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# Transcriptional responses to exposure to the brassicaceous defence metabolites camalexin and allyl-isothiocyanate in the necrotrophic fungus *Alternaria brassicicola*

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## SUMMARY

*Alternaria brassicicola* is the causative agent of black spot disease of Brassicaceae belonging to the genera *Brassica* and *Raphanus*. During host infection, *A. brassicicola* is exposed to high levels of antimicrobial defence compounds such as indolic phytoalexins and glucosinolate breakdown products. To investigate the transcriptomic response of *A. brassicicola* when challenged with brassicaceous defence metabolites, suppression subtractive hybridization (SSH) was performed to generate two cDNA libraries from germinated conidia treated either with allyl isothiocyanate (Al-ITC) or with camalexin. Following exposure to Al-ITC, *A. brassicicola* displayed a response similar to that experienced during oxidative stress. Indeed, a substantial subset of differentially expressed genes was related to cell protection against oxidative damage. Treatment of *A. brassicicola* conidia with the phytoalexin camalexin appeared to activate a compensatory mechanism to preserve cell membrane integrity and, among the camalexin-elicited genes, several were involved in sterol and sphingolipid biosynthesis. The transcriptomic analysis suggested that protection against the two tested compounds also involved mechanisms aimed at limiting their intracellular accumulation, such as melanin biosynthesis (in the case of camalexin exposure only) and drug efflux. From the Al-ITC and the camalexin differentially expressed genes identified here, 25 were selected to perform time-course studies during interactions with brassicaceous hosts. *In planta*, up-regulation of all the selected genes was observed during infection of *Raphanus sativus* whereas only a subset were over-expressed during the incompatible interaction with *Arabidopsis thaliana* ecotype Columbia.

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## INTRODUCTION

Plants activate a variety of defensive mechanisms in response to challenges by fungal pathogens. Chemical defence mediated by host antimicrobial secondary metabolites has been demonstrated to be an efficient response to kill or inhibit the growth of invading fungi (Morrissey and Osbourn, 1999). These antimicrobial metabolites may be either constitutive compounds, also called phytoanticipins (Osbourn, 1996; Van Etten *et al.*, 1994), or biosynthesized *de novo* such as phytoalexins (Brooks and Watson, 1985). Both constitutive and induced chemical defences have been well documented in the plant model *Arabidopsis thaliana*. The indolic secondary metabolite camalexin is the major phytoalexin of this plant species. Mutation in the gene *PAD3*, encoding a cytochrome P450, abolishes the biosynthesis of camalexin (Glazebrook and Ausubel, 1994), and results in enhanced susceptibility to necrotrophic fungi (Thomma *et al.*, 1999). The phytoanticipins glucosinolates (GS) are sulphonated thioglycosides comprising a common glycone moiety with a variable aglycone side chain and are considered as the major secondary metabolites of Brassicaceae (Fahey *et al.*, 2001). Upon tissue damage, GS come into contact with myrosinases, a specific class of β-thioglucosidases, which are stored separately in the cell. Hydrolysis of GS by myrosinases yields isothiocyanates (ITC), nitriles and epithionitriles. The most common breakdown products, ITC, exhibit toxicity towards several plant pathogens including bacteria, fungi, insects and nematodes (Fahey *et al.*, 2001). It has been demonstrated that ITC produced by *Arabidopsis thaliana* significantly inhibit *in planta* growth of some fungal pathogens (Tierens *et al.*, 2001) although recent results suggest that apart from their direct toxic effects, GS breakdown products may also act by modulation of plant defence signalling (Brader *et al.*, 2006).

*Alternaria brassicicola*, a necrotrophic ascomycete, causes black spot disease on a wide range of Brassicaceae including weeds and crops. The cultivated plants *Brassica* and *Raphanus* spp. are susceptible to this disease, which is therefore economically important. During host infection, *A. brassicicola* is exposed

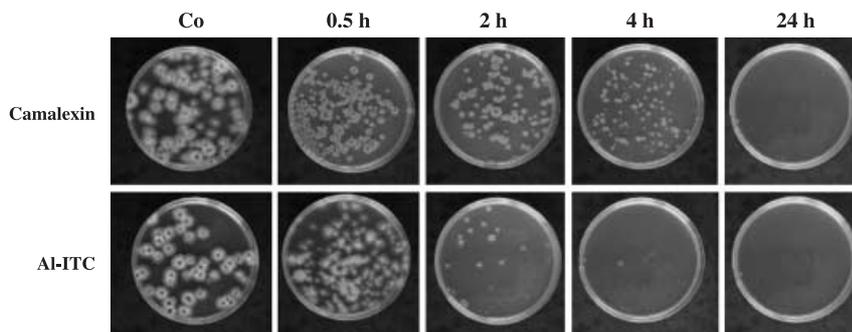|  | Co | 0.5 h | 2 h | 4 h | 24 h |
|---|---|---|---|---|---|

**Camalexin**

**Al-ITC**



**Fig. 1** Lethal effect of camalexin (125 μM) and Al-ITC (2.5 mM) on germinated *Alternaria brassicicola* conidia. The conidia ($2 \times 10^4$ mL$^{-1}$) were germinated for 24 h in PDB, incubated for various times in the presence of the tested compound at the desired concentration and then applied on the Petri dishes containing PDA media. After 48 h of incubation colonies were visualized. Control plates (Co) were prepared with conidia incubated for up to 24 h with methanol or DMSO (1% v/v final concentration).

to high levels of phytoalexins and GS-breakdown products. Their ability to detoxify such chemical defence compounds may therefore constitute a key determinant of their virulence, as has been shown for several other fungi (Bowyer *et al.*, 1995; Quidde *et al.*, 1998). Cramer and Lawrence (2004) have shown that *CyhAB*, a cyanide hydratase-encoding gene, was up-regulated during the interaction of *A. brassicicola* with *Arabidopsis thaliana*, and suggested that this enzyme may be a candidate for the detoxification of GS-breakdown products. Recently, we have demonstrated that the ITC differentially expressed gene *AbGst1*, encoding a glutathione transferase, exhibiting high transferase activity with ITC as substrates, is also a candidate for ITC detoxification during host infection (Sellam *et al.*, 2006b).

To explore further *A. brassicicola* detoxification mechanisms against the major GS breakdown products ITC and brassicaceous phytoalexins, we have focused on the analysis of the transcriptional response in germinating conidia exposed either to the indolic phytoalexin camalexin produced by *Arabidopsis thaliana* and some brassicaceous weeds or to allyl-ITC (Al-ITC), a breakdown product of the aliphatic GS sinigrin produced by a variety of cruciferous vegetables. The identification of differentially expressed genes was performed with the suppression subtractive hybridization (SSH) technique and *in vitro* or *in planta* increased expression in response to plant compounds of several selected genes was confirmed by real-time quantitative PCR. The generated data provide insights on the cellular mechanisms by which the two studied compounds exert their toxicity and on the strategies used by the fungus to protect itself against these defence metabolites.

## RESULTS AND DISCUSSION

### Construction of the subtracted cDNA libraries

SSH was used to generate populations of cDNA corresponding to mRNA transcripts whose levels were increased after the exposure of *A. brassicicola* germinated conidia to sublethal concentrations of either the phytoalexin camalexin or the sinigrin breakdown product Al-ITC. To ensure that the observed changes in gene expression not only reflected a non-specific response of the fungus to severe stress conditions, these treatments were performed using short periods of exposure (0.5 h) to concentrations of camalexin and Al-ITC that do not exceed the IC$_{50}$ previously determined for this isolate by measuring their inhibitory effects on mycelial growth (Sellam *et al.*, 2006a). Under these conditions, cell viability did not differ from untreated controls, as revealed by plating assays (Fig. 1).

Subtraction efficiencies of the two cDNA libraries were very similar, as estimated using real-time PCR quantification of the housekeeping gene β-tubulin on the subtracted cDNA relative to the unsubtracted one. The cycle threshold (Ct) in the unsubtracted samples was obtained at ~15 cycles as opposed to ~27 cycles in the subtracted samples, thereby providing evidence for the efficiency and reproducibility of the SSH procedure. PCR analysis of 333 and 379 recombinant clones from the Al-ITC and camalexin libraries, respectively, revealed insert sizes ranging from ~200 to ~1500 bp (data not shown). Differential screening of the two libraries was performed using probes generated from the forward- and reverse-subtracted cDNA pools to select for positive clones, i.e. those exhibiting signal intensities more than five-fold higher with the forward-subtracted probes compared with the reverse-subtracted one. A total of 234 clones from the Al-ITC cDNA library and 280 clones from the camalexin cDNA library were thus selected for sequencing. After expressed sequence tag (EST) cluster analysis to remove sequence redundancies, a total of 76 and 112 unique ESTs were finally obtained for the Al-ITC and camalexin cDNA libraries, respectively.

Real-time quantitative PCR was performed to confirm further the differential expression of the identified sequences. From the Al-ITC cDNA library, a subset of 18 sequences was selected and their expressions were evaluated after exposure of conidia to Al-ITC and also to the aromatic ITC benzyl-ITC (Bz-ITC), a breakdown product of the GS glucotropaeolin (Table S1). As shown in Fig. 2, these experiments confirmed that the expression of all of the selected sequences was induced by Al-ITC following 0.5 h of exposure. After 2 h the expression of almost all the selected genes decreased back to the basal control levels, probably reflecting the strong inhibitory effect recorded for longer exposures to Al-ITC
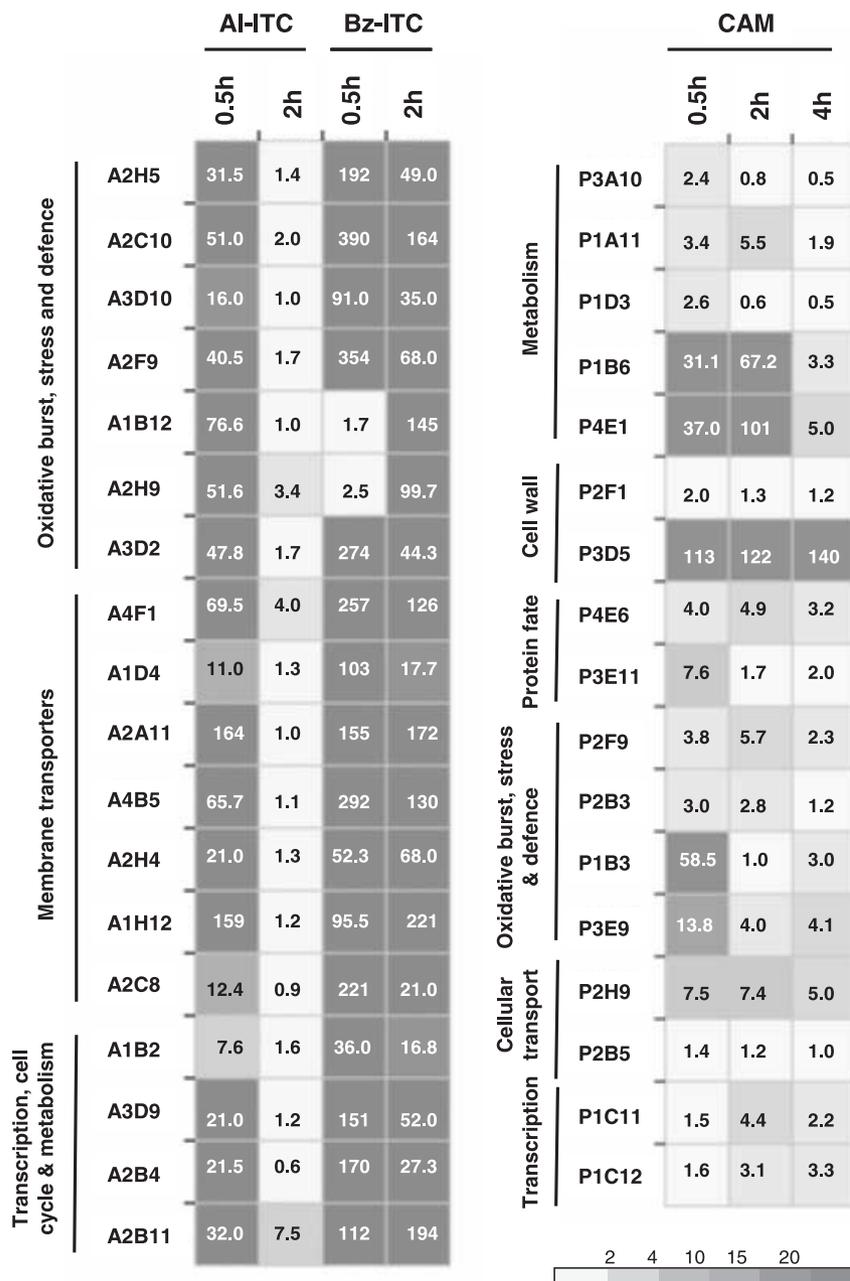
| | AI-ITC 0.5h | AI-ITC 2h | Bz-ITC 0.5h | Bz-ITC 2h |
|---|---|---|---|---|
| **Oxidative burst, stress and defence** | | | | |
| A2H5 | 31.5 | 1.4 | 192 | 49.0 |
| A2C10 | 51.0 | 2.0 | 390 | 164 |
| A3D10 | 16.0 | 1.0 | 91.0 | 35.0 |
| A2F9 | 40.5 | 1.7 | 354 | 68.0 |
| A1B12 | 76.6 | 1.0 | 1.7 | 145 |
| A2H9 | 51.6 | 3.4 | 2.5 | 99.7 |
| A3D2 | 47.8 | 1.7 | 274 | 44.3 |
| **Membrane transporters** | | | | |
| A4F1 | 69.5 | 4.0 | 257 | 126 |
| A1D4 | 11.0 | 1.3 | 103 | 17.7 |
| A2A11 | 164 | 1.0 | 155 | 172 |
| A4B5 | 65.7 | 1.1 | 292 | 130 |
| A2H4 | 21.0 | 1.3 | 52.3 | 68.0 |
| A1H12 | 159 | 1.2 | 95.5 | 221 |
| A2C8 | 12.4 | 0.9 | 221 | 21.0 |
| **Transcription, cell cycle & metabolism** | | | | |
| A1B2 | 7.6 | 1.6 | 36.0 | 16.8 |
| A3D9 | 21.0 | 1.2 | 151 | 52.0 |
| A2B4 | 21.5 | 0.6 | 170 | 27.3 |
| A2B11 | 32.0 | 7.5 | 112 | 194 |

| | CAM 0.5h | CAM 2h | CAM 4h |
|---|---|---|---|
| **Metabolism** | | | |
| P3A10 | 2.4 | 0.8 | 0.5 |
| P1A11 | 3.4 | 5.5 | 1.9 |
| P1D3 | 2.6 | 0.6 | 0.5 |
| P1B6 | 31.1 | 67.2 | 3.3 |
| P4E1 | 37.0 | 101 | 5.0 |
| **Cell wall** | | | |
| P2F1 | 2.0 | 1.3 | 1.2 |
| P3D5 | 113 | 122 | 140 |
| **Protein fate** | | | |
| P4E6 | 4.0 | 4.9 | 3.2 |
| P3E11 | 7.6 | 1.7 | 2.0 |
| **Oxidative burst, stress & defence** | | | |
| P2F9 | 3.8 | 5.7 | 2.3 |
| P2B3 | 3.0 | 2.8 | 1.2 |
| P1B3 | 58.5 | 1.0 | 3.0 |
| P3E9 | 13.8 | 4.0 | 4.1 |
| **Cellular transport** | | | |
| P2H9 | 7.5 | 7.4 | 5.0 |
| P2B5 | 1.4 | 1.2 | 1.0 |
| **Transcription** | | | |
| P1C11 | 1.5 | 4.4 | 2.2 |
| P1C12 | 1.6 | 3.1 | 3.3 |

Scale: 2  4  10  15  20

**Fig. 2** Expression levels of sequences randomly selected from the AI-ITC (A) and camalexin (B) cDNA libraries in *Alternaria brassicicola* exposed to AI-ITC, Bz-ITC or camalexin. First-strand cDNAs were prepared from RNA samples extracted from germinated conidia exposed to AI-ITC (2.5 mM), Bz-ITC (0.3 mM) or camalexin (125 μM) for the indicated times and used as template for real-time PCR. For each gene, induction of expression is represented as a ratio (fold induction) of its relative expression (studied gene transcript abundance/β-tubulin transcript abundance) in each inductive condition to its relative expression in the corresponding control (spores germinated in medium amended with the solvent only). Each value is the mean of two independent experiments each with three replicates. For easier visualization of the results, numerical data were transformed into colour-grid representations using the JColorGrid software (Joachimiak *et al.*, 2006) in which fold induction of gene expression is represented by a grey scale.

(see Fig. 1). Irrespective of the length of the treatment with Bz-ITC, all the selected genes were induced and their relative induction levels by this compound were higher compared with AI-ITC. It should be noted that, at the concentration used in this study (0.3 mM), no apparent lost of viability was observed after up to 2 h of exposure (data not shown). A total of 17 sequences were selected from the camalexin cDNA library and their differential expression levels were tested following exposure of conidia to camalexin for 0.5, 2 and 4 h (Table S2). The results of this assay revealed that transcript levels of most of the selected genes increased following exposure to camalexin (Fig. 2).

## Functional classification of *A. brassicicola* genes expressed in response to ITC

To assign potential functions to the putative proteins encoded by SSH-generated cDNA fragments, the 76 AI-ITC unique EST sequences were analysed by comparing them to the GenBank non-redundant database sequences and the current complete genomes in the Fungal Genome Initiative project database using the BLASTX algorithm. Fifty-three sequences (71%) isolated from the AI-ITC cDNA library exhibited significant protein similarity to previously identified or putative proteins, and nine clones (11%)

**Table 1** Summary of *Alternaria brassicicola* clones from the AI-ITC cDNA library. The presented ESTs correspond to those with significant matches (*e*-value < 1e-05) to proteins of known or predicted function.

| Clone | GenBank accession no.* | Size† | Best BLAST match Accession no. | Organism | Annotation | *e*-value |
|---|---|---|---|---|---|---|
| **1. Oxidative burst, stress and defence** | | | | | | |
| A2H5 | DY542653 | 724 | CAC18210 | *Neurospora crassa* | Putative microsomal glutathione S-transferase | 6e-25 |
| A1F1 | DY542654 | 521 | EAL87849 | *Aspergillus fumigatus* | Putative glutathione S-transferase | 1e-15 |
| A2C1 | DY542655 | 742 | EAL87157 | *A. fumigatus* | Putative glutathione S-transferase | 4e-61 |
| A2C10 | DY542656 | 916 | AAX07321 | *A. fumigatus* | Similar to the glutathione S-transferase gstA | 4e-48 |
| A4D12 | DY542657 | 886 | BAB68404 | *Gibberella fujikuroi* | Putative glutathione S-transferase | 2e-39 |
| A3D10 | DY542658 | 808 | O59858 | *Schizosaccharomyces pombe* | Similar to the glutathione peroxidase gpx1 | 2e-49 |
| A2F9 | DY542659 | 713 | CAA59379 | *S. pombe* | Similar to the γ-glutamylcysteine synthetase Gcs1 | 1e-65 |
| A3B11 | DY542660 | 748 | EAL92160 | *A. fumigatus* | Putative γ-glutamylcysteine synthetase | 8e-71 |
| A3H11 | DY542661 | 344 | SNU08948.1 | *Stagonospora nodorum* | Putative thioredoxin | 4e-12 |
| A2G8 | DY542662 | 713 | EAL89878 | *A. fumigatus* | Putative thioredoxin | 3e-23 |
| A3G5 | DY542663 | 555 | EAL91252 | *A. fumigatus* | Putative thioredoxin | 1e-27 |
| A1B12 | DY542664 | 645 | EAL88433 | *A. fumigatus* | Putative DSBA-like thioredoxin | 5e-22 |
| A4D11 | DY542665 | 711 | P51978 | *N. crassa* | Similar to the thioredoxin reductase cys9 | 1e-29 |
| A3C8 | DY542666 | 465 | EAL87526 | *A. fumigatus* | Putative NADH-dependent flavin oxidoreductase | 2e-27 |
| A2H9 | DY542667 | 800 | AAD41159 | *S. pombe* | Similar to the sulphide-quinone oxidoreductase cad1 | 5e-46 |
| A4B11 | DY542668 | 948 | SNU05420.1 | *S. nodorum* | Putative Pyridine nucleotide-disulphide oxidoreductase | 5e-07 |
| A2B7 | DY542669 | 326 | SNU13823.1 | *S. nodorum* | Putative Pyridine nucleotide-disulphide oxidoreductase | 5e-08 |
| A4A12 | DY542670 | 735 | BAC20562 | *Penicillium citrinum* | Similar to the oxidoreductase mlcG | 6e-18 |
| A3A6 | DY542671 | 700 | CAF05989 | *N. crassa* | Putative Hsc70-interacting protein | 4e-16 |
| A3G8 | DY542672 | 600 | S50131 | *Aspergillus nidulans* | Similar to the heat-shock protein HSP30 | 6e-20 |
| A4F3 | DY542673 | 554 | P19882 | *Saccharomyces cerevisiae* | Similar to the heat-shock protein HSP60 | 4e-09 |
| A3D2 | DY542674 | 482 | AAN74815 | *G. moniliformis* | Similar to the Cytochrome P450 monooxygenase Fum12p | 2e-37 |
| **2. Membrane transporters** | | | | | | |
| A4F1 | DY542675 | 698 | XP_365840 | *M. grisea* | Putative ABC-type $Fe^{3+}$ transporter | 2e-62 |
| A1D4 | DY542676 | 684 | CAC41639 | *Botryotinia fuckeliana* | Similar to the PDR ABC transporter BcAtrD | 4e-29 |
| A2A11 | DY542677 | 486 | CAA93140 | *A. nidulans* | Similar to the PDR ABC transporter AtrA | 3e-15 |
| A4B5 | DY542678 | 539 | CAB46279 | *Mycosphaerella graminicola* | Similar to the PDR ABC transporter MgAtr1 | 5e-42 |
| A2H4 | DY542679 | 462 | Q06598 | *S. cerevisiae* | Similar to the Arsenite transporter ACR3 | 2e-35 |
| A1H12 | DY542680 | 423 | AAC44819 | *Methanothermobacter thermautotrophicus* | Similar to the Formate/nitrite transporter FdhC | 5e-20 |
| A2C8 | DY542681 | 710 | AAC64976 | *S. pombe* | Similar to the MFS hexose transporter ght6p | 3e-64 |
| A3H9 | DY542682 | 373 | XP_363585 | *M. grisea* | Putative MFS transporter | 1e-13 |
| A4B2 | DY542683 | 451 | EAL92997 | *A. fumigatus* | Putative MFS transporter | 5e-14 |
| A2A12 | DY542684 | 492 | EAL92132 | *A. fumigatus* | Putative MFS transporter | 4e-44 |
| **3. Transcription and cell cycle** | | | | | | |
| A4F6 | DY542685 | 726 | AAM08677 | *A. fumigatus* | Similar to the Cell division control protein Cdc48p | 2e-103 |
| A1B2 | DY542686 | 865 | T37963 | *S. pombe* | Similar to the caffeine-induced death protein Cid1 | 3e-14 |
| A3D9 | DY542687 | 614 | O94684 | *S. pombe* | Similar to the RNA polymerase II transcription factor pmh1 | 2e-35 |
| A2B4 | DY542688 | 1124 | CAB98237 | *N. crassa* | Similar to the positive-acting sulphur transcription factor CYS-3 | 1e-26 |
| **4. Metabolism** | | | | | | |
| A2B11 | DY542689 | 1083 | AAM88292 | *Cochliobolus heterostrophus* | Similar to the Oxidoreductase RED1 | 6e-24 |
| A4A8 | DY542690 | 763 | AAD09811 | *A. nidulans* | Similar to the pantothenate kinase PanK | 3e-57 |
| A2A7 | DY542691 | 423 | CAA81612 | *Geobacillus stearothermophilus* | Similar to the alcohol dehydrogenase ADH-HT | 9e-12 |

*Twenty-three additional sequences that match to unclassified or hypothetical proteins have been registered under GenBank accession nos. DY542692 to DY542713.
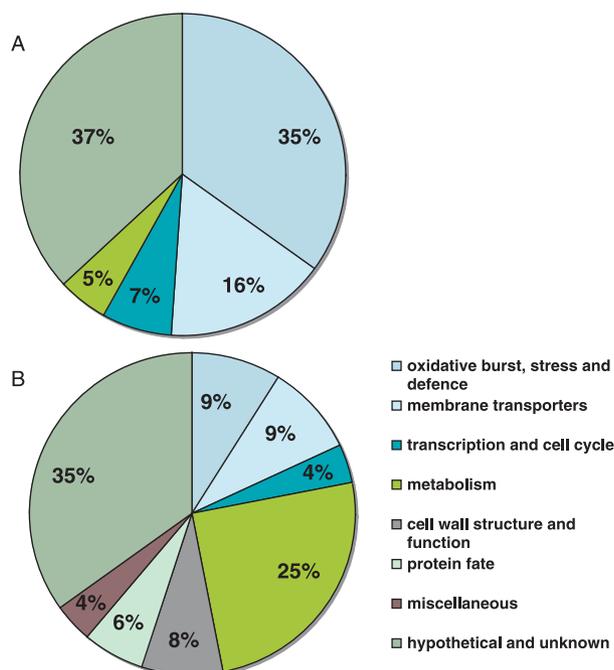†In base pairs.

**Fig. 3** Functional classification of up-regulated genes in *Alternaria brassicicola* Al-ITC- (A) and camalexin- (B) treated conidia according to their putative biological function.

contained inserts that showed similarity to conserved hypothetical proteins. No apparent similarity to known sequences was found for the inserts from the 14 remaining clones (18%). The set of sequences with significant matches to proteins of known or predicted function is listed in Table 1. These were assigned to four functional categories (Fig. 3A).

A significant portion of genes transcriptionally induced by Al-ITC such as glutathione transferases (GST), glutathione peroxidase, γ-glutamylcysteine synthetases, thioredoxins, thioredoxin reductase and oxidoreductases are involved in the oxidative stress response. The generation of reactive oxygen species (ROS) by ITC has already been reported in experimental animals (Nakamura *et al.*, 2000; Paolini *et al.*, 2004), resulting in the induction of the phase II enzymes NAD(P)H:Quinone oxidoreductase and GST activities and also the γ-glutamylcysteine synthetases catalysing the rate-limiting step in the formation of reduced gluthatione (GSH) (McWalter *et al.*, 2004). Redox imbalance is probably caused by the depletion of the antioxidant GSH that reacts with ITC, resulting therefore in alterations of the oxidants/antioxidants ratio. An *A. brassicicola* cytochrome P450-encoding gene was induced by ITC. Interestingly, an EST corresponding to the same gene has already been detected in an *A. brassicicola–Brassica oleracea* SSH library and was shown to be induced *in planta* at 2 days post-infection (Cramer *et al.*, 2006). Similar phase I enzyme was also reported to be induced in animals by some dietary carcinogens including ITC (Paolini *et al.*, 2004). It therefore

appears that a redox imbalance occurs following the exposure of fungal cells to ITC, resulting in the activation of the conserved ITC-detoxification mechanism mediated by phase I and II enzymes. These results suggest, for the first time in fungi, that ITC causes oxidative stress, which is likely to be the origin of their cytotoxicity. Alternatively, ITC may induce antioxidant genes in *A. brassicicola* by direct activation of upstream signalling pathways without creating cellular oxidative stress. However, the results presented below (Fig. 4) showing the detection of intracellular reactive oxygen intermediates (ROI) after exposure of germinated conidia to ITC strongly support the first hypothesis.

Ten membrane transporters were induced by Al-ITC including four PDR-type ABC transporters and four MFS transporters. In fungi, ABC and MFS transporters represent different multidrug efflux pumps associated with resistance to xenobiotics and antibiotics including plant defence metabolites (Schoonbeek *et al.*, 2001; Urban *et al.*, 1999). The induction of these potential 'non-degradative' detoxification systems suggests that ITC is either removed directly from fungal cells or after being conjugated to GSH. In the yeast *Saccharomyces cerevisiae*, two ABC transporters have been shown to be GS-X pumps that catalyse the transport of glutathione conjugates into the vacuole (Li *et al.*, 1996; Sharma *et al.*, 2002). However, no apparent homology was found between the four isolated ITC-induced ABC transporters and the yeast GX pumps.

Interestingly, a gene encoding a positive-acting sulphur regulatory protein was identified from the Al-ITC cDNA library. In fungi, this transcription factor is required to turn on the expression of the sulphur-related metabolic enzymes (Natorff *et al.*, 2003). The transcriptional activation of this gene suggests that ITC, which are sulphur-containing metabolites, could be metabolized by *A. brassicicola* and used as a source of sulphur. Indeed, a stimulation of radial growth of *A. brassicicola* on solid medium containing low concentrations of Al-ITC or Bz-ITC was recorded compared with ITC-free medium (data not shown). Similarly, Giamoustaris and Mithen (1997) demonstrated that the level of *Alternaria* spp. infection was positively correlated with the GS content of leaves and pods of oilseed rape.

## Functional classification of *A. brassicicola* genes expressed in response to camalexin

Details of the camalexin ESTs sequences that had significant matches to proteins of known or predicted function are given in Table 2. Among the 112 unique ESTs, 29 (25%) exhibited similarity to hypothetical proteins and 33 (30%) had no apparent similarity to known sequences. The remaining 50 ESTs (45%) that show homology to known or putative proteins were classified into six functional categories (Fig. 3B).

Genes responsive to camalexin included sphingolipid and ergosterol biosynthesis genes, which are essential compounds of
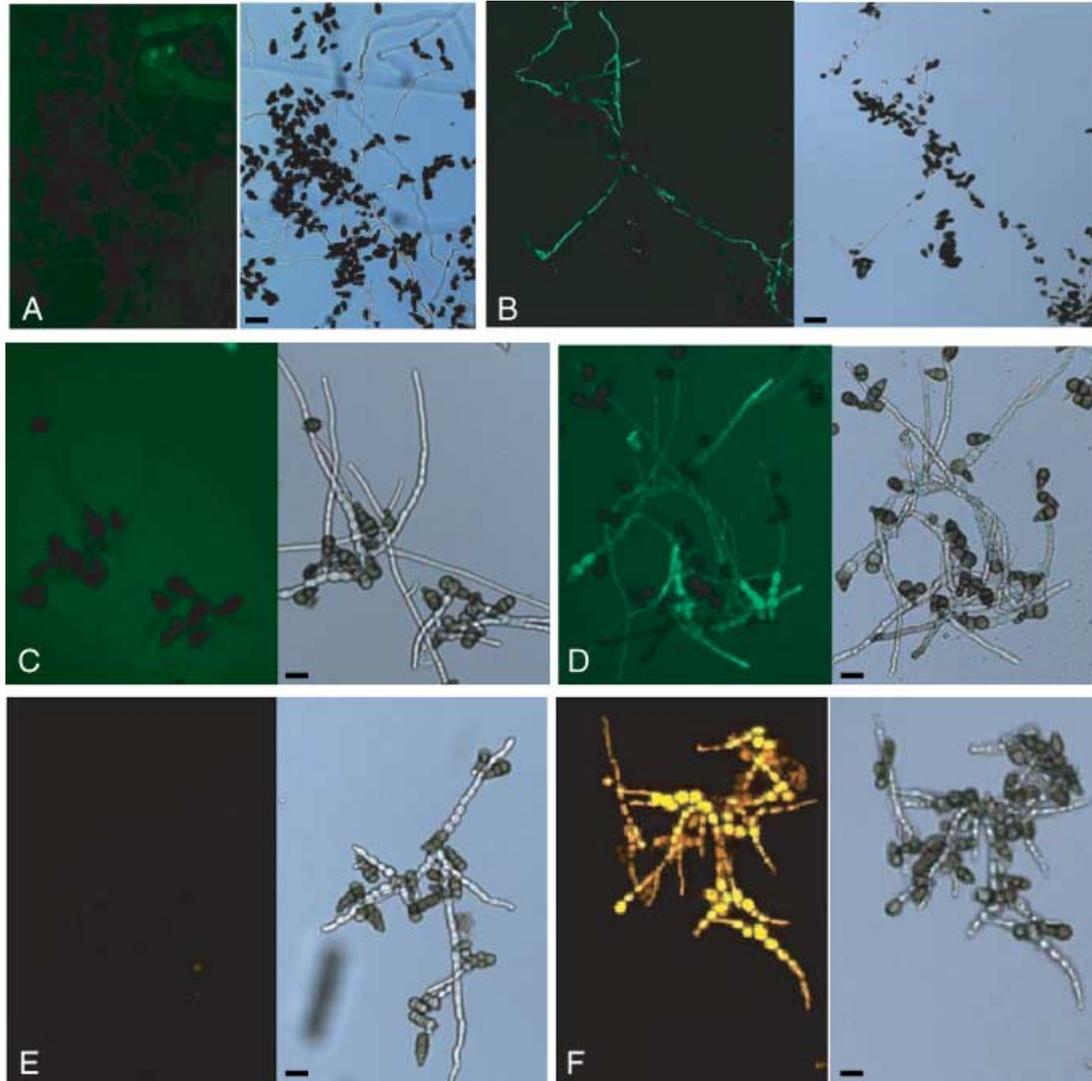
**Fig. 4** Determination of membrane damage and oxidative stress symptoms in *Alternaria brassicicola* treated with camalexin and ITC. Membrane integrity was checked using SYTOX green uptake assays in 24-h germinated conidia treated for 24 h with DMSO 1% v/v (A) or 125 $\mu$M camalexin (B). ROS within hyphae of germinated conidia treated for 4 h with methanol 1% v/v (C,E) or 2.5 mM Al-ITC (D,F) were detected using the fluorescent dyes $H_2$DCFDA (C,D) and DHE (E,F). For each panel, the left part corresponds to fluorescence microscopy and the right part to light-field microscopy. Scale bars = 20 $\mu$m.

the fungal plasma membrane (Dickson and Lester, 2002; Parks and Casey, 1995). The induction of these genes in response to camalexin is somewhat similar to what was observed with *S. cerevisiae* and *Candida albicans* treated with azole fungicides, which are known to inhibit ergosterol biosynthesis (Bammert and Fostel, 2000; Liu *et al.*, 2005). Induction of the expression of the *A. brassicicola ERG3* gene encoding the sterol $\Delta$5,6-desaturase could be the consequence of ergosterol depletion, as demonstrated in the yeast *S. cerevisiae* after exposure to ergosterol biosynthesis-inhibitors (Liu *et al.*, 2005; Smith *et al.*, 1996). Several links between the biosynthetic pathway of ergosterol and other pathways involved in the biosynthesis of lipids have been already

highlighted in *S. cerevisae* and *C. albicans* (Bammert and Fostel, 2000; Mukhopadhyay *et al.*, 2004; Veen and Lang, 2005) and these interactions probably constitute important determinants of drug susceptibility. By extrapolating to the present study, changes in the expression of these metabolic genes in response to camalexin are suggestive of alterations in the membrane integrity of the fungus. The damage caused by camalexin to the fungal cell surface may thus trigger a compensatory mechanism that would result in the activation of genes involved in membrane maintenance to ensure the stability of the cell. This would be consistent with the mechanism of action of camalexin on phytopathogenic bacteria reported by Rogers *et al.* (1996).

**Table 2** Summary of *Alternaria brassicicola* clones identified from the camalexin cDNA library. The presented ESTs correspond to those with significant matches (*e*-value < 1e-05) to proteins of known or predicted function.

| Clone | GenBank accession no.* | Size† | Best BLAST match | | | *e*-value |
|---|---|---|---|---|---|---|
| | | | Accession no. | Organism | Annotation | |
| **1. Metabolism** | | | | | | |
| **1.1. Lipid, fatty acid and sterol metabolism** | | | | | | |
| P3A10 | DY543047 | 758 | AAB41115 | *S. cerevisiae* | Similar to the Sphinganine C4-hydroxylase Sur2p | 4e-22 |
| P1A11 | DY543048 | 636 | EAL87310 | *A. fumigatus* | Putative delta 8-sphingolipid desaturase | 2e-35 |
| P2F2 | DY543049 | 200 | EAA74448 | *G. zeae* | Putative sphingolipid hydroxylase | 2e-05 |
| P1H8 | DY543050 | 1217 | CAA84977 | *S. cerevisiae* | Similar to the pyridoxine phosphate oxidase PDX3 | 2e-07 |
| P1D3 | DY543051 | 505 | AAN27998 | *Leptosphaeria maculans* | Similar to the sterol delta 5,6-desaturase ERG3 | 4e-42 |
| **1.2. Melanin biosynthesis** | | | | | | |
| P1B6 | DY543052 | 788 | BAA36503 | *Alternaria alternata* | Similar to the 1,3,8-trihydroxynaphthalene reductase BRM2 | 9e-116 |
| P4E1 | DY543053 | 228 | BAC79365 | *Bipolaris oryzae* | Similar to the scytalone dehydratase BSCD1 | 2e-05 |
| **1.3. Other metabolism-linked genes** | | | | | | |
| P4E11 | DY543054 | 1028 | AAS91580 | *S. nodorum* | Putative glyoxysomal malate synthase | 2e-69 |
| P3A9 | DY543055 | 595 | P87208 | *A. nidulans* | Similar to the pyruvate decarboxylase pdcA | 5e-21 |
| P3A3 | DY543056 | 1112 | SNU10756.1 | *S. nodorum* | Putative zinc-binding dehydrogenase | 2e-26 |
| P1H4 | DY543057 | 541 | EAL86321 | *A. fumigatus* | Putative D-xylulose 5-phosphate/D-fructose 6-phosphate phosphoketolase | 3e-81 |
| P4F3 | DY543058 | 1161 | AAN87355 | *Paracoccidioides brasiliensis* | Putative formamidase | 1e-23 |
| P4C2 | DY543059 | 620 | EAL84490 | *A. fumigatus* | Putative flavohemoprotein | 2e-12 |
| P2E7 | DY543060 | 734 | NP_014798 | *S. cerevisiae* | Similar to the IMP-specific 5′-nucleotidase Isn1p | 6e-45 |
| P3D12 | DY543061 | 625 | EAL92794 | *A. fumigatus* | Putative mitochondrial succinate dehydrogenase | 4e-13 |
| P4D8 | DY543062 | 450 | Q8SHP8 | *Hypocrea jecorina* | Similar to the NADH-ubiquinone oxidoreductase ND4L | 1e-10 |
| P2C2 | DY543063 | 294 | EAL91367 | *A. fumigatus* | Putative oxidoreductase, 2OG-Fe (II) oxygenase family | 4e-15 |
| P3D8 | DY543064 | 405 | SNU13647.1 | *S. nodorum* | Putative purine permease | 3e-14 |
| P3F12 | DY543065 | 481 | AAZ73168 | *G. moniliformis* | Putative alpha-amylase 1 | 1e-06 |
| P4D4 | DY543066 | 645 | CAA51009 | *A. nidulans* | uricase | 4e-28 |
| **2. Cell-wall structure and function** | | | | | | |
| P2F1 | DY543067 | 460 | AAC35942 | *A. fumigatus* | Similar to the beta (1–3) glucanosyltransferases GEL1 | 2e-13 |
| P4C8 | DY543068 | 701 | HJU89991 | *H. jecorina* | Similar to the mannose-1-phosphate guanylyltransferase MPG1 | 7e-08 |
| P3D5 | DY543069 | 550 | O74254 | *C. albicans* | Putative cell wall mannoprotein glycosyl hydrolase | 2e-19 |
| P2B11 | DY543070 | 367 | EAL91687 | *A. fumigatus* | Putative polysaccharide deacetylase | 1e-54 |
| P4A12 | DY543071 | 561 | EAL92848 | *A. fumigatus* | Putative polysaccharide deacetylase | 2e-11 |
| P3C1 | DY543072 | 1003 | AAS07042 | *Chlamydomonas reinhardtii* | Similar to the minus agglutinin SAD1 | 5e-07 |
| **3. Protein fate** | | | | | | |
| P4A11 | DY543073 | 732 | CAA70219 | *P. janthinellum* | Similar to the fructosyl amino acid oxidase FaoP | 2e-30 |
| P3F5 | DY543074 | 489 | YP_188090 | *Staphylococcus epidermidis* | Putative cysteine desulphurase | 2e-06 |
| P4E6 | DY543075 | 1118 | Q9Y7P1 | *S. pombe* | Similar to the endoplasmic oxidoreductin 1-like protein A ERO1p | 2e-14 |
| P3E11 | DY543076 | 496 | CAA07773 | *G. pulicaris* | Similar to the polyubiquitin Ubi1 | 1e-38 |
| P3H11 | DY543077 | 450 | SNU02705.1 | *S. nodorum* | Putative proteasome non-ATPase regulatory subunit Nin1/mts3 | 3e-09 |
| **4. Oxidative burst, stress and defence** | | | | | | |
| P2F9 | DY543078 | 400 | EAL85706 | *A. fumigatus* | Similar to the MFS drug transporter TRI12 | 9e-06 |
| P2B3 | DY543079 | 488 | EAA75163 | *G. zeae* | Putative MFS Transporter | 2e-16 |
| P1B3 | DY543080 | 1108 | AAC49410 | *Nectria haematococca* | Similar to the flavin-containing mono-oxygenase MAK1 | 8e-17 |
| P3E9 | DY543081 | 578 | ABC33908 | *Tuber borchii* | Putative dehydrin | 4e-09 |
| P2D12 | DY543082 | 626 | P41747 | *A. flavus* | Similar to the alcohol dehydrogenase adh1 | 1e-64 |
| P2C10 | DY543083 | 889 | AAO73810 | *P. brasiliensis* | Similar to the heat shock protein CLPA | 1e-41 |
| P2D5 | DY543084 | 290 | SNU00278.1 | *S. nodorum* | Putative cation efflux transporter | 1e-07 |
| **5. Cellular transport, transport facilitation and transport routes** | | | | | | |
| P2H9 | DY543085 | 1031 | AAN08046 | *A. nidulans* | Similar to the siderophore iron transporter mirC | 9e-55 |
| P2B5 | DY543086 | 829 | P38865 | *S. cerevisiae* | Similar to the copper transporter CTR2 | 8e-13 |

**Table 2** *continued.*

| Clone | GenBank accession no.* | Size† | Best BLAST match | | | e-value |
| | | | Accession no. | Organism | Annotation | |
|---|---|---|---|---|---|---|
| P3C4 | DY543087 | 382 | AAW42114 | *Cryptococcus neoformans* | Putative cation transport-related protein | 4e-06 |
| P2C7 | DY543088 | 483 | ABB90287 | *G. zeae* | Similar to the calcium-translocating P-type ATPase | 1e-40 |
| P3F9 | DY543089 | 1089 | EAL86522 | *A. fumigatus* | Putative FAD dependent oxidoreductase | 3e-46 |
| P3E10 | DY543090 | 530 | SNU12213.1 | *S. nodorum* | Putativethiamine pyrophosphate protein | 1e-17 |
| P2E6 | DY543091 | 239 | SNU15080.1 | *S. nodorum* | Putative vacuolar protein sorting-associated protein 26 | 4e-11 |
| 6. Transcription | | | | | | |
| P1C11 | DY543092 | 818 | EAL87704 | *A. fumigatus* | Putative fungal specific transcription factor | 3e-39 |
| P1C12 | DY543093 | 998 | XP_893708 | *Mus musculus* | Putative Histone H2A-Bbd | 5e-11 |
| P2B4 | DY543094 | 464 | O43003 | *S. pombe* | Similar to the endoribonuclease L-PSP; translation initiation inhibitor Mmf1p | 6e-15 |

*Thirty-one additional sequences that match to unclassified or hypothetical proteins have been registered under GenBank accession nos. DY543095 to DY543125.
†In base pairs.

In addition to the ergosterol and sphingolipid biosynthesis genes, camalexin induced the expression of two melanin biosynthesis genes. An EST similar to the sequence from clone P1B6 encoding a 1,3,8-trihydroxynaphtalene reductase has already been derived from an *A. brassicicola*–*B. oleracea* SSH library and has been shown to be induced *in planta* as soon as 12 h after infection (Cramer *et al.*, 2006). The second EST (P4E1) from our camalexin SSH library is similar to the scytalone dehydratase, another key gene in the synthesis of melanin, which is a constitutive compound of the *Alternaria* conidia wall, located in the septa and outer primary wall (Carzaniga *et al.*, 2002). The induction of these genes occurred probably in an attempt to decrease cell-wall permeability and thus to prevent the intracellular accumulation of camalexin. The relationship between wall impermeability and protection against antifungal products has indeed already been demonstrated in *Cryptococcus neoformans* (Wang and Casadevall, 1994). Camalexin also induced the expression of several genes involved in cell-wall maintenance, including a gene encoding a β(1–3)-glucanosyltransferase, which is homologous to the *Aspergillus fumigatus GEL1* gene involved in the elongation of β-1.3-glucan (Mouyna *et al.*, 2000), and the mannose-1-phosphate guanylyltransferase and mannoprotein glycosyl hydrolase genes implicated in cell-wall mannoprotein synthesis (Zakrzewska *et al.*, 2003; Zhao *et al.*, 2000). In addition to the induction of genes encoding proteins involved in melanin biosynthesis, changes in the expression of cell-wall biogenesis genes are also suggestive of a reinforcement of the physical barriers against the cellular penetration of toxic compounds.

As observed after Al-ITC challenge, exposure to camalexin resulted in enhanced expression of two efflux pumps belonging to the MFS superfamily that could actively participate to maintain low concentrations of this compound inside the fungal cell.

Interestingly, a gene encoding the flavin-containing mono-oxygenase homologous to the phytoalexin maackiain detoxifying enzyme *MAK1* was up-regulated by camalexin. In *Nectria haema-*tococca the *MAK1* gene product converts a chick-pea phytoalexin maackiain into the less toxic compound 1a-hydroxy-maackiain (Covert *et al.*, 1996). Pedras and Khan (1997) reported that camalexin detoxification in *Rhizoctonia solani* may result from its conversion to the less toxic compound 5-hydroxycamalexin. It is therefore possible that camalexin hydroxylation could be also a potential detoxification mechanism in *A. brassicicola*.

## Camalexin-induced membrane permeabilization

To check whether camalexin had an effect on fungal membrane integrity, an assay based on the uptake of the fluorigenic dye SYTOX green was used. This substance can only penetrate cells that have compromised plasma membranes, and it fluoresces upon binding to DNA. *A. brassicicola* germinated conidia were incubated with or without camalexin for various times and then the fluorescent probe was added. As shown in Fig. 4B, green fluorescence in hyphae was observed after treatment of germinated conidia exposed for 24 h with camalexin. Virtually no green fluorescence was detected in control samples treated with the fluorescent probes only (Fig. 4A). Samples incubated with camalexin for short time exposures, i.e. for times where the cell viability was not significantly affected by the treatment, did not significantly differ from controls (data not shown). These data indicated that camalexin probably causes membrane damage.

## ITC-induced production of cellular reactive oxygen intermediates

To obtain additional support for the generation of an oxidative stress by ITC in fungal cells, the production of ROI was assessed using 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA), which is hydrolysed within the cells to 2′,7′-dichlorodihydrofluorescein (H$_2$DCF), which reacts with peroxides to generate the fluorescent

compound 2′,7′-dichlorofluorescein (DCF). After 4 h of incubation with either Al- or Bz-ITC (Fig. 4D), DCF-dependent fluorescence was observed in hyphae whereas no signal was recorded in untreated controls (Fig. 4C). To confirm these data further, dihydroethidium (DHE), a redox-sensitive probe that is oxidized to ethidium by superoxide anions, was also used to detect intracellular ROI after exposure to ITC. Fluorescence intensity of hyphae was strongly increased in samples exposed for 4 h to either Al- or Bz-ITC (Fig. 4F) compared with untreated controls (Fig. 4E). These observations confirmed that ITC are potential inducers of intracellular ROI in fungal cells, as was shown for animal cells (Nakamura *et al.*, 2000).

### Expression of candidate genes during *Arabidopsis thaliana* and *Raphanus sativus* infection

In order to address the question of expression of the plant-compound-responsive genes in compatible vs. incompatible interactions, 25 candidate genes were selected from the two cDNA libraries on the basis of their similarity to known proteins potentially involved in cell protection, detoxification and transcription regulation. Expression time-course studies during *A. brassicicola* interaction with the resistant weed *Arabidopsis thaliana* and also with the susceptible host *R. sativus* were then performed.

Results of the time-course experiment during interaction with *Arabidopsis thaliana* (Fig. 5A; Table S3) revealed that among the ITC-up-regulated candidate genes, the expression levels of all transporters were induced as well as the thioredoxin and the sulphide-quinone oxidoreductase encoding cDNA. *In planta* increased expression levels of camalexin-induced genes were recorded for the sequences P1C11, P1C12, P2F9 and P4E1 encoding homologues of MFS transporter, histone H2A-binding, fungal-specific transcription factor and scytalone dehydratase, respectively. During interaction with the host *R. sativus*, the expression of all the candidate genes was significantly induced, except for the camalexin-induced sequence P2H9 encoding a putative siderophore transporter (Table S4).

Fungal biomass evolution during the interaction with both *Arabidopsis thaliana* (Fig. 5B) and *R. sativus* (Fig. 5C) was also followed by measuring the amount of *A. brassicicola* DNA in plant tissues. Fungal biomass remained stable during the incompatible interaction with *Arabidopsis thaliana* Co, which is consistent with the fact that this accession is resistant to this fungus. During infection of the compatible host *R. sativus*, *A. brassicicola* biomass increased significantly during the time-course of the experiment, indicating an invasive growth of the fungus in this susceptible plant.

These results demonstrated that almost all the selected ITC and camalexin candidate genes were up-regulated during the compatible interaction with *R. sativus*, whereas only a few genes were induced during the incompatible interaction with *Arabidopsis*

*thaliana*. However, a possible transient expression, not covered by our time-course experiment, cannot be ruled out. It should be noted that the selected camalexin-induced genes were up-regulated during interaction with *R. sativus* while this plant is not known to produce this phytoalexin. This suggests that these genes may also be activated in response to other derivatives of this indolic phytoalexin, such as 6-methoxycamalexin, which is produced by *R. sativus* (Pedras *et al.*, 2000).

### CONCLUSION

The present study reports the results of an analysis of the responses of *A. brassicicola* to short time exposures to brassicaceous metabolites at concentrations that are likely to be found in plant tissues during infection in the area surrounding necrotic lesions (Kliebenstein *et al.*, 2005). Two distinct gene induction profiles were obtained for the two tested compounds, implying that the recorded responses were not simply a general response of the fungus to severe stress conditions. This was further supported by the *in planta* induction of almost all the selected candidate genes during the compatible interaction with *R. sativus*. The data generated using both SSH and cellular fluorescent probes suggest possible mechanisms of cytotoxicity for ITC and camalexin on the fungus and shed light on the differences in cellular responses to these two different defence compounds. In the presence of ITC, fungal cells displayed responses similar to those experienced during oxidative stress, while camalexin activated the so-called 'compensatory mechanism' aimed at preserving membrane integrity. Furthermore, the expression of some efflux pumps was remarkably increased in response to ITC and camalexin, suggesting that efflux mechanisms could also be a potential detoxification pathway in *A. brassicicola*. Some of the genes identified here, by allowing the fungus to overcome the toxic effects of plant defence compounds, should be added to the growing list of potential *A. brassicicola* virulence factors together with the recently published expressed sequence tags derived from an *A. brassicicola*—*B. oleracea* interaction (Cramer *et al.*, 2006). Thanks to the forthcoming *A. brassicicola* genome annotation and the recent development of an efficient method for gene disruption in this fungus (Cho *et al.*, 2006), functional analyses of several of these candidate genes may soon be performed.

### EXPERIMENTAL PROCEDURES

#### Antimicrobial metabolites

Al-ITC and Bz-ITC were purchased from Aldrich Chemical Co. (Milwaukee, WI). The phytoalexin camalexin was synthesized according to Ayer *et al.* (1992). Stock solutions (100-fold concentrated) were prepared using methanol and dimethyl sulphoxide (DMSO) as solvents for ITC and camalexin, respectively.
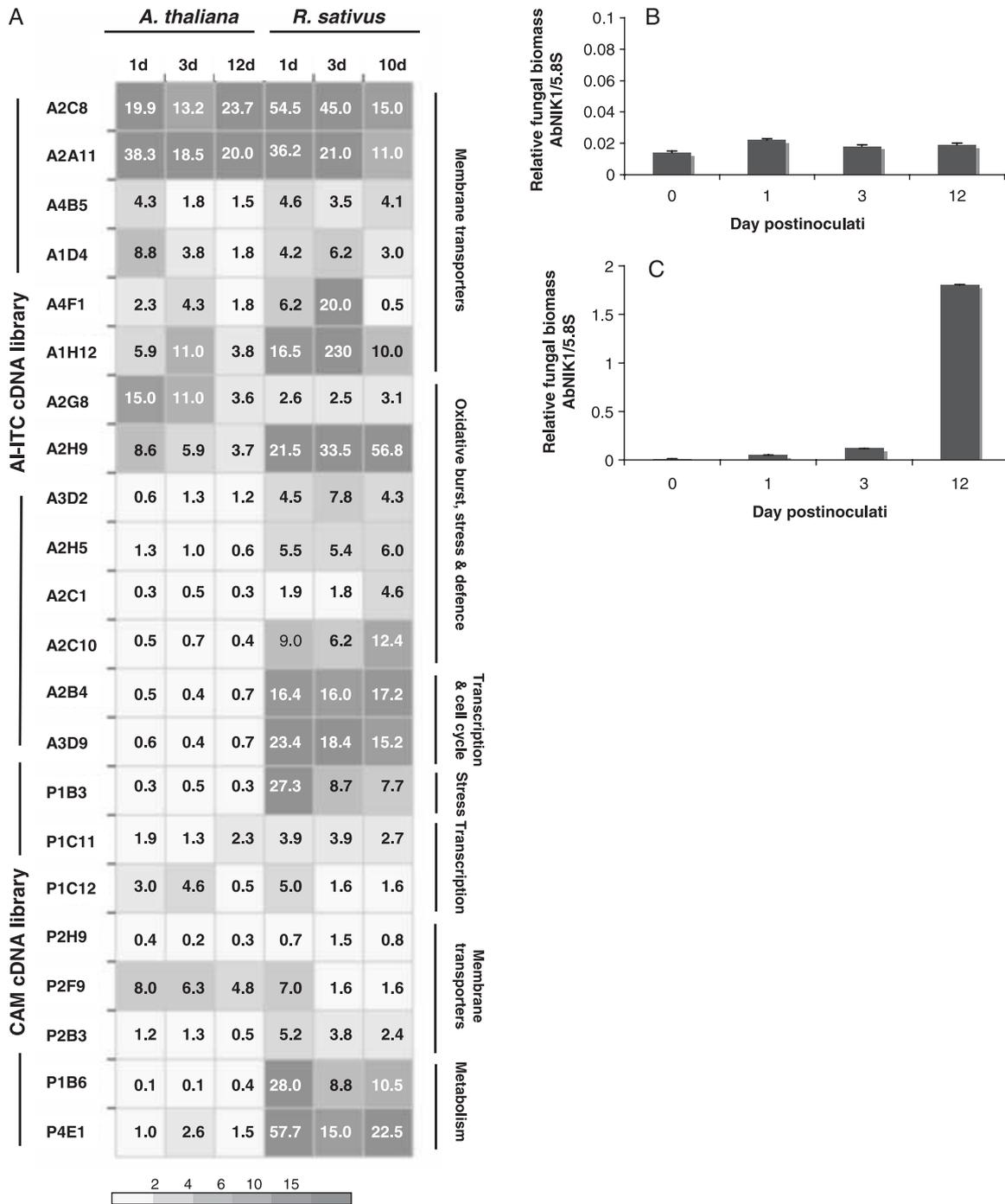
**Fig. 5** Expression levels of sequences selected from the AI-ITC and camalexin cDNA libraries during the interaction between *Alternaria brassicicola* and *Arabidopsis thaliana* or *Raphanus sativus*. (A) First-strand cDNAs were prepared from RNA samples extracted from plant tissues at the indicated times after inoculation and used as template for real-time PCR. For each gene, induction of expression is represented as a ratio (fold induction) of its relative expression (studied gene transcript abundance/β-tubulin transcript abundance) in each inoculated sample to its relative expression in free-living fungal control cultures. Each value is the mean of two independent experiments each with three replicates. For easier visualization of the results, numerical data were transformed into colour-grid representations using the JColorGrid software (Joachimiak *et al.*, 2006) in which fold induction of gene expression is represented by a grey scale. (B,C) Fungal growth quantification during interaction with *Arabidopsis thaliana* and *R. sativus*, respectively. The abundance of *AbNIK1* was quantified in infected leaves, normalized with plant 5.8S ribosomal DNA and expressed by the ratio of the fungal *AbNIK1* DNA amount to plant 5.8S ribosomal DNA (AbNIK1/5.8S). The data are the mean of three repetitions. The star indicates the beginning of conidia germination and leaf tissue penetration.

## Fungal strain, growth conditions, plant infection and pathogenicity assay

The *A. brassicicola* wild-type strain Abra43 used in this study was isolated from *R. sativus* seeds. Its taxonomic status has already been confirmed, using both morphological and molecular criteria (Iacomi-Vasilescu *et al.*, 2002). For routine culture, *A. brassicicola* was grown and maintained on potato dextrose agar (PDA). Antimicrobial compounds were added at concentrations equivalent to or below the $IC_{50}$ for mycelial growth (2.5 mM Al-ITC, 300 μM Bz-ITC, 125 μM camalexin), to 2-day-old germinating conidia grown in potato dextrose broth (PDB). Incubation in the presence of the fungus was then carried out for 0.5, 2, 4 and 24 h. Controls were performed by adding the relevant solvent (1% v/v final concentration) instead of the tested compound. Assays for viability were performed at each time point by spreading aliquots of treated or control conidia suspension on to PDA plates. Mycelial colonies developed after 48 h of incubation were scored. All culturing was performed at 24 °C.

*Arabidopsis thaliana* Co plants were grown to the eight- to 12-leaf stage in controlled environment rooms (21/19 °C day/night temperature, respectively) and an 8-h light photoperiod. For inoculations, 5-μL drops of a freshly prepared *A. brassicicola* spore suspension ($5 \times 10^6$ spores/mL in water) were pipetted on to six leaves per plant (two drops per leaf). The plants were then maintained under saturating humidity (100% relative humidity). Control plants were not inoculated, but were otherwise treated in the same way.

To assess fungal development and disease severity during the interaction, fungal DNA was quantified by real-time quantitative PCR as described by Sellam *et al.* (2006b) using the *A. brassicicola* histidine kinase *AbNIK1* gene (AY700092) as target sequence.

## Suppression-subtractive hybridization

SSH cDNA was synthesized from 1 μg total RNA, isolated from *A. brassicicola* as previously described (Guillemette *et al.*, 2004), using the Super SMART PCR cDNA synthesis kit (Clontech, Palo Alto, CA) as described in the user manual. The cDNA populations were then subtracted using the PCR-Select cDNA subtraction kit (Clontech) following the manufacturer's instructions. The PCR products derived from the final SSH step were ligated into the pGEM-T vector (Promega, Charbonnières, France) and transferred into *Escherichia coli* JM109 cells to generate libraries of SSH-derived fragments.

## Differential screening of the subtracted library by reverse dot-blot

cDNA inserts of putative recombinant clones were amplified from 1 μL of an overnight culture using nested primers and PCR conditions as described in the manual of the PCR-Select cDNA subtraction kit. After amplification, the PCR products were analysed by gel electrophoresis to confirm the presence of an insert in each recombinant clone. Two microlitres of the denaturized PCR products were blotted on to duplicate Hybond-N+ nylon membranes (Amersham, Buckinghamshire, UK) using a Minifold® dot-blotter (Schleicher & Schuell BioScience, Dassel, Germany). Forward and reverse subtracted probes were labelled with $^{32}$P using a random primer labelling kit (Amersham). The labelled cDNA probes were hybridized to the dot-blot membranes at 72 °C overnight with continuous agitation. After hybridization, membranes were washed with low-stringency (2× SSC and 0.5% SDS) solution at 68 °C followed by high-stringency (0.2× SSC and 0.5% SDS) solution at the same temperature for 30 min each. The wet membranes were then exposed to Kodak K-screens and blots were visualized on a phosphoimager (Molecular Imager FX, Bio-Rad, Hercules, CA) and analysed using Quantity one software (Bio-Rad).

## Clone sequencing and analysis

The selected positive clones were all sequenced with T7 primer by the automated sequencing facility of Ouest-Génopole (Roscoff, France) using the AB3130 genetic analyser (Applied Biosystems, Foster City, CA). Unique ESTs were selected and annotated on the basis of the existing annotation of non-redondant GenBank database sequences using the BlastX algorithm. Functional classification was carried out according to the functional categories of the yeast *S. cerevisiae* proteins (http://www.yeastgenome.org/). EST sequences have been submitted to the GenBank EST database under the accession numbers given in Tables 1 and 2.

## Expression analysis by real-time quantitative PCR

Traces of genomic DNA contaminants were removed by treating total RNA with TURBO™ DNase (Ambion, Huntingdon, UK). The absence of contaminating genomic DNA in the RNA samples was determined by direct amplification of non-reverse transcribed samples. cDNA was synthesized from 5 μg of total RNA using the reverse-transcription system (50 mM Tris-HCl, 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl$_2$, 400 nM oligo(dT)15, 1 μM random hexamers, 0.5 mM dNTP, 200 units M-MLV reverse transcriptase; Promega). The total volume was adjusted to 30 μL and the mixture was then incubated for 60 min at 42 °C. Aliquots of the resulting first-strand cDNA were used for real-time PCR amplification experiments. Real-time PCRs were performed with the ABI Prism 7000 sequence detection system (Applied Biosystems) using the SYBR green PCR master mix, according to the manufacturer's instructions. After 10 min denaturation at 95 °C, the reactions were cycled 40 times at 95 °C for 15 s and 60 °C for 1 min. To verify that only the specific product was amplified, a melting point analysis was performed after the last cycle by cooling samples to 55 °C and then increasing the temperature to 95 °C at 0.2 °C/s. A single product at a specific melting temperature was found for

**Table 3** Primer sets used in real-time quantitative PCR.

| Gene | 5′ primer | 3′ primer |
| --- | --- | --- |
| β-Tubulin | TTCAACGAAGCCTCCAACAAC | GTGCCGGGCTCGAGAT |
| A2H5 | GTACCCATACGAATACGCATCCTA | GCATTGCGGCTCTGGATT |
| A2C10 | GCCAACCACTTCAACCGTTT | TCGCCTGTATAACGCTGCAT |
| A3D10 | GGCTCCCCCTACGATCTCA | TTCGAGGCGGTGTTGACA |
| A2F9 | CCGACATCAAGCAGCGTCTA | AGCAAAATGGGTCGCTAGGA |
| A1B12 | GAAAAGAGCGCACAGCAATG | AAAATTGATCCCCTCGTCTTTG |
| A2H9 | CGACAACTGCTTGACGCTACA | GATGCCTGGCGCAATGAC |
| A3D2 | GCGGCTTGCATCAAGGAA | GGCGCGGAAGTCCAAGT |
| A4F1 | TCCAGACGCTGCACGAGTT | AACGGCGCGTAGTTCATCA |
| A1D4 | CTTGACAGCGCCCTTGTGT | GCCCAAGCCCAAAAAGGA |
| A2A11 | GAGAAGGGTGCGCAACAAG | GGTGCCAGCATCCTTCTCA |
| A4B5 | ACATCAAAGCCAGCGACAAGT | CCCAATTGCTGACGCAGAA |
| A2H4 | CCAGGCCCACCAAAATCAG | TTGCATGGGCGTTCTTACC |
| A1H12 | CACCTTTGCGCGTCTCTTC | CCACAGCCCAGACAATTGGT |
| A2C8 | CGCCCGGTGGTATCTTGTC | TCGGCTGTGATCTTGGTTGTT |
| A1B2 | CGCCCGGTGGTATCTTGTC | TCGGCTGTGATCTTGGTTGTT |
| A3D9 | TCGTGATCCGTGGACTCAAA | CGGGTCGAAGGGTGCTT |
| A2B4 | CTCCGTCTGCAGTTGCTGTATC | AGTCCCAGAAGGAGGCACAA |
| A2B11 | GCGCTAATATTGCTATGCTTTGC | ACGGAATCTACCCGGGAATATACT |
| P3A10 | CGGGCTTGCCTGTTGAGA | CAACGATGATCTTCCTAACCACAA |
| P1A11 | GCGCTCATGAACCGCTATC | GGCGGTAGGAAGTTGATCCA |
| P1D3 | TCGCGATACGTCGTGTGTTT | TCCATCCCATCACCAGTCTTTT |
| P1B6 | GACGACGTTGTTGCCCATT | AGAAACGACACCGGAGTTTGA |
| P4E1 | GGCGCCGTAGGAAATGTG | CCCCATCATCATTCGTATTGC |
| P2F1 | CCCCCGGGCTGGTAGTC | CCGTCACGGTCAAAGGAAA |
| P3D5 | GCGCTTGGCAACGTGACTAT | TTGTCTCCCCATTCAACTTAACC |
| P4E6 | ATTTCCGAAGCCGCAAAAG | CAAGCCCCCCCAAAGAAG |
| P3E11 | GCAGGGCAAATCATCCTATGA | GTCCCCTGATGGTCGATGAC |
| P2F9 | GGTCCTGCGCCGTGATTA | CCAATTAGCCCACCCGTATTT |
| P2B3 | TCGACCTCGCTTTATCAAAGG | GCGTAGCATATCCAAGCAAACC |
| P1B3 | CGCTACGAGGCAGATGTAGTCA | CAACAAGCTTCCGGCTCTTC |
| P3E9 | CGTGGACCATCGTGCTGAT | GCATCGTCTATCTACGTGTCTGGTA |
| P2H9 | CCGGCAGAATAACAGCTCAGA | GCAGGCGATTTACACCAACA |
| P2B5 | TCAGCCAGTCTTCGTCAACAA | CGCGCCAGCTGTCTAACTTT |
| P1C11 | GCTTTTCCATCACACCATGTGT | CTTGACGGGCGCATTGA |
| P1C12 | GGTGCCGTATGAGTGGAGCTA | CGTGGAAAGAGCGCAGTGA |

each target. All samples were tested in triplicate and the mean was determined for further calculations. Each run included a no-template control to rule out test reagent contamination.

The relative quantification analysis was performed using the comparative ΔΔCt method as described by Winer *et al.* (1999). To evaluate the gene expression level, the results were normalized using Ct values obtained from β-tubulin RNA amplifications run on the same plate. Primer sequences used in real-time quantitative PCR are summarized in Table 3.

### SYTOX green uptake assay and intracellular detection of oxidative products

For membrane permeabilization assays, 2-day-old germinating conidia were exposed to either 125 μM camalexin or 1% (v/v) DMSO as negative control as described for viability tests, for the indicated times. After treatment, 20 μL of incubation mixtures were mixed with a SYTOX green solution (1 μM final concentration) and immediately observed with a Zeiss fluorescence microscope. Essentially the same procedure was used to detect intracellular ROI after exposure to 2.5 mM Al-ITC or 0.3 mM Bz-ITC or 1% (v/v) methanol. After treatment, 100 μL of incubation mixtures were mixed with $H_2DCF$-DA or DHE solutions (5 μM final concentration) and observations were performed as above after 30 min of incubation.

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## SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

**Table S1** Expression levels of sequences randomly selected from the Al-ITC cDNA library in *Alternaria brassicicola* exposed to Al-ITC and Bz-ITC. Numerical data.

**Table S2** Expression levels of sequences randomly selected from the camalexin cDNA library in *Alternaria brassicicola* exposed to camalexin. Numerical data.

**Table S3** Expression levels of sequences selected from the Al-ITC and camalexin cDNA libraries during the interaction between *Alternaria brassicicola* and *Arabidopsis thaliana*. Numerical data.

**Table S4** Expression levels of sequences selected from the Al-ITC and camalexin cDNA libraries during the interaction between *Alternaria brassicicola* and *Raphanus sativus*. Numerical data.

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