764 | CV921681 | 1 | 0.33 ± 0.05 | estExt_fgenesh1_pg.C_400066(r) | TMD-NBF | 618 |
| | PiABCB24 | PYrpcy_5877 | CV963934 | 1 | 0.07 ± 0.01 | estExt_fgenesh1_pm.C_70015 | TMD-NBF | 600 |
| | PiABCB25 | pinb_37766.z1 | CW807202 | 1 | 0.29 ± 0.04 | gwEuk.41.80.1(r) | TMD-NBF | 616 |
| C | PiABCC1 | PB028B2 | CV899689 | 2 | 0.09 ± 0.02 | scaffold 269 : 18042–20860 | $(TMD\text{-}NBF)_2$ | 1320 |
| | PiABCC2 | PD003A5 | DR783197 | 1 | 0.02 ± 0.01 | estExt_fgenesh1_pg.C_1280003(r) | $(TMD\text{-}NBF)_2$ | 1273 |
| | PiABCC3 | PL002G8 | CV927820 | 1 | — | estExt_fgenesh1_pg.C_450041 | $(TMD\text{-}NBF)_2$ | 1179 |
| | PiABCC4 | rpch_1529 | CV922296 | 2 | 0.02 ± 0.00 | estExt_fgenesh1_pg.C_160125(r) | $(TMD\text{-}NBF)_2$ | 1323 |
| | PiABCC5 | rpineb_1068 | CW820139 | 1 | 0.02 ± 0.00 | fgenesh1_pm.C_scaffold_700004(r) | $(TMD\text{-}NBF)_2$ | 1064 |
| | PiABCC6 | rpinea_30465 | CW808438 | 1 | 0.26 ± 0.03 | estExt_fgenesh1_pg.C_450041 | $(TMD\text{-}NBF)_2$ | 1179 |
| | PiABCC7 | rpcm0–000287 | CV938420 | 1 | 0.08 ± 0.02 | estExt_Genewise1.C_800048(r) | $(TMD\text{-}NBF)_2$ | 1346 |
| | PiABCC8 | pinb_2884 | CW804633 | 1 | 0.03 ± 0.01 | estExt_fgenesh1_pg.C_170032(r) | $(TMD\text{-}NBF)_2$ | 1288 |
| | PiABCC9 | PMrpcm_4755 | CV935400 | 1 | 0.11 ± 0.02 | estExt_fgenesh1_pg.C_740034 | $(TMD\text{-}NBF)_2$ | 1384 |
| | PiABCC10 | PM058E11 | CV931760 | 2 | 0.05 ± 0.01 | estExt_fgenesh1_pm.C_840008 | $(TMD\text{-}NBF)_2$ | 1264 |
| | PiABCC11 | PVrpvb_5587 | CV951795 | 1 | 0.04 ± 0.01 | estExt_fgenesh1_pg.C_850049 | $(TMD\text{-}NBF)_2$ | 1063 |
| G | PiABCG1 | PYrpcy_2444 | CV963331 | 1 | 0.18 ± 0.02 | estExt_fgenesh1_pg.C_280140(r) | $(NBF\text{-}TMD)_2$ | 1766 |
| | PiABCG2 | PMrpcm_5726 | CV936321 | 3 | 0.04 ± 0.01 | estExt_fgenesh1_pg.C_720049 | NBF-TMD | 636 |
| | PiABCG3 | pine_42549 | CW816085 | 2 | — | estExt_fgenesh1_pg.C_280127 | NBF-TMD | 634 |
| | PiABCG4 | PJ041B10 | CV926077 | 2 | 0.11 ± 0.00 | estExt_fgenesh1_pg.C_850027 | NBF-TMD | 987 |
| | PiABCG5 | rpvb_13289 | CV949302 | 2 | 0.04 ± 0.01 | estExt_fgenesh1_pg.C_280132 | NBF-TMD | 915 |
| | PiABCG6 | rpcd_7852 | DR783198 | 2 | 0.03 ± 0.01 | estExt_fgenesh1_pg.C_720047 | NBF-TMD | 748 |
| | PiABCG7 | rpineb_35678 | DR783199 | 1 | — | estExt_fgenesh1_pg.C_280126 | NBF-TMD | 621 |
| | PiABCG8 | PMrpct_3853 | CV941468 | 1 | — | estExt_fgenesh1_pg.C_190002 | NBF-TMD | 622 |

*Sequence read anchoring the contig.

†Rough estimate of copy number based on DNA blots.

‡Abundance relative to actin in non-treated hyphal culture, showing average of strains H1 and L1. Genes for which signals were not significantly above background in hyphae are indicated by a dash.

§Closest gene model in assembly 1.0 of the *P. sojae* genome, based on BLASTN searches, with the following exceptions. In cases marked by '(r)', the machine annotation was judged incorrect and a revised model was developed. For the *PiABCC1* relative, an apparent homologue was detected in *P. sojae* for which no gene model had been predicted, at the site noted, although its C-terminus is truncated due to a gap in the assembly. For *PiABCC4* and *PiABCB25*, homologues were not detected in *P. sojae* and therefore the closest relative from *P. ramorum* is listed. For *PiABCA10*, no homologues were detected.

¶Predicted from gene model in the previous column.

Additional characterizations were performed to aid later studies of the ABC transporters in *P. infestans*, for example to see if coordinately regulated genes are related. One analysis entailed determining to which eukaryotic TMD-containing ABC family each *P. infestans* gene belonged, using the human scheme (Dean *et al.*, 2001) which classifies proteins into Families A (MDR/TAP), B (CFTR/MRP), D (ALD) and G (White). BLASTP searches against GenBank using the entire *P. infestans*, *P. sojae* and *P. ramorum* sequences, and analyses of the NBF (pfam00005) domains alone, gave consistent results. As shown in Table 2, the name of each transporter includes this family assignment (ABCA, ABCB, etc.). Ten proteins are in Family A. All are full transporters, having two NBF and two TMD domains in either forward and reverse orientations. Interestingly, this is the same as in humans but unlike *Arabidopsis thaliana* where most are half transporters, having only one NBF and TMD domain, and unlike *Dictyostelium discoideum* where full and half transporters are equally common; true fungi such as *Saccharomyces cerevisiae* lack ABCA proteins (Anjard and Loomis, 2002; Bauer *et al.*, 1999; Davies and Coleman, 2000; Dean *et al.*, 2001). Family B was the largest in *P. infestans*, with 25 members. Nineteen are full transporters of the type linked in other species with multiple drug resistance, and six are half transporters that are often associated with mitochondrial transport. Eleven *P. infestans* proteins are in Family C, all being full transporters as in other eukaryotes. Eight are in Family G, in which seven are half transporters. This resembles the situation in humans where half transporters also predominate, but is unlike true fungi where full transporters are more common. No members of Family D were detected. Most eukaryotes have a small number of such proteins, which are typically peroxisomal lipid transporters (Footitt *et al.*, 2002). Possibly, a *P. infestans* ABCD gene exists but is not expressed strongly enough to have been detected as an EST. This is not a problem in terms of the objectives of this project, as peroxisomal transporters are probably not involved in fungicide efflux.

The individual NBF domains were sorted in phylogenetic analyses according to the eukaryotic ABC families without exception, although some families such as B appear polyphyletic (Fig. 3). Because full transporters have two NBF domains, they are shown twice in the tree, for example as A1-1 and A1-2 in the case of *ABCA1*. This type of analysis enables distinct patterns of evolution to be discerned. For example, Family A, which exclusively contains full transporters, appears to have resulted from the ancient duplication of a half transporter as the two NBF domains form distinct clades rooted in a common ancestor. A similar pattern is seen for Family C. By contrast, in Family B, half and full transporters reside in the same clades.

The majority of *Phytophthora* ABC transporters most closely resembled plant proteins, based on their best BLASTX hits in GenBank. Of the 54 transporters, 34 had closest matches against proteins from plants, 13 from animals, two from a protozoan, three from a slime mould, one from an ascomycete and one from a bacterium.

DNA blot analyses were used to estimate the copy number of the genes. As shown in Table 2 and Fig. 4, some genes were single copy, e.g. *PiABCC8*, others such as *PiABCB4* were members of small families (2–3 members), and genes such as *PiABCB10* belonged to larger families (more than five members). Large families were most common in Family B, reflecting its expansion into the largest ABC transporter group in *P. infestans.*

### Transcription of ABC transporter genes in *P. infestans*

A cDNA macroarray approach (Fig. 5) was employed to test the hypothesis that the lower sensitivities of some *P. infestans* strains was due to enhanced ABC transporter expression, as in many non-oomycetes (Hayashi *et al.*, 2001; Laing *et al.*, 1998; Lyons and White, 2000; Nakaune *et al.*, 1998; Slaven *et al.*, 2002). Polymerase chain reaction (PCR)-amplified fragments of the 54 ABC transporter genes plus actin (*ActA*) were spotted on membranes, which were hybridized with $^{32}$P-cDNA from four $1114 \times 510$ *mex*/*mex* offspring. These included two offspring (H1, H2; see Fig. 2A) with the highest and two with the lowest (L1, L2) combined insensitivity to metalaxyl and trifloxystrobin; this was expected to maximize the likelihood of detecting differences in ABC transporter expression. Probes were made from hyphae grown in unamended media, or media containing the fungicides dimethomorph, metalaxyl or trifloxystrobin. As a spotting control, the membranes were later probed with an oligonucleotide on each DNA fragment. The mRNA abundance of each gene, relative to actin, was then calculated by phosphorimager analysis. To select the most informative treatments, preliminary tests were performed using several concentrations of fungicide ($IC_5$, $IC_{10}$, $IC_{50}$) and times of treatment (1, 2, 6 and 24 h). Based on those data, 2-h treatments at $IC_{50}$ were compared with cultures lacking fungicides.

Of the 54 genes, 44 yielded reliable signals in the arrays (Table 2). Values are not presented for ten genes yielding signals below 2.5× background, but their expression seemed similarly low in all experiments. Many of those are transcribed specifically in developmental stages not tested here, such as asexual sporulation or mating (data not shown), so presumably do not influence the fungicide sensitivity of hyphae.

The data failed to support the hypothesis that some ABC transporters would be expressed at higher levels in strains that were less sensitive to metalaxyl and trifloxystrobin. This is illustrated in Fig. 6 in comparisons between H1 and L1, and H2 and L2, in non-amended rye broth (Fig. 6A,B) and media containing trifloxystrobin (Fig. 6C), metalaxyl (Fig. 6D) or a third fungicide, dimethomorph (Fig. 6E). However, five genes (*PiABCB5*, *PiABCB14*, *PiABCC6*, *PiABCC9*, *PiABCG5*) were induced by both dimethomorph and trifloxystrobin in all strains (Fig. 6, Table 3). Three

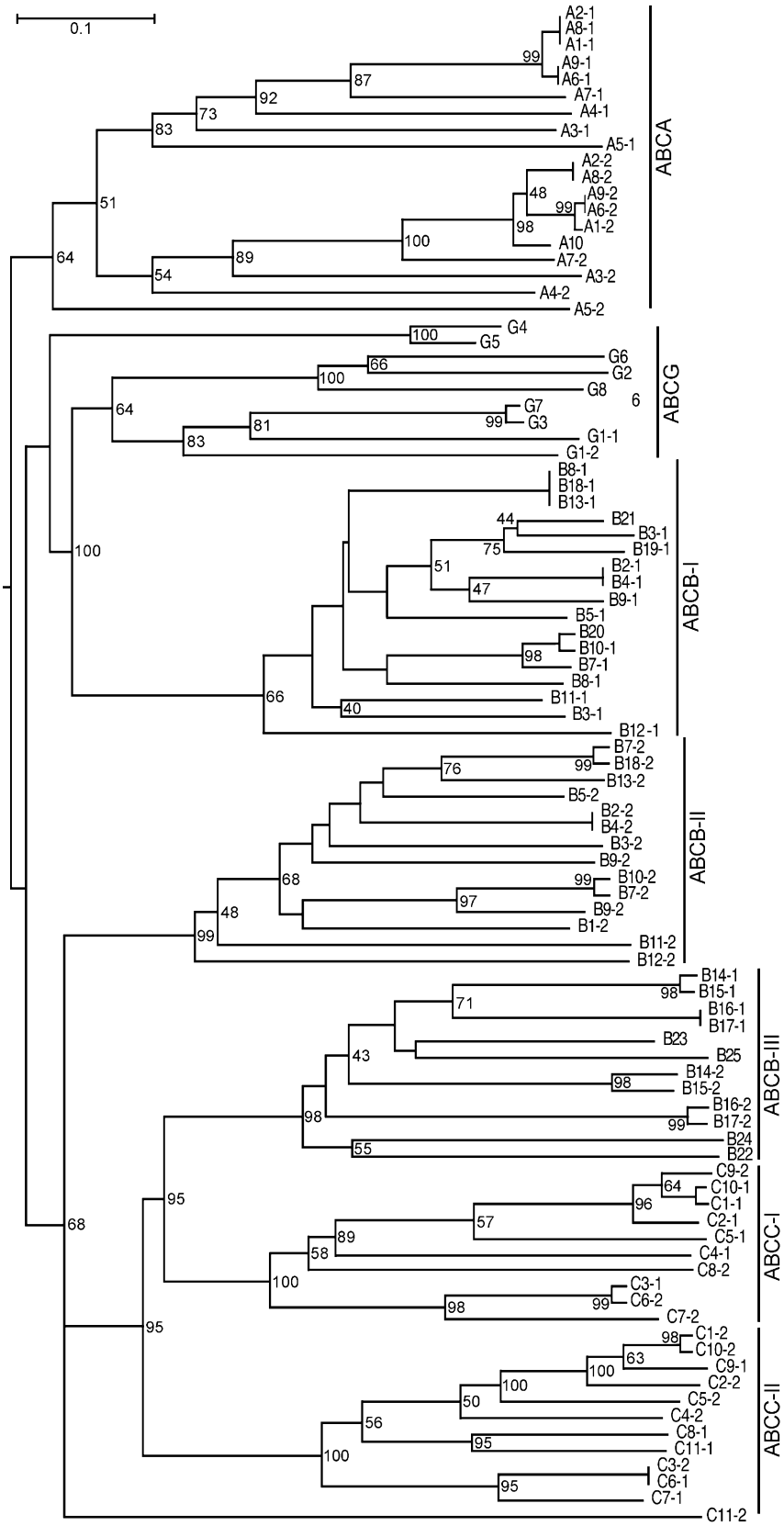


**Fig. 3** Phylogenetic analysis of ABC transporters. Shown is a consensus neighbour-joining tree based on NBF domains, constructed using CLUSTALW. For full transporters, N- and C-terminal domains are denoted by the '-1' and '-2' suffixes, respectively. Numbers at nodes denote their percentage occurrence in 1000 bootstrap replicates; only values greater than 40% are shown. Major clades encompassing families A, B, C and G are marked.

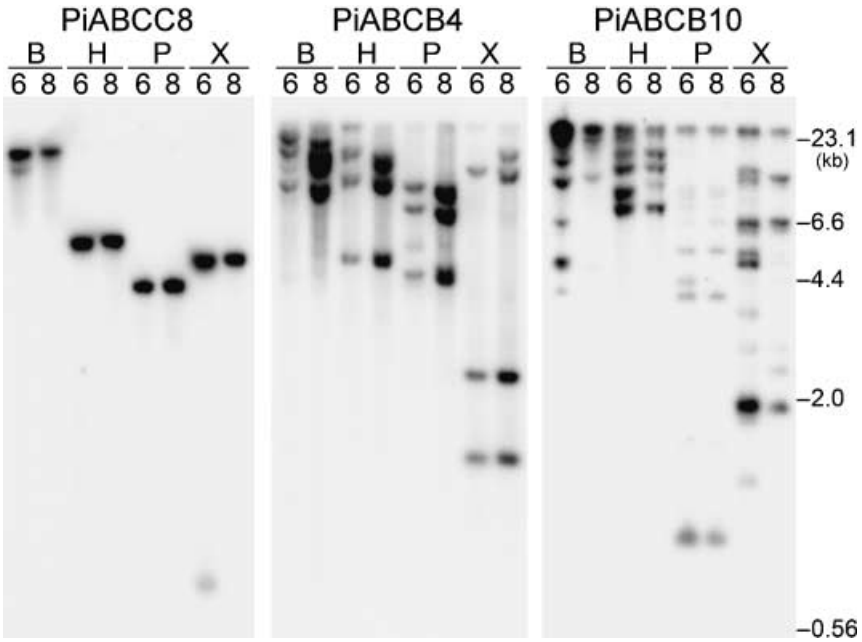


**Fig. 4** Representative DNA blots of ABC transporters. Genomic DNA from isolates 618 (6) and 8811 (8) were digested with *Bam*HI (B), *Hin*dIII (H), *Pst*I (P) or *Xba*I (X), electrophoresed, and hybridized with probes for genes *PiABCC8*, *PiABCB4* or *PiABCB10*.

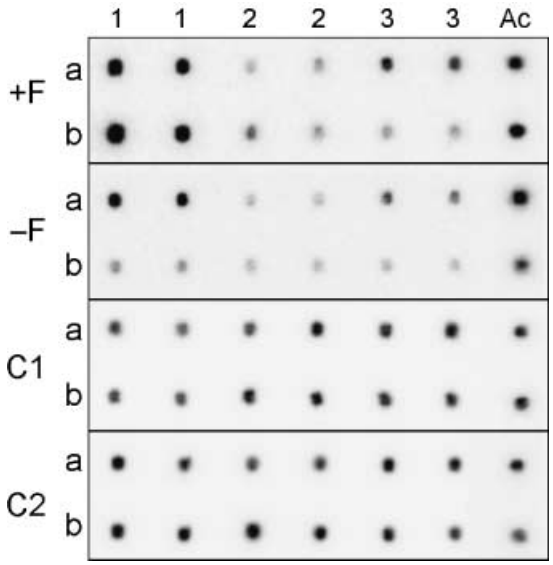


**Fig. 5** Example of dot-blot approach. Shown are portions of two representative filters corresponding to six ABC transporters. These were hybridized with $^{32}$P-cDNA from cultures grown in media containing trifloxystrobin (+F) or lacking fungicide (–F). C1 and C2 are the same filters, but hybridized with T7 oligonucleotide as a control. The genes include one that is induced by fungicide (*PiABCC6*, spotted in duplicate at coordinates b1) and five that are not induced (*PiABCB20*, *PiABCA4*, *PiABCB2*, *PiABCB13* and *PiABCB4*, spotted at a1, a2, a3, b2 and b3, respectively).

genes also exhibited some induction by metalaxyl. The other genes showed no significant differences between strains, in either amended or fungicide-containing media. To save space, comparisons of treated and non-treated L1 and L2 are not shown. However, it can be surmised from the data presented that L1 and L2 behaved similarly to H1 and H2.

The induction of *PiABCB5*, *PiABCB14* and *PiABCC6* by fungicides was confirmed by RNA blot analysis using RNA samples independent of those used in the arrays (Fig. 7). This up-regulation was not a general stress response, as their mRNAs did not increase during starvation. As a control, *PiABCB6* was also tested, which based on array data was not induced by fungicide; the RNA blots confirmed this pattern.

By comparing the positions of the fungicide-induced genes in the phylogenetic tree shown in Fig. 3, it is apparent that they are not closely related. In addition, near relatives of those genes are not induced. For example, the closest relative of *PiABCB14* is *PiABCB15* (clade ABCB-III). However, only the former is induced by fungicide.

## DISCUSSION

Understanding the basis of strain-to-strain variation in fungicide sensitivity, outside the role of major genes such as *MEX*, is of practical importance for controlling microbes in agricultural and clinical settings. The reduced sensitivity of some strains may be inadequate to cause 'practical resistance', as defined as an inability to achieve control in the field (McGrath, 2001). However, continued fungicide use may select for mutations that reduce sensitivity. This would require the use of higher dosages, which has negative economic and environmental consequences. This would resemble the situation with demethylation-inhibiting (DMI) fungicides, where progressive mutations decreased the sensitivities of many pathogens (McGrath, 2001). Knowing what genes

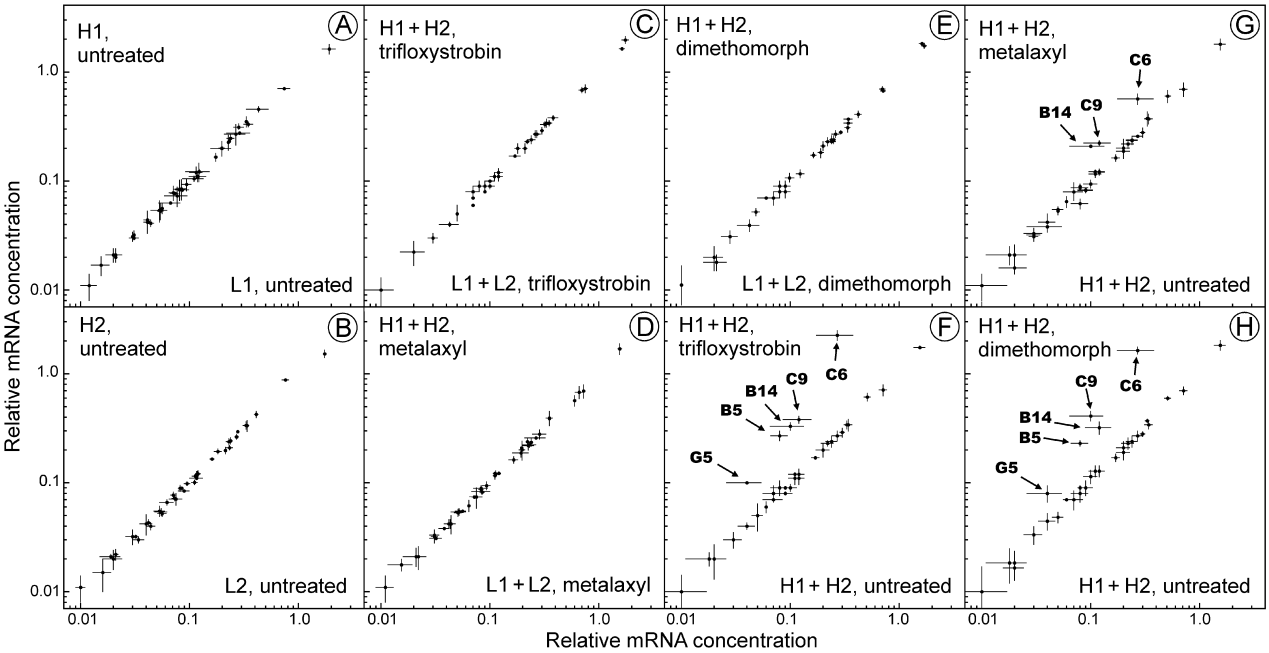


**Fig. 6** RNA levels of ABC transporters based on arrays. Four 1114 × 510 $F_1$ strains of *mex*/*mex* genotype showing relatively high (H1 and H2) or low (L1 and L2) growth in metalaxyl, as noted in Fig. 2, were analysed. Shown are RNA levels of each transporter gene in rye broth (untreated) or after 2 h with fungicides, normalized to *actA*. Panels A and B show comparisons of individual strains, with error bars representing replicates of the same strain. Panels C–H show values averaged from arrays hybridized separately with cDNA from H1, H2, L1 and L2; error bars represent the range between H1 and H2 (or L1 and L2). In panels C–H, averaged values are shown to save space, but similar trends were seen between individual strains. Data points representing fungicide-induced genes (including *PiABCG5*, *PiABCB5*, *PiABCB14*, *PiABCC9* and *PiABCC6*) are marked in panels F–H.

**Table 3** Relative mRNA levels of ABC transporters showing significant changes in transcript abundance during fungicide treatments.

| | Unamended media | | Trifloxystrobin | | Dimethomorph | | Metalaxyl | |
|---|---|---|---|---|---|---|---|---|
| Gene | H1 + H2 | L1 + L2 | H1 + H2 | L1 + L2 | H1 + H2 | L1 + L2 | H1 + H2 | L1 + L2 |
| *PiABCB5* | 0.08 ± 0.02 | 0.07 ± 0.01 | 0.27 ± 0.03 | 0.25 ± 0.02 | 0.23 ± 0.01 | 0.26 ± 0.04 | 0.07 ± 0.01 | 0.06 ± 0.01 |
| *PiABCB14* | 0.10 ± 0.04 | 0.11 ± 0.02 | 0.33 ± 0.03 | 0.32 ± 0.03 | 0.41 ± 0.04 | 0.42 ± 0.03 | 0.22 ± 0.04 | 0.20 ± 0.04 |
| *PiABCC6* | 0.27 ± 0.12 | 0.28 ± 0.03 | 2.26 ± 0.24 | 2.17 ± 0.16 | 1.62 ± 0.13 | 1.72 ± 0.10 | 0.56 ± 0.07 | 0.60 ± 0.04 |
| *PiABCC9* | 0.12 ± 0.04 | 0.12 ± 0.02 | 0.36 ± 0.05 | 0.39 ± 0.04 | 0.32 ± 0.04 | 0.35 ± 0.04 | 0.21 ± 0.03 | 0.23 ± 0.03 |
| *PiABCG5* | 0.04 ± 0.01 | 0.04 ± 0.01 | 0.10 ± 0.02 | 0.11 ± 0.03 | 0.09 ± 0.02 | 0.10 ± 0.02 | 0.04 ± 0.01 | 0.04 ± 0.01 |

Hyphae were treated with fungicides as described in the Experimental procedures. Values represent averages ± SD of the indicated strains, normalized to actin (ActA).

influence insensitivity may also be helpful by indicating ways to enhance control. In theory, reductions in application rates can result by mixing inhibitors of the defence proteins with fungicides. For example, cellular concentrations of drugs can increase in fungal and animal cells in the presence of inhibitors of ABC transporters (Di Pietro *et al.*, 2002; Yamamoto *et al.*, 2005).

This study has shown that a process exists in *P. infestans* that coordinately influences sensitivity against fungicides with discrete structures and modes of action. Cross-resistance to related fungicides by a single cellular pathway is well documented in other phytopathogens, but not to dissimilar molecules (Diriwachter *et al.*, 1987; Ito *et al.*, 2004). It is interesting to note that the *P. infestans* isolates studied here pre-date the use of strobilurins, reflecting natural diversity in the mechanism(s) affecting their sensitivities. It is reasonable to propose that ABC transporters are involved owing to similarities with the phenomenon of multidrug resistance. This often results from increased transcription of ABC transporters as a consequence of promoter mutations or gene amplification (Hayashi *et al.*, 2001; Laing *et al.*, 1998; Lyons and White, 2000; Nakaune *et al.*, 1998; Slaven *et al.*, 2002). In addition, over-expressing ABC transporters in transgenic *Magnaporthe grisea* and *Aspergillus nidulans* decreased their sensitivities to diverse toxicants (Andrade *et al.*, 2000; Lee *et al.*, 2005).

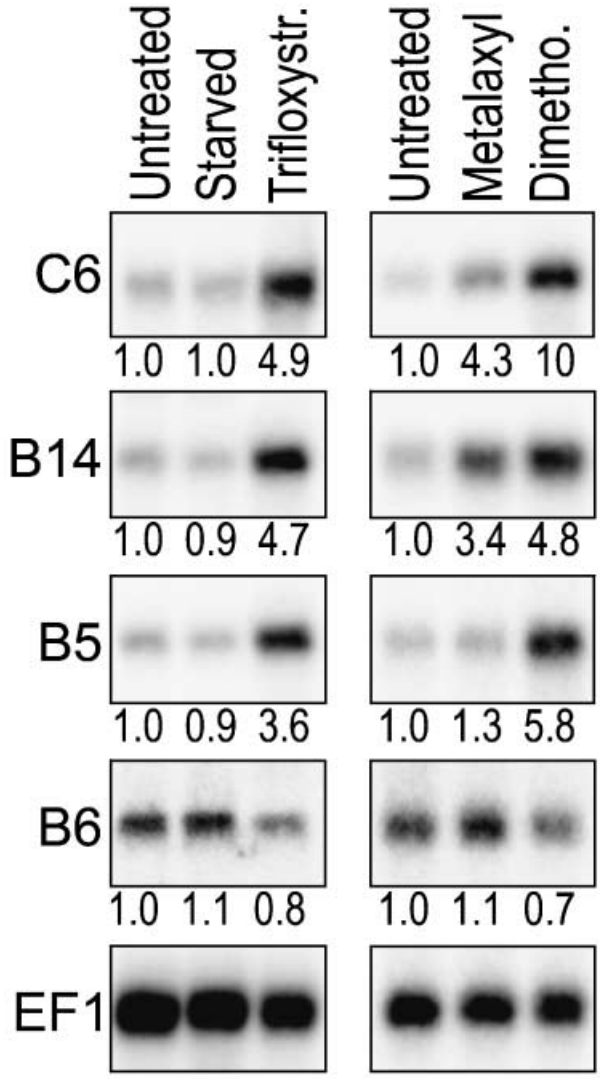


**Fig. 7** RNA blot analysis of representative ABC transporters. RNA was prepared from hyphae of strain H1 in rye broth treated for 2 h with trifloxystrobin, metalaxyl or dimethomorph; untreated rye broth; or starved hyphae grown in a carbon-limiting defined media. After electrophoresis, these were hybridized with probes for (top to bottom) *PiABCC6*, *PiABCB14*, *PiABCB5*, *PiABCB6* or elongation factor-1α (EF-1). Numbers below panels show intensities relative to untreated controls.

Nevertheless, this study failed to demonstrate that *P. infestans* strains with higher intrinsic levels of insensitivity to metalaxyl and trifloxystrobin transcribe any ABC transporter gene at increased levels compared with more-sensitive siblings. Some explanations for this include the possibility that not all ABC transporters of *P. infestans* were in the sequence database and therefore not tested, that analysing four offspring (H1, H2, L1, L2) was insufficient to detect correlations between fungicide insensitivity and mRNA levels, or that differences exist at levels other than transcription. For example, variation in splice junctions and translational efficiency can regulate the level and activity of ABC transporters (Gerk and Vore, 2002; He *et al.*, 2004). Differences may also be manifested in the substrate specificity or reaction rate of the transporter due to changes in the coding region, as described in the parasite *Plasmodium*, cancer cells and yeast (Egner *et al.*, 2000; Honjo *et al.*, 2001; Mu *et al.*, 2003).

Small differences in the expression of multiple genes with overlapping substrates could also contribute to the variation in *P. infestans*. This would greatly increase the complexity of an expression-profiling approach for linking phenotype with specific genes, along the lines of associating expression level polymorphisms with quantitative trait loci (Doerge, 2002). Proteins other than ABC transporters may also be involved, acting alone or synergistically with an ABC protein. Candidates for such factors include the passive transporters of the major facilitator superfamily, and metabolism-based enzymes such as cytochrome P450 enzymes (Pao *et al.*, 1998; Scott, 1999).

Several of the *P. infestans* ABC transporters were induced by metalaxyl, trifloxystrobin or dimethomorph. This probably reflects a mechanism to defend against natural toxins, as successful pathogens must resist xenobiotics produced by both plants and microbial competitors (Schoonbeek *et al.*, 2002). The induction response is probably not specific to those three compounds, given that the ability of a chemical to induce an transporter is not restricted to its substrates (Lee *et al.*, 2005; Schoonbeek *et al.*, 2001; Urban *et al.*, 1999) and coordinate induction of transporters by toxicants is reported in agricultural, clinical and model fungi (Bauer *et al.*, 1999; De Backer *et al.*, 2001; Semighini *et al.*, 2002). In *S. cerevisiae*, single transcription factors induce ABC transporters from different structural groups, plus other defence proteins (Bauer *et al.*, 1999; DeRisi *et al.*, 2000). Interestingly, in *P. infestans* the five induced genes fell into discrete families (A, B, C, G), implying that their promoters have evolved independently of their coding sequences.

Other interesting aspects of the evolution of the ABC transporters of *P. infestans* were revealed by this study, such as the expansion of Family B, which is most commonly associated with multidrug resistance in other species. In addition, the phylogenetic analysis indicated that some groups had simple evolutionary histories, such as Family C, which contains only full $(TMD\text{-}NBF)_2$ transporters. Family A was slightly more complex, containing both $(TMD\text{-}NBF)_2$ and $(NBF\text{-}TMD)_2$ proteins, with the domain reversal apparently due to a recent rearrangement. The most complex history was observed for Family B, especially subfamily ABCB-III, which contains both full and half transporters that possibly evolved through both ancient duplications and recent deletions. The detection of 54 ABC transporters also continues a trend of an upward expansion in the size of the superfamily in eukaryotic plant pathogens. This compares (according to the latest annotations on their genome-project websites, and excluding non-transporter ABC proteins) to 35 and 60 in the phytopathogens

*M. grisea* and *Fusarium graminearum*, respectively. By comparison, *S. cerevisiae* contains 24 ABC transporters. However, our analysis of the *P. infestans* family may be considered incomplete because it was not based on a completed genome sequence, and no effort was made to characterize genes from *P. ramorum* or *P. sojae* that lack homologues in our *P. infestans* set. Further analysis of the structure and function of the family will await completed oomycete genomes and gene silencing studies.

## EXPERIMENTAL PROCEDURES

### Strains of *P. infestans*

Isolates 8811, 115.11, 93H3, 618, 1114, E13a, 1306, 510, Ca65 and 550 of *P. infestans* were described previously (Judelson and Roberts, 1999). Cultures were maintained at 18 °C on rye A agar and genetic crosses were made as described (Judelson *et al.*, 1995), including the use of RAPD markers to genotype offspring to ensure that they are sexual hybrids. Crosses were between 1114 and 510, 216 (an $F_1$ of 8811 and E13a) and Ca65, and 3029 (an $F_1$ of 115.11 and 510) and 550.

### Fungicide treatments

Sensitivities were determined by measuring the radial growth of strains on rye agar, or dry mass measurements of cultures in clarified rye broth. For the latter, hyphal mats initiated from a 2-mm inoculum disc were removed from cultures, placed on planchets, baked at 160 °C and weighed. Measurements were made in quadruplicate after 9 days. Toxicant concentrations were selected to allow about 40–50% growth of 'average' strains. These were 10 p.p.m. chlorothalonil [Bravo™, 40% a.i. (active ingredient)], 5 p.p.m. cymoxanil (Curzate™, 60% a.i.), 2.5 p.p.m. dimethomorph (90% a.i.), 30 p.p.m. trifloxystrobin (Flint™, 50% a.i.), 30 p.p.m. azoxystrobin (Quadris™, 23% a.i.), 10 p.p.m. hymexazol (99% a.i.) and 5 p.p.m. metalaxyl (Ridomil™, 99% a.i.). Cultures for RNA analysis were prepared by growing non-sporulating hyphal mats in clarified rye broth for about 4 days. Toxicants were added for varying times, 2 h in the case of the experiments detailed in Figs 6 and 7. For inducing starvation, hyphal mats were moved to carbon-deficient minimal media (Randall *et al.*, 2005).

### Bioinformatics

*P. infestans* unigenes (Randall *et al.*, 2005) were compared to the SwissProt database on a local BLAST server using BLASTX. Matches were mined for hits to ABC transporters and Pfam motif PF00005. BLASTN searches of the *P. ramorum* and *P. sojae* genome databases (assembly 1.0) were performed using on-line tools at the Joint Genome Institute of the US Department of Energy (Walnut Creek, CA); when multiple hits were obtained, the strongest was chosen as the homologue of the *P. infestans* sequence. Gene models with multiple or unusually long introns (> 150 nt), or which were flanked by sequences matching the NBF or TMD domains of typical transporters, were manually examined for alternate splice or transcription start sites that would result in a more appropriate protein.

### RNA and DNA hybridization

Nucleic acids were extracted from *P. infestans*, electrophoresed, blotted and hybridized as described (Judelson and Roberts, 2002). Expression profiling arrays were analysed as described (Kim and Judelson, 2003). Briefly, clones to be spotted either were cDNAs in pSPORT1 (Invitrogen, Carlsbad, CA) or genomic DNA cloned into pGEMT-Easy (Promega, Madison, WI). These were amplified by PCR using SP6 and T7 primers, purified, adjusted to 100 ng/μL and spotted on nylon membranes using a 96-pin tool (V & P Scientific, San Diego, CA). Also spotted were an *actA* cDNA plus controls for non-specific hybridization. Membranes were hybridized with $^{32}$P-labelled cDNA primed using oligo-dT and random hexamers. Later, to control for DNA spotting the filters were reprobed with $^{32}$P-labelled T7 primer. Hybridization signals were analysed by phosphorimager analysis using exposures in the linear range. After correction for background, data were normalized to actin (for cDNA arrays) or elongation factor-1α (for RNA blots).

## ACKNOWLEDGEMENTS

We thank A. Ah Fong for valuable assistance in bioinformatics analyses, P. Morris for helpful discussions, R. Narayan for providing comments on an earlier version of the manuscript, S. Roberts for technical support, the US Department of Agriculture for financial support, Syngenta Corp. for providing access to a *P. infestans* sequence database, and the US Department of Energy-Joint Genome Institute for sharing *P. ramorum* and *P. sojae* sequence data.

## REFERENCES

**Andrade, A.C., Del Sorbo, G., Van Nistelrooy, J.G.M. and De Waard, M.A.** (2000) The ABC transporter AtrB from *Aspergillus nidulans* mediates resistance to all major classes of fungicides and some natural toxic compounds. *Microbiology*, **146**, 1987–1997.

**Anjard, C. and Loomis, W.F.** (2002) Evolutionary analyses of ABC transporters of *Dictyostelium discoideum*. *Eukaryot. Cell*, **1**, 643–652.

**Bartlett, D.W., Clough, J.M., Godwin, J.R., Hall, A.A., Hamer, M. and Parr-Dobrzanski, B.** (2002) The strobilurin fungicides. *Pest Manag. Sci.* **58**, 649–662.

**Bauer, B.E., Wolfger, H. and Kuchler, K.** (1999) Inventory and function of yeast ABC proteins: about sex, stress, pleiotropic drug and heavy metal resistance. *Biochim. Biophys. Acta*, **1461**, 217–236.

**Ben-Yaacov, R., Knoller, S., Caldwell, G.A., Becker, J.M. and Koltin, Y.** (1994) *Candida albicans* gene encoding resistance to benomyl and methotrexate is a multidrug resistance gene. *Antimicrob. Agents Chemother.* **38**, 648–652.

**Borst, P., Evers, R., Kool, M. and Wijnholds, J.** (1999) The multidrug resistance protein family. *Biochim. Biophys. Acta*, **1461**, 347–357.

**Davidse, L.C., Gerritsma, O.C.M., Ideler, J., Pie, K. and Velthuis, G.C.M.** (1988) Antifungal modes of action of metalaxyl, cyprofuram, benalaxyl and oxadixyl in phenylamide-sensitive and phenylamide-resistant strains of *Phytophthora megasperma* f. sp. *medicaginis* and *Phytophthora infestans*. *Crop Protect.* **7**, 347–355.

**Davies, T.G.E. and Coleman, J.O.D.** (2000) The *Arabidopsis thaliana* ATP-binding cassette proteins: an emerging superfamily. *Plant Cell Environ.* **23**, 431–443.

**Davies, J. and Wright, G.D.** (1997) Bacterial resistance to aminoglycoside antibiotics. *Trends Microbiol.* **5**, 234–240.

**De Backer, M.D., Ilyina, T., Ma, X.-J., Vandoninck, S., Luyten, W.H.M.L. and Bossche, H.V.** (2001) Genomic profiling of the response of *Candida albicans* to itraconazole treatment using a DNA microarray. *Antimicrob. Agents Chemother.* **45**, 1660–1670.

**Dean, M., Rzhetsky, A. and Allikmets, R.** (2001) The human ATP-binding cassette (ABC) transporter superfamily. *Genome Res.* **11**, 1156–1166.

**DeRisi, J., van den Hazel, B., Marc, P., Balzi, E., Brown, P., Jacq, C. and Goffeau, A.** (2000) Genome microarray analysis of transcriptional activation in multidrug resistance yeast mutants. *FEBS Lett.* **470**, 156–160.

**Di Pietro, A., Conseil, G., Perez-Victoria, J.M., Dayan, G., Baubichon-Cortay, H., Trompier, D., Steinfels, E., Jault, J.M., de Wet, H., Maitrejean, M, *et al.*** (2002) Modulation by flavonoids of cell multidrug resistance mediated by P-glycoprotein and related ABC transporters. *Cell. Molec. Life Sci.* **59**, 307–322.

**Diriwachter, G., Sozzi, D., Ney, C. and Staub, T.** (1987) Cross-resistance in *Phytophthora infestans* and *Plasmopara viticola* against different phenylamides and unrelated fungicides. *Crop Protect.* **6**, 250–255.

**Doerge, R.W.** (2002) Mapping and analysis of quantitative trait loci in experimental populations. *Nat. Rev. Genet.* **3**, 43–52.

**Egner, R., Bauer, B.E. and Kuchler, K.** (2000) The transmembrane domain 10 of the yeast Pdr5p ABC antifungal efflux pump determines both substrate specificity and inhibitor susceptibility. *Mol. Microbiol.* **35**, 1255–1263.

**Fabritius, A.-L., Shattock, R.C. and Judelson, H.S.** (1997) Genetic analysis of metalaxyl insensitivity loci in *Phytophthora infestans* using linked DNA markers. *Phytopathology*, **87**, 1034–1040.

**Footitt, S., Slocombe, S.P., Larner, V., Kurup, S., Wu, Y., Larson, T., Graham, I., Baker, A. and Holdsworth, M.** (2002) Control of germination and lipid mobilization by COMATOSE, the Arabidopsis homologue of human ALDP. *EMBO J.* **21**, 2912–2922.

**Franke, M.D., Brenneman, T.B., Stevenson, K.L. and Padgett, G.B.** (1998) Sensitivity of isolates of *Sclerotium rolfsii* from peanut in Georgia to selected fungicides. *Plant Dis.* **82**, 578–583.

**Fry, W.E. and Goodwin, S.B.** (1997) Re-emergence of potato and tomato late blight in the United States. *Plant Dis.* **81**, 1349–1357.

**Gaudet, R. and Wiley, D.C.** (2001) Structure of the ABC ATPase domain of human TAP1, the transporter associated with antigen processing. *EMBO J.* **20**, 4964–4972.

**Gerk, P.M. and Vore, M.** (2002) Regulation of expression of the multidrug resistance-associated protein 2 (MRP2) and its role in drug disposition. *J. Pharmacol. Exper. Therap.* **302**, 407–415.

**Gisi, U., Hermann, D., Ohl, L. and Steden, C.** (1997) Sensitivity profiles of *Mycosphaerella graminicola* and *Phytophthora infestans* populations to different classes of fungicides. *Pest. Sci.* **51**, 290–298.

**Hayashi, K., Schoonbeek, H.-J., Sugiura, H. and De Waard, M.A.** (2001) Multidrug resistance in *Botrytis cinerea* associated with decreased accumulation of the azole fungicide oxpoconazole and increased transcription of the ABC transporter gene BcatrD. *Pestic. Biochem. Physiol.* **70**, 168–179.

**He, X., Ee, P.L., Coon, J.S. and Beck, W.T.** (2004) Alternative splicing of the multidrug resistance protein 1/ATP binding cassette transporter subfamily gene in ovarian cancer creates functional splice variants and is associated with increased expression of the splicing factors PTB and SRp20. *Clin. Cancer Res.* **10**, 4652–4660.

**Honjo, Y., Hrycyna, C.A., Yan, Q.-W., Medina-Perez, W.Y., Robey, R.W., van de Laar, A., Litman, T., Dean, M. and Bates, S.E.** (2001) Acquired mutations in the MXR/BCRP/ABCP gene alter substrate specificity in MXR/BCRP/ABCP-overexpressing cells. *Cancer Res.* **61**, 6635–6639.

**Ito, Y., Muraguchi, H., Seshime, Y., Oita, S. and Yanagi, S.O.** (2004) Flutolanil and carboxin resistance in *Coprinus cinereus* conferred by a mutation in the cytochrome b560 subunit of succinate dehydrogenase complex (Complex II). *Mol. Genet. Genom.* **272**, 328–335.

**Judelson, H.S. and Roberts, S.** (1999) Multiple loci determining insensitivity to phenylamide fungicides in *Phytophthora infestans*. *Phytopathology*, **89**, 754–760.

**Judelson, H.S. and Roberts, S.** (2002) Novel protein kinase induced during sporangial cleavage in the oomycete *Phytophthora infestans*. *Eukaryot. Cell*, **1**, 687–695.

**Judelson, H.S., Spielman, L.J. and Shattock, R.C.** (1995) Genetic mapping and non-Mendelian segregation of mating type loci in the oomycete, *Phytophthora infestans*. *Genetics*, **141**, 503–512.

**Kim, K.S. and Judelson, H.S.** (2003) Sporangia-specific gene expression in the oomyceteous phytopathogen *Phytophthora infestans*. *Eukaryot. Cell*, **2**, 1376–1385.

**Laing, N.M., Belinsky, M.G., Kruh, G.D., Bell, D.W., Boyd, J.T., Barone, L., Testa, J.R. and Tew, K.D.** (1998) Amplification of the ATP-binding cassette 2 transporter gene is functionally linked with enhanced efflux of estramustine in ovarian carcinoma cells. *Cancer Res.* **58**, 1332–1337.

**Lee, T.Y., Mizubuti, E. and Fry, W.E.** (1999) Genetics of metalaxyl resistance in *Phytophthora infestans*. *Fungal Genet. Biol.* **26**, 118–130.

**Lee, Y.-J., Yamamoto, K., Hammamoto, H., Nakaune, R. and Hibi, T.** (2005) A novel ABC transporter gene ABC2 involved in multidrug susceptibility but not pathogenicity in rice blast fungus, *Magnaporthe grisea*. *Pestic. Biochem. Physiol.* **81**, 13–23.

**Leroux, P., Fritz, R., Debieu, D., Albertini, C., Lanen, C., Bach, J., Gredt, M. and Chapeland, F.** (2002) Mechanisms of resistance to fungicides in field strains of *Botrytis cinerea*. *Pest Manag. Sci.* **58**, 876–888.

**Lyons, C.N. and White, T.C.** (2000) Transcriptional analyses of antifungal drug resistance in *Candida albicans*. *Antimicrob. Agents Chemother.* **44**, 2296–2303.

**McGrath, M.** (2001) Fungicide resistance in cucurbit powdery mildew: experiences and challenges. *Plant Dis.* **85**, 236–245.

**Mu, J., Ferdig, M.T., Feng, X., Joy, D.A., Duan, J., Furuya, T., Subramanian, G., Aravind, L., Cooper, R.A., Wootton, J.C., Xiong, M. and Su, X.Z.** (2003) Multiple transporters associated with malaria parasite responses to chloroquine and quinine. *Molec. Microbiol.* **49**, 977–989.

**Nakaune, R., Adachi, K., Nawata, O., Tomiyama, M., Akutsu, K. and Hibi, T.** (1998) A novel ATP-binding cassette transporter involved in multidrug resistance in the phytopathogenic fungus *Penicillium digitatum*. *Appl. Environ. Microbiol.* **64**, 3983–3988.

**Ochiai, N., Fujimura, M., Motoyama, T., Ichiishi, A., Usami, R., Horikoshi, K. and Yamaguchi, I.** (2001) Characterization of mutations in the two-component histidine kinase gene that confer fludioxonil resistance and osmotic sensitivity in the os-1 mutants of *Neurospora crassa*. *Pest Manag. Sci*. **57**, 437–442.

**Olaya, G. and Koller, W.** (1999) Diversity of kresoxim-methyl sensitivities in baseline populations of *Venturia inaequalis*. *Pesticide Sci*. **55**, 1083–1088.

**Orbach, M.J., Porro, E.B. and Yanofsky, C.** (1986) Cloning and characterization of the gene for b-tubulin from a benomyl-resistant mutant of *Neurospora crassa*. *Molec. Cell. Biol*. **6**, 2452–2461.

**Orelle, C., Dalmas, O., Gros, P., Di Pietro, A. and Jault, J.M.** (2003) The conserved glutamate residue adjacent to the Walker-B motif is the catalytic base for ATP hydrolysis in the ATP-binding cassette transporter BmrA. *J. Biol. Chem*. **278**, 47002–47008.

**Pao, S.S., Paulsen, I.T. and Saier, M.H.** (1998) Major facilitator superfamily. *Microbiol. Molec. Biol. Rev*. **62**, 1–34.

**Randall, T.A., Dwyer, R.A., Huitema, E., Beyer, K., Cvitanich, C., Kelkar, H., Ah Fong, A.M.V., Gates, K., Roberts, S., Yatzkan, E., Gaffney, T., Law, M., Testa, A., Torto, T., Zhang, M., Zheng, L., Mueller, E., Windass, J., Binder, A., Birch, P.R.J., Gisi, U., Govers, F., Gow, N., Mauch, F., van West, P., Waugh, M., Yu, J., Boller, T., Kamoun, S., Lam, S.T. and Judelson, H.S.** (2005) Large-scale gene discovery in the oomycete *Phytophthora infestans* reveals likely components of phytopathogenicity shared with true fungi. *Mol. Plant–Microbe Interact*. **18**, 229–243.

**Samoucha, Y. and Cohen, Y.** (1984) Differential sensitivity to mancozeb of metalaxyl-sensitive and metalaxyl-resistant isolates of *Pseudoperonospora cubensis*. *Phytopathology*, **74**, 1437–1439.

**Schoonbeek, H., Del Sorbo, G. and De Waard, M.A.** (2001) The ABC transporter BcatrB affects the sensitivity of *Botrytis cinerea* to the phytoalexin resveratrol and the fungicide fenpiclonil. *Mol. Plant–Microbe Interact*. **14**, 562–571.

**Schoonbeek, H.-J., Raaijmakers, J.M. and De Waard, M.A.** (2002) Fungal ABC transporters and microbial interactions in natural environments. *Mol. Plant–Microbe Inter*. **15**, 1165–1172.

**Scott, J.G.** (1999) Cytochromes P450 and insecticide resistance. *Insect Biochem*. **29**, 757–777.

**Semighini, C.P., Marins, M., Goldman, M.H.S. and Goldman, G.H.** (2002) Quantitative analysis of the relative transcript levels of ABC transporter Atr genes in *Aspergillus nidulans* by real-time reverse transcription-PCR assay. *Appl. Environ. Microbiol*. **68**, 1351–1357.

**Shattock, R.C.** (1988) Studies on the inheritance of resistance to metalaxyl in *Phytophthora infestans*. *Plant Pathol*. **37**, 4–11.

**Slaven, J.W., Anderson, M.J., Sanglard, D., Dixon, G.K., Bille, J., Roberts, I.S. and Denning, D.W.** (2002) 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**, 199–206.

**Sujkowski, L.S., Fry, B.A., Power, R.J., Goodwin, S.B., Peever, T.L., Hamlen, R.A. and Fry, W.E.** (1995) Sensitivities of Mexican isolates of *Phytophthora infestans* to chlorothalonil, cymoxanil, and metalaxyl. *Plant Dis*. **79**, 1117–1120.

**Urban, M., Bhargava, T. and Hamer, J.E.** (1999) An ATP-driven efflux pump is a novel pathogenicity factor in rice blast disease. *EMBO J*. **18**, 512–521.

**Voglmayr, H. and Greilhuber, J.** (1998) Genome size determination in peronosporales (Oomycota) by Feulgen image analysis. *Fungal Genet. Biol*. **25**, 181–195.

**Whittaker, S.L., Shattock, R.C. and Shaw, D.S.** (1991) Inheritance of DNA contents in sexual progenies of *Phytophthora infestans*. *Mycol. Res*. **95**, 1094–1100.

**Yamamoto, S., Hiraga, K., Abiko, A., Hamanaka, N. and Oda, K.** (2005) A new function of isonitrile as an inhibitor of the Pdr5p multidrug ABC transporter in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun*. **330**, 662–668.

## NOTE ADDED IN PROOF

Some information in Table 2 may have inadvertently propagated errors in the annotations of other transporters in a previous publication and in GenBank. Consequently, *P. infestans* genes *PiABCB1* to *PiABCB12* may be classified more appropriately in Family G.