# A cDNA microarray approach to decipher sunflower (*Helianthus annuus*) responses to the necrotrophic fungus *Phoma macdonaldii*

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## Summary

• To identify the genes involved in the partial resistance of sunflower (*Helianthus annuus*) to the necrotrophic fungus *Phoma macdonaldii*, we developed a 1000-element cDNA microarray containing carefully chosen genes putatively involved in primary metabolic pathways, signal transduction and biotic stress responses.

• A two-pass general linear model was used to normalize the data and then to detect differentially expressed genes. This method allowed us to identify 38 genes differentially expressed among genotypes, treatments and times, mainly belonging to plant defense, signaling pathways and amino acid metabolism.

• Based on a set of genes whose differential expression was highly significant, we propose a model in which negative regulation of a dual-specificity MAPK phosphatase could be implicated in sunflower defense mechanisms against the pathogen. The resulting activation of the MAP kinase cascade could subsequently trigger defense responses (e.g. thaumatin biosynthesis and phenylalanine ammonia lyase activation), under the control of transcription factors belonging to MYB and WRKY families. Concurrently, the activation of protein phosphatase 2A (PP2A), which is implicated in cell death inhibition, could limit pathogen development.

• The results reported here provide a valuable first step towards the understanding and analysis of the *P. macdonaldii*–sunflower interaction.

**Key words:** cDNA microarray, *Helianthus annuus* (sunflower), necrotrophic fungus, partial resistance, *Phoma macdonaldii*, plant defense.

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## Introduction

Many advances in plant–microbe interactions have been achieved in the past decade. This includes cloning and characterization of plant resistance genes (for a review, see Martin *et al.*, 2003) and identification of components involved in the regulation of defense response (Feys & Parker, 2000; Glazebrook, 2001). Upon pathogen attack, plants develop a set of defense mechanisms: expression of defense-related proteins such as pathogenesis-related (PR) proteins, thionins and plant defensins, synthesis of antibiotics such as phytoalexins, reinforcement of the cell wall, or production of rapid active oxygen species (Dangl & Jones, 2001; May *et al.*, 1996). Biochemical and molecular evidence suggests that defense responses do not result from a linear signal transduction cascade, but rather from complex interactions among multiple signaling pathways acting in parallel (Kunkel & Brooks, 2002). As a consequence, the signaling pathways leading to active defense in the host could differ depending on the lifestyle of the pathogen. A model which classifies pathogens into necrotrophs and biotrophs proposes that the lifestyle of a pathogen might be a predictor of whether the pathogen will be affected by the jasmonate response or not (McDowell & Dangl, 2000). In support of this model, it has been

demonstrated in *Arabidopsis* that the salicylic acid-dependent pathway is required for defense against *Peronospora parasitica* and *Erysiphe orontii*, two biotrophic fungi. In contrast, a jasmonate/ethylene-dependent pathway has been shown to be effective against *Botrytis cinerea*, via the functional ethylene insensitive 2 (*EIN2*) gene (Thomma *et al.*, 1999a), whereas a jasmonic acid (JA)-dependent but ethylene-independent pathway, in parallel with a pathway leading to camalexin production, provides resistance against *Alternaria brassicicola*. *B. cinerea* and *A. brassicicola* are necrotrophic fungal pathogens (for a review, see Thomma *et al.*, 2001). Little is known about the genes involved in resistance to diseases in sunflower (*Helianthus annuus*). Mazeyrat *et al.* (1999) have investigated the resistance of sunflower to the biotrophic oomycete *Plasmopara halstedii*, the causal agent of downy mildew of sunflower. They showed that transcript levels of phenylalanine ammonia lyase (PAL), chitinase and ubiquitin were rapidly and strongly increased after infection of sunflower in incompatible interactions, but not in compatible ones, suggesting that regulation of these mRNAs is an important component of resistance mechanisms in sunflower (Mazeyrat *et al.*, 1999).

*Phoma macdonaldii* (Boerema, 1970) and its teleomorph *Leptosphaeria lindquistii* (Frezzi, 1968) are responsible for the black stem disease of sunflower. This disease is characterized by the formation of black spots on the stem, around the petiole insertion point. At the bases of stems, coalescing spots develop into a wide black sleeve (Peres & Lefol, 1996). Black stem disease is the second most important sunflower disease in France after downy mildew. To date, sunflower genotypes with contrasted levels of resistance to this disease have been identified, but no fully resistant genotypes have been described. The inheritance of partial resistance of sunflower to *P. macdonaldii* was reported to be quantitative under additive and dominant effects (Roustaee *et al.*, 2000). In two independent studies, a significant number of quantitative trait loci (QTLs) for *P. macdonaldii* resistance were identified (Rachid Al-Chaarani *et al.*, 2002; Bert *et al.*, 2004), confirming the polygenic nature of this partial resistance. Currently, little is known about the identity of genes involved in the partial resistance of sunflower to this necrotrophic fungus.

Although analysis of gene expression using the microarray approach has already provided significant insights into the signaling processes involved in defense responses in model plants (Dowd *et al.*, 2004; Maleck *et al.*, 2000; Schenk *et al.*, 2000; Scheideler *et al.*, 2002), there are few reports describing the global changes in transcription activities in response to pathogen infection in cultivated crops. In most cases, these studies deal with monogenic resistance. Surprisingly, no microarray analysis of quantitative resistance has been published to date. In this context, investigation of metabolic pathways, signal transduction and defense mechanisms involved in partial resistance could be fruitful, not only to facilitate recognition of patterns of gene expression during resistance responses, but also to elucidate the mechanism by which the plant overcomes the pathogen. Analysis of expression profiles of genes involved in metabolic pathways will, for example, facilitate identification of metabolic changes in response to pathogen infection. This could contribute to the development of new strategies in order to control disease infections in agronomy.

In this study, we developed and used a 1000-element cDNA nylon microarray. Taking into account the fact that use of thematic arrays limits the range of genes that can be detected, we have carefully chosen for this array a set of representative genes putatively involved in primary metabolic pathways, signal transduction and response to biotic stress, identified on the basis of their homology to *Arabidopsis thaliana* genes. We employed this thematic array to investigate transcriptional changes that occur during the activation of multigenic resistance in partially resistant and susceptible sunflower lines inoculated with *P. macdonaldii*, at two time-points. In addition to well-known defense-related genes, we identified a number of genes involved in primary metabolic and signaling pathways, with putative roles in *P. macdonaldii* partial resistance. These results provide insights into key regulators and cellular processes involved in sunflower resistance to black stem disease, and make a significant contribution to the clarification of the mechanisms developed by the plant against this pathogen.

## Materials and Methods

### Plant material and experimental design

Lines of sunflower (*Helianthus annuus* L.) partially resistant (C146) and susceptible (C106) to *Phoma macdonaldii* (Boerema) were selected and used in this investigation. These lines, provided by F. Vear (INRA, Clermont Ferrand, France), have been developed by single seed descent (SSD) from a cross between PAC-2 and RHA-266. Seeds were sown in plastic containers filled with horticulture substrate (Hawita Flor, Germany) and transferred to a growth chamber with a 14 h : 10 h light:dark photoperiod, a $24 \pm 1°C : 17 \pm 1°C$ light:dark temperature regime, a light intensity of 200 $\mu E\ m^{-2}\ s^{-1}$ provided by daylight fluorescent tubes (Philips, Eindoven, the Netherlands), and a relative humidity of 75–85%. MP6, an aggressive monopycniospore isolate of *P. macdonaldii*, was used (Roustaee *et al.*, 2000). The MP6 isolate was grown on potato dextrose agar medium at $25 \pm 1°C$ with a 12 h : 12 h light:dark photoperiod and a light intensity of 37 $\mu E\ m^{-2}\ s^{-1}$. After a 10-d incubation period, a pycniospore suspension was obtained by the addition of sterile water to the surface of the culture and mechanical mixing.

Twenty microliters of spore suspension containing $10^6$ pycniospores $ml^{-1}$ in water, 0.5% orange juice and 0.25% gelatine was deposed at the intersection of the cotyledon petiole and the hypocotyl of two-leaf-stage sunflower seedlings. During the first 48 h following contamination, the containers in which plants were grown were covered with a transparent top to maintain nearly saturated humidity, which is favorable

 

for pathogen development. For control treatments, 20 µl of the same solution without spores was used. The plants were grown in parallel under the same conditions and petiole samples were collected at the same time as for the treated plants. Inoculated plants of both C106 and C146 lines were grown for 1 wk in the same conditions as described above, in order to control the efficiency of contamination. Then, cotyledon petioles of the seedlings were scored according to the percentage of petiole area exhibiting disease symptoms (necrosis). A rating scale from 1 to 9, based on the percentage of necrosed area, was used (Roustaee *et al.*, 2000).

### Preparation of microarrays

We constructed a thematic sunflower cDNA microarray containing 911 cDNA clones, representing high sequence similarity with known or predicted *A. thaliana* genes related to primary metabolic and signal transduction pathways. We added to this array all the putative defense-related cDNAs (116) available for sunflower. The full list of the selected clones, as well as their potential functions, can be found in the supplementary data. The sunflower expressed sequences tag (EST) clones were selected from different cDNA libraries (Tamborindeguy *et al.*, 2004; Ben *et al.*, 2005) based on sequence homology to already known or predicted genes involved in major primary metabolic pathways, signal transduction and defense responses in *A. thaliana* using the BLASTN and BLASTX programs (Altschul *et al.*, 1990). Sequences with significant similarities (*E*-value < 1.0 $e^{-10}$ or BLAST score > 100) were selected.

The selected cDNA clones were collected from different cDNA libraries and incubated in 96-well microplates (Greiner bio-one, Courtaboeuf, France), containing 200 µl of culture medium (LB) with 100 mg $l^{-1}$ ampicillin, at 37 or 31°C and 200 r.p.m. in a shaking incubator. Five microliters of bacterial suspension was transferred to Thermowell 96-well plates (Costar) containing 95 µl of MilliQ water (Yvelines, France). The plates were then heated to 95°C for 10 min in a laboratory oven, followed by centrifugation at 1200 ***g*** for 3 min to remove the cellular debris. Eight microliters of the supernatant was used to amplify the cDNA insert using primer pairs specific for the vector ends. For amplification of clones in pSport1, pCR4 and pGMT the universal M13 forward primer 5′-GTTTTCCCAGTCACGACGTTG-3′ and M13 reverse primer 5′-TGAGCGGATAACAATTTCACACAG-3′ were used. However, for inserts in Triplex2, the forward and reverse oligonucleotides were 5′-TCCGAGATCTGCACGAGC-3′ and 5′-TAATACGACTCACTATAGGG-3′, respectively.

The PCR reaction was performed in 100 µl containing 10 µl of 10X buffer, 2.5 mM $MgCl_2$, 200 µM dNTP, 0.2 µM of each primer and 3 U of Taq DNA polymerase (Qiagene, Paris, France). DNA samples were denaturated at 95°C for 2 min followed by 40 cycles of amplification at 95°C for 30 s, 52°C for 30 s and 72°C for 2 min followed by a final extension step at 72°C for 7 min. The size and quality of PCR products were tested by agarose gel electrophoresis. PCR samples showing double bands were removed or replaced by another cDNA clone. The PCR solutions were then concentrated by evaporation using a speed vacuum at 70°C for 90 min and the products were then suspended in 40 µl of water to obtain a DNA concentration of *c.* 300–500 ng $\mu l^{-1}$. Finally, the concentrated PCR products were arrayed on Hybond N+ nylon membranes (Amersham, Saclay, France), using MicroGrid II (Biorobotics, Cambridge, UK) with eight microarraying pins. A 12 × 12 grid pattern and a distance of 0.5 mm between spots were used. To increase the reliability of signal, each PCR sample was arrayed twice in randomized spot to yield 2304 datapoints including positive and negative controls. After spotting, the nylon membranes were placed face up onto Whatman paper moistened with denaturation solution (1.5 M NaCl and 0.5 M NaOH) followed by neutralization solution (1.5 M NaCl and 1 M Tris HCl, pH 7.4) for 20 min each. The treated membranes were then dried at 80°C for 2 h and UV cross-linked with UV Stratalinker 1800 (Stratagene, Strasbourg, France).

### Sample collection, RNA isolation and probe preparation

For both treated and control plants, cotyledon petioles were collected at 6 and 48 h after contamination and total RNA was extracted as described by Verwoerd *et al.* (1989). For the two independent biological replicates, cotyledon petioles collected from at least 15 plants were pooled before probe preparation. Single-stranded probes were synthesized from DNase-treated RNA using the Advantage RT-for-PCR Kit (BD Biosciences, Pont-de-Claix, France). The reaction mixture containing 10 µg of total RNA and 40 pmol oligo (dT18) was heated at 70°C for 2 min. Then, 8 µl of 5 × reaction buffer, 2 µl of dNTP mix (0.625 mM each of dATP, dTTP and GTP and 0.625 µM dCTP), 4 µl of [α-33P] dCTP (40 µCi), 1 µl (1 unit) of RNase inhibitor and 2 µl (400 units) of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase were added. The reaction was incubated at 42°C for 1 h. An additional 200 units of MMLV reverse transcriptase was added and the mixture was incubated for another 60 min at 42°C followed by heating at 94°C for 5 min to stop the synthesis reaction and to destroy any DNAse activity. To remove unincorporated labeled nucleotides, the radiolabeling reaction was purified by passing the reaction mixture through Probe Quant G-50 Micro Columns (Amersham). The purified radiolabeled cDNA was then used for hybridization.

Hybridization was performed at 65°C for 24 h in a buffer containing 0.5% sodium dodecyl sulphate (SDS), 5 × saline sodium citrate (SSC), 0.1% each of Ficoll 400, polyvinylpyrrolidone and bovine serum albumin and 100 µg $ml^{-1}$ of salmon sperm DNA (Sigma, St Louis, MO, USA). Membranes were washed twice with buffer containing 0.1% SSC and 0.1% SDS at 65°C for 15 min each. The membranes were then exposed to Fuji film imaging plate as a radioactive energy

sensor. The radioactive intensity of the spots was captured using Bio-Imaging Analyzer BAS-5000 (Fujifilm, St Quentin Yveline, France) with a density gradation of 16 bits pixel$^{-1}$ at a resolution of 25 μm pixel$^{-1}$. Signal quantification was performed using ArrayGauge V.1.3 (Fujifilm) and the numeric values were used for statistical analysis.

For microarray hybridization, 16 different radiolabeled probes corresponding to two lines (partially resistant and susceptible), two treatments (inoculation and control), two time-points (6 and 48 h after inoculation; based on reverse transcriptase–polymerase chain reaction (RT-PCR) experiments, data not shown) and two independent biological replicates for each combination were synthesized and used to hybridize with the nylon filters.

### Oligonucleotide hybridization

In order to estimate the quantity of each cDNA clone spotted onto nylon membrane, filters were hybridized with a mix of M13 and Triplex reverse primers. Five hundred ng of each primer was end-labeled with 3 μl of [α-33P] dATP (30 μCi), using 200 units of polynucleotide kinase (Invitrogen). The reaction was incubated at 37°C for 20 min followed by heating at 65°C for 20 min. Purification of the radiolabeling reaction was performed as described before (Hewezi *et al.*, 2006). Hybridization was achieved overnight at 42°C in the same buffer as described above. Filters were washed in 2 × SSC containing 0.1% SDS at room temperature for 10 min followed by 5 min at 42°C. Scanning and quantification of signal intensities were as already described (see 'Sample collection' section).

### Data normalization and analysis of variance (ANOVA)

To correct for unequal cDNA loading across the array, for each experiment, the absolute raw hybridization signal for each spot was divided by the corresponding value obtained through hybridization with the nonspecific oligos. This scaled value was considered as the crude expression level of a spot. We used ANOVA models both to estimate relative gene expression and to account for other sources of variation in microarray data (Wolfinger *et al.*, 2001; Kerr *et al.*, 2000). A two-pass general linear model as described by Wolfinger *et al.* (2001) was used to normalize the data and then to detect differentially expressed genes. After background correction, we subjected the $\log_{10}$-transformed scores for all spot measures ($y_{xijklm}$ being the measured intensity for gene $x$ subject to treatment $i$, at time $j$, in genotype $k$, for spot $m$, on array $l$) to a normalization model based on a four-way ANOVA, of the form

$$y_{xijklm} = \mu + Tr_i + Tm_j + Gn_k + Tr_iTm_j + Tr_iGn_k + Tm_jGn_k + Tr_iTm_jGn_k + S_l(T_{\pi i}Tm_jGn_k) + \varepsilon_{xijklm}$$

[$Tr_i$, treatment effect (infected, noninfected, i.e. $i$ = 1;2); $Tm_j$, time effect (6 h, 48 h, i.e. $j$ = 1;2); $Gn_k$, genotype effect (susceptible, partially resistant, i.e. $k$ = 1;2); $S_l$, membrane effect within combination of the factors; $\varepsilon_{xijklm}$, stochastic errors.]

The residuals from this model can be regarded as a crude indicator of relative expression level and are referred to as 'normalized expression levels'. We retained as 'above background genes' the genes for which the normalized expression levels of the two spots on a given slide are above the maximum of the empirical distribution of normalized expression levels for the control spots. The gene-specific models were of the form

$$r_{xijklm} = G_x + G_xTr_i + G_xTm_j + G_xGn_k + G_xTr_iTm_j + G_xTr_iGn_k + G_xTm_jGn_k + \varepsilon_{xijklm}$$

($r_{xijklm}$, normalized expression levels of gene $x$.)

The $G_xTr_i$, $G_xTm_j$ and $G_xGn_k$ effects quantify the overall variability of a gene as a function of treatment, time or genotype, respectively. The effects of particular interest are the $G_xTr_iTm_j$ and $G_xTr_iGn_k$ interactions, measuring the effect of a gene as a function of time and treatment, and the effect of a gene as a function of treatment and genotype, respectively. A test for heterogeneous variances for the normalized expression levels among tissues was performed using the Levene test, for each gene model. The Bonferroni method was used to conservatively control the Type I statistical error rate resulting from multiple testings. 'Volcano plots' of significance against magnitude of effects were drawn for each main effect, whereas interaction plots were drawn for genes with significant interaction effects. Computations were performed on a PC running GNU/Linux (Suse 9.0) and the R 1.9.0 statistical system.

### Real-Time RT-PCR

The expression levels of six differentially expressed ESTs were determined by real-time RT-PCR to confirm the results obtained from microarray experiments. Moreover, the expression patterns of 11 cDNA clones were also determined by real-time RT-PCR at different time-points after inoculation in the partially resistant line. First-strand cDNA was reverse-transcribed from 5 μg of DNase-treated RNA as described in the 'Material and Methods' section. Gene-specific primers were designed using PRIMER EXPRESS software, version 2.0 (Applied Biosystems, Courtaboeuf, France). The reaction was performed in 20 μl containing 10 μl of 2X Syper Green Mastermix (Applied Biosystems), 300 nM of each primer and 1 μl of 5-fold-diluted RT products. The PCR reactions were run in the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using the following program: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. Following PCR amplification, the reactions were subjected to a temperature ramp to create the dissociation curve, measured as changes in fluorescence measurements as a function of temperature, using which the nonspecific products can be

 

detected. The dissociation program was 95°C for 15 s and 60°C for 15 s followed by 20 min of slow ramp from 60 to 95°C. Three replications of each reaction were performed and an elongation factor of 1 α (EF1α), for a putative constitutively expressed gene, was used as an internal control for normalization. Quantification of the relative changes in gene expression was performed using the $2^{-\Delta\Delta CT}$ method as described by Livak & Schmittgen (2001).

The construction of our cDNA microarray, the hybridization protocol, the statistical analysis and the validation of results meet the MIAME criteria described by Brazma *et al.* (2001).


**Fig. 1** Phenotypes of susceptible and partially resistant sunflower (*Helianthus annuus*) lines. Note the area of necrotic lesions on cotyledon petioles in susceptible line C106 (a) compared with those in the partially resistant line C146 (b), 1 wk after inoculation of 2- week-old plants.

## Results

### Evaluation of lines

Line C146 was assigned a necrosis rating of 2 (± 1.8), corresponding to a very localized necrosis; this necrosis did not correspond to a hypersensitive response as it spread slightly with time. By contrast, line C106 had a necrosis rating of 7.4 (± 1.5), with the necrotic area spreading over 65–80% of the petiole surface (Fig. 1).

### Genotype effect

At a *P*-value cut-off of $10^{-3}$, and after excluding the ESTs that showed significant interaction effects, we identified 11 cDNA clones showing significant genotype-specific variation in expression levels across all treatments. Results are shown in Table 1 and presented as 'volcano plots' ( Jin *et al.*, 2001) in Fig. 2a. The low number of ESTs (11) that showed statistically significant changes in the levels of mRNA accumulation among partially resistant and susceptible lines reflects the fact that these lines are derived from the same parents and have close genetic backgrounds. Interestingly, seven of these differentially expressed genes were found to be induced in the partially resistant line C146, whereas the other four cDNA clones displayed preferential expression patterns in the susceptible line C106 (Table 1). The significantly differentially induced genes in the partially resistant line are likely to be related to disease resistance.

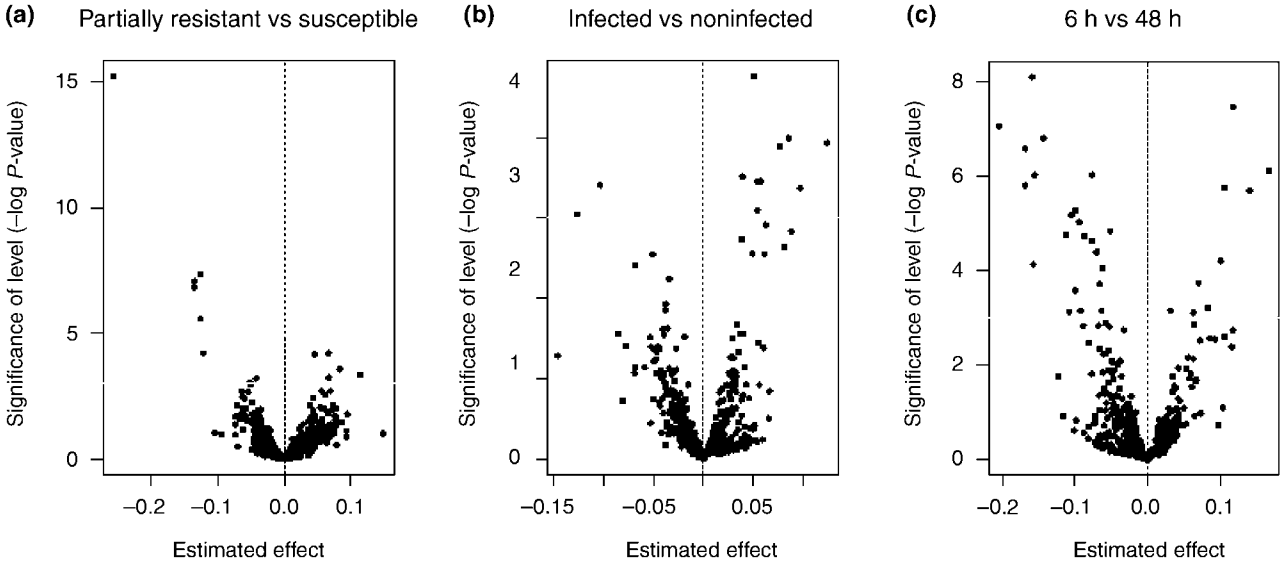


**Fig. 2** Volcano plots of significance against difference in expression. The plots show differences in transcript abundance between: (a) partially resistant and susceptible lines, (b) infected and control plants, and (c) plants 6 and 48 h after treatment. Significance is indicated as the negative logarithm of the *P*-value. Each of the 918 expressed sequences tags (ESTs), including those with interaction effects, is plotted as a point. The horizontal line corresponds to the significance scale. Genes with highly significant differences in expression are located at the top of the graph.

**Table 1** Differentially expressed genes

| Sequence name | Putative function | Functional category | Main effects: Fold change: Partially resistant vs susceptible | Main effects: Fold change: Infected vs control | Main effects: Fold change: 48 h vs 6 h | Interaction effects: Fold change: Genotype/ treatment | Interaction effects: Fold change: Treatment/ time |
|---|---|---|---|---|---|---|---|
| CD847018 | Glutamate decarboxylase 1; EC:4.1.1.15 | Amino acid metabolism | 1.79 | | –1.31 | | |
| CX944830 | Pyruvate dehydrogenase; EC:1.2.4.1 | Amino acid metabolism | 1.79 | | | | |
| CD855351 | Glutamate dehydrogenase 1 (GDH1); EC:1.4.1.3 | Amino acid metabolism | | 1.56 | 2.82 | | |
| CD851608 | Glutamine synthetase (GS1); EC:6.3.1.2 | Amino acid metabolism | | | 1.89 | | |
| CD846465 | Glutamate:glyoxylate aminotransferase 2; EC:2.6.1.2 | Amino acid metabolism | | | 2.14 | | |
| CD852550 | Pyruvate dehydrogenase E1 component α subunit; EC:1.2.4.1 | Amino acid metabolism | | – | – | | 1.83 |
| CD852523 | Glyceraldehyde 3-phosphate dehydrogenase, putative; EC:1.2.1.12 | Carbohydrate metabolism | | 1.20 | | | |
| AJ541069 | 6-phosphogluconate dehydrogenase family protein; EC:1.1.1.44 | Carbohydrate metabolism | | | 2.14 | | |
| AJ828522 | Fructose-bisphosphate aldolase, putative; EC:4.1.2.13 | Carbohydrate metabolism | | | 1.41 | | |
| CX944948 | *Arabidopsis thaliana* class IV chitinase (CHIV) | Defense | –1.69 | | 1.72 | | |
| CD853264 | AMPKBI β subunit, complex-interacting region domain family | Defense | 1.22 | | | | |
| CX944894 | Thaumatin-like protein, putative | Defense | | 1.77 | 3.00 | | |
| AJ540097 | Beta-1,3-glucanase 2 | Defense | | | 2.46 | | |
| CD857615 | Peroxidase, putative; EC:1.11.1.7 | Defense | | | 1.76 | | |
| CD849150 | Phenylalanine ammonia lyase 2 (PAL2); EC:4.3.1.5 | Defense | | 1.48 | 2.35 | | |
| AJ828267 | F-type $H^+$-transporting ATPase subunit C; EC:3.6.3.14 | Energy metabolism | 1.27 | | –1.84 | | |
| CX943975 | Vacuolar $H^+$-ATPase subunit B, putative; EC:3.6.3.14 | Energy metabolism | 1.87 | | | | |
| CX943940 | Photosystem II 47-kDa protein | Energy metabolism | | –1.79 | | | |
| CD846156 | Photosystem I subunit IX | Energy metabolism | | | 1.47 | | |
| CD847301 | Carbonate dehydratase 1 (CA1); EC:4.2.1.1 | Energy metabolism | | | 1.55 | | |
| AJ828896 | Acetyl-CoA C-acyltransferase, putative; EC:2.3.1.9 | Fatty acid metabolism | | | –1.36 | | |
| CD851371 | Omega-6 fatty acid desaturase | Fatty acid desaturation | | | 1.63 | | |
| CD855909 | Esterase/lipase/thioesterase family; EC:3.1.1.3 | Glycerolipid metabolism | 1.75 | –1.61 | –2.42 | | |
| CD847070 | Coline-phosphate cytidylyltransferase; EC 2.7.7.15 | Glycerolipid metabolism | | | | | –1.43 |
| CD854873 | Adenylate kinase, putative; EC:2.7.4.3 | Protein kinase | | | –1.84 | | |
| AJ829107 | Phosphoenolpyruvate carboxykinase (ATP); EC:4.1.1.49 | Protein kinase | | | 2.40 | | |
| BU026754 | CBL-interacting protein kinase 18 (CIPK18) | Protein kinase | | | –1.47 | | |
| CD857614 | Protein kinase family protein; EC:2.7.1. | Protein kinase | | | 1.67 | | |
| AJ412383 | Tyrosine/serine/threonine protein phosphatase-related | Protein phosphatase | | | | –1.89 | |
| AJ539881 | Protein phosphatase 2A | Protein phosphatase | | | – | | 2.13 |
| CD853200 | Transcription factor-related | Transcription factor | 1.86 | | | | |
| CD848175 | MYB-related transcription factor (CCA1) | Transcription factor | –1.36 | 1.30 | 1.52 | | |
| CD850032 | MYB family transcription factor | Transcription factor | –1.47 | | | | |
| CD848631 | CCAAT-binding transcription factor subunit A | Transcription factor | –1.36 | | | | |
| AJ412452 | WRKY family transcription factor | Transcription factor | | – | – | | 2.15 |
| CX946549 | AP2 domain transcription factor RAP2.7 | Transcription factor | | – | – | | 1.74 |
| CX946945 | HD-Zip transcription factor | Transcription factor | – | – | – | | 1.59 |
| CD855464 | Isopentenyl diphosphate:dimethylallyl diphosphate isomerase; EC:5.3.3.2 | Vitamin E biosynthesis | | | 1.82 | | |
| CD845816 | Dimethyllallyl pyrophosphate isomerase; EC:5.3.3.2 | Vitamin E biosynthesis | | | 1.39 | | |

Thirty-nine cDNA clones showed significant variation in expression level under the conditions tested. Putative functions are based on sequence homology to already known or predicted genes in the *Arabidopsis thaliana* functional category based on the Kyoto Encyclopedia of Genes and Genomes database (http://www.genome.jp/kegg/). The normalized expression levels, as computed from the normalization model, are logarithmic values of the expression values. The fold change between two conditions is thus computed as $10^{\log(x1)-\log(x2)}$, where *x*1 and *x*2 are the expression values in conditions 1 and 2, respectively.

AMPKBI, 5′-AMP-actvated protein kinase, complex-interactive region; AP, activating enhancer binding protein; CBL, Cas-Br-M ecotropic retroviral transforming sequence; HD-Zip, homeobox leucine zipper; MYB, myeloblastosis oncogene homolog.

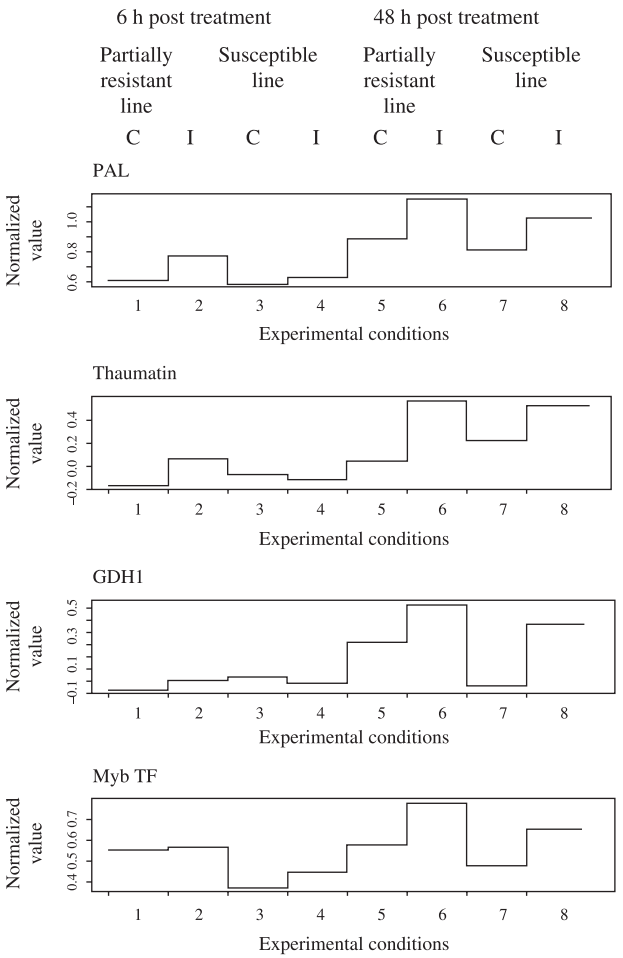


**Fig. 3** Changes in the expression profiles of four genes across all experimental conditions. The abscissa shows the experimental conditions (C, control; I, inoculated). The ordinate shows the normalized values of the expression level.

## Time effect

After removal of six cDNA clones showing significant interaction effects, 24 ESTs showing significant differences in expression among the time-points at $P < 0.001$ were identified. The majority of these clones (18) were up-regulated at 48 h after inoculation when compared with the 6-h time-point. The low number of induced genes at the 6-h time-point may reflect the delayed response of plant genes after *P. macdonaldii* inoculation. A complete list of genes showing significant time-point main effects, as well as their putative functions, is provided in Table 1. Many of the differentially expressed genes encode enzymes involved in primary metabolic pathways (14 ESTs). Interestingly, all clones classified as defense-related genes were found to be induced later, 48 h after inoculation.

## Treatment effect

After the exclusion of four ESTs showing significant interaction effects, only seven cDNA clones were identified as showing treatment-specific expression patterns across all experiments (Fig. 2b). Five of these genes were induced in the inoculated plants (located in the upper right square of Fig. 2b) while two clones were found to be repressed (located in the upper left square). These regulated genes belonged to various functional categories and had *P*-values < 0.001 (summarized in Table 1). It is important to mention that two out of the five clones that were found to be up-regulated in inoculated plants are potentially involved in amino acid metabolism. These cDNA clones are thought to be involved in the biosynthesis of glutamate, arginine and proline (CD855351; EC:1.4.1.3), and tyrosine and phenylalanine (CD849150; EC:4.3.1.5). These results indicate that the metabolism of these amino acids as well as gluconeogenesis (glyceraldehyde 3-phosphate dehydrogenase; CD852523; EC:1.2.1.12) might be associated with plant responses to *P. macdonaldii* infection.

A search for genes showing significant main effects, together with treatment and time factors, revealed four clones encoding glutamate dehydrogenase 1 (CD855351; EC:1.4.1.3), phenylalanine ammonia lyase 2 (CD849150; EC:4.3.1.5), thaumatin-like protein (CX944894) and MYB-related transcription factor (CD848175). The expression of these genes, induced 48 h post inoculation and showing approximately the same expression profiles across all experiments (Fig. 3), suggests coregulation mechanisms. More interestingly, two clones encoding the aforementioned MYB-related transcription factor and an esterase/lipase/thioesterase protein also showed significant genotype effects, suggesting that they could directly modulate the response of sunflower to *P. macdonaldii* infection.

## Genotype response to infection reveals genes associated with susceptibility

Only one clone, encoding tyrosine/serine/threonine protein phosphatase (AJ412383), was found to show significant genotype × treatment interactions as deduced from the ANOVA table. This clone exhibited different patterns of gene expression in the two lines under control and infected conditions. mRNA accumulation was abundant in the partially resistant line in the control treatment compared with the susceptible line. However, after contamination, expression was largely down-regulated in the partially resistant line and up-regulated in the susceptible line (Fig. 4). These results suggest that this gene could function as a negative regulator of defense signaling pathways, and could be considered as a susceptibility-associated factor.

## Time course of defense reaction

ANOVA analysis identified six ESTs showing significant interaction between treatment and time-point. Of these, three clones putatively encode transcription factors belonging to the WRKY (AJ412452), activating enhancer binding protein

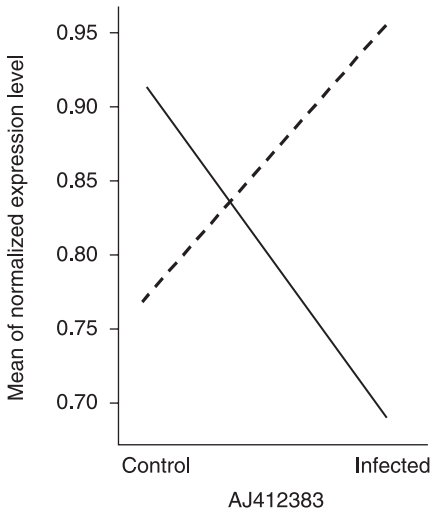


**Fig. 4** Expression profiles of a cDNA clone encoding tyrosine/serine/threonine protein phosphatase (AJ412383) in the two investigated sunflower (*Helianthus annuus*) lines (solid line, partially resistant sunflower line; dashed line, susceptible sunflower line) under infected and control conditions. mRNA accumulation was greater in the partially resistant line in the control treatment compared with the susceptible line. However, after contamination, the expression level was largely down-regulated in the partially resistant line and up-regulated in the susceptible line.

(AP2) (CX946549) and homeobox leucine zipper (HD-Zip) (CX946945) superfamilies. Two clones are involved in glycerolipid and amino acid metabolism. One cDNA clone also exhibited significant interaction between treatment and time. In the absence of infection, these clones exhibited the same expression profiles at the different time-points (Fig. 5). This observation, which indicates that these genes are not stress-related, shows that the major components of interaction effects can be attributed to inoculation treatment. With the exception of choline-phosphate cytidylyltransferase (CD847070; EC 2.7.7.15), five ESTs exhibited high levels of transcript accumulation in infected tissues at 48 h when compared with the expression levels at 6 h post inoculation (Fig. 5). These results indicate that these genes act later in infection, and suggest functional coordination between these regulators to control the expression of defense-related genes.

## Verification of differentially expressed genes

To confirm the microarray results, six clones were randomly selected from different expression profiles and functional categories, and subjected to real-time RT-PCR analysis. These genes were differentially expressed in microarray experiments. The expression levels of clones corresponding to the AP2 transcription factor (CX946549), choline-phosphate cytidylyltransferase (CD847070; EC 2.7.7.15) and the pyruvate dehydrogenase E1 component alpha subunit (CD852550; EC:1.2.4.1) showed significant treatment–time interactions; those of thaumatin-like protein (CX944894) and phenylalanine ammonia lyase 2 (CD849150, EC:4.3.1.5) showed significant main treatment effects and time effects; and the MYB-related transcription factor (CD848175) showed significant main genotype, treatment and time effects. The expression levels of these genes were tested in all conditions using the same RNA samples that were used for microarray hybridization. They were normalized using EF1 α as an internal control, and fold-change values, representing changes in mRNA abundance in inoculated samples vs control, were calculated using the $2^{-\Delta\Delta CT}$ method. As shown in Table 2, the results obtained from quantitative RT-PCR were in good agreement with those obtained by microarray analysis. In all cases, genes that showed high expression levels in microarray experiments showed high expression levels in quantitative RT-PCR analysis.

Moreover, in order to follow the expression profiles of some genes that were differentially expressed according to the

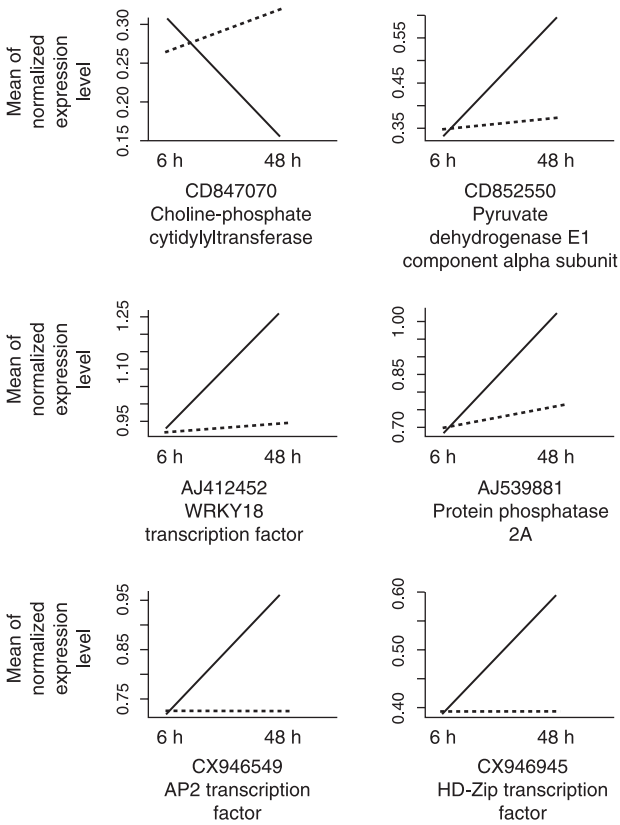


**Fig. 5** Expression profiles of genes displaying a significant interaction between time-points and treatments (solid line, infected; dotted line, control). Three clones, AJ412452, CX946549 and CX946945, putatively encode transcription factors belonging to the WRKY, AP2 and HD-Zip superfamilies, respectively. Two clones, putatively encoding choline-phosphate cytidylyltransferase (CD847070) and the pyruvate dehydrogenase E1 component α subunit (CD852550), are involved in glycerolipid and amino acid metabolism, respectively. One cDNA clone, encoding protein phosphatase 2A (AJ539881), also exhibited a significant interaction between treatment and time. In the absence of infection, these clones exhibited the same expression profiles.

 

**Table 2** Comparison of gene expression levels of six differentially expressed clones using quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR) with microarray results

| | Partially resistant | | | | Susceptible | | | |
|---|---|---|---|---|---|---|---|---|
| | 6 h | | 48 h | | 6 h | | 48 h | |
| Sequence name | Microarray | QPCR | Microarray | QPCR | Microarray | QPCR | Microarray | QPCR |
| CX946549 | –1.04 | 1.20 | 1.66 | 2.39 | 1.00 | 1.06 | 1.70 | 2.18 |
| CD847070 | 1.12 | 1.60 | –1.26 | –1.17 | 1.13 | –1.04 | –1.43 | –1.01 |
| CD852550 | –1.05 | 1.21 | 2.37 | 1.34 | –1.01 | 1.07 | 1.22 | 1.67 |
| CX944894 | –1.09 | –3.89 | 1.88 | 2.37 | 1.72 | 4.47 | 3.33 | 4.80 |
| CD849150 | 1.11 | 1.00 | 1.63 | 2.09 | 1.45 | 1.51 | 1.84 | 3.42 |
| CD848175 | 1.19 | 1.04 | 1.49 | 1.21 | 1.03 | –1.21 | 1.59 | 1.68 |

Six clones displaying different expression profiles and belonging to different functional categories were subjected to real-time RT-PCR analysis to confirm microarray results. The expression levels of the target genes derived from real-time RT-PCR were normalized using EF1 α as an internal control and the fold-change values were calculated using the $2^{-\Delta\Delta CT}$ method and represent changes of mRNA abundance in inoculated samples vs control. The fold-change values derived from microarray analysis represent changes of mRNA abundance in inoculated samples vs control and are computed as 10 to the difference between normalized expression values.
QPCR, quantitative PCR.

microarray experiment results, we investigated the kinetics of transcript accumulation in the partially resistant line. These transcripts included those of the six clones described above, and of five additional clones selected for their potential role in defense. The time course was defined in order to obtain results complementary (expression levels at 3, 24 and 72 h) to those obtained with the microarray experiment (6 and 48 h). The data were collected in independent biological replicates, as in the microarray analyses. All gene transcripts (AJ539881, CX946549, CX946945, AJ412383, CD847070, CX944894, CD849150, CD855909, AJ412452, CD848175 and CD850032) followed the same patterns. The expression levels of these transcripts increased between 3 and 6 h post inoculation, followed by a temporary decrease between 6 and 24 h, and finally increased again between 24 and 72 h after contamination (Fig. 6).

## Discussion

The aim of this work was to begin to elucidate the responses of sunflower to the necrotrophic fungus *P. macdonaldii*, using a microarray approach. On the basis of a contamination test previously described (Roustaee *et al.*, 2000), we chose two lines of sunflower, a partially resistant line (C146) and a susceptible line (C106). We constructed a thematic sunflower cDNA microarray containing 911 clones representing high sequence similarity with known or predicted *A. thaliana* genes related to primary metabolic pathways and signal transduction, and all the putative defense-related cDNAs (116) available for sunflower (Fig. 7).

For most global gene expression studies, the identification of genes whose expression is altered under given conditions is based on fold-change criteria. The major drawbacks of fold-change-based microarray analysis are that experimental variations for all genes are considered to be equivalent and there is no quantitative estimate of false positive or false negative rates. In contrast, the ANOVA method, which takes into account all sources of variability affecting gene expression values, permits ranking of differentially expressed genes according to their significance threshold using *P*-value scores. Moreover, using ANOVA, which is based on well-developed statistical theories, changes in gene expression as small as 1.2-fold can be detected as highly significant (Jin *et al.*, 2001). As illustrated in Fig. 2, volcano plots demonstrate that the expression of many clones may show low variation among treatments which is highly significant (points close to the dotted line and above the significance threshold line), whereas the expression of other genes may tend to show higher variation among treatments which is not significant (point at the edges of the graph but below the line of significance scale).

The ANOVA method allowed us to identify differentially expressed genes among genotypes, treatments and time-points, as well as the interactions among these factors. From our point of view, the most interesting genes identified in this analysis were those showing significant effects of both genotype and infection. Genes that showed only a genotype effect may have reflected the genetic background, whereas genes exhibiting only a treatment effect may have reflected the overall plant response to infection. It is therefore conceivable that genes showing significant effects of both genotype and treatment might play a role in the mediation of defense mechanisms rather than simply respond to the pathogen. In this context, a MYB-related transcription factor and an esterase/lipase/thioesterase protein have been identified, with opposite expression patterns. The transcript level of the lipase was found to be largely decreased after fungal inoculation. Lipases are hydrolytic enzymes that break down triacylglycerol into fatty acids and glycerol. The *A. thaliana* genes enhanced disease

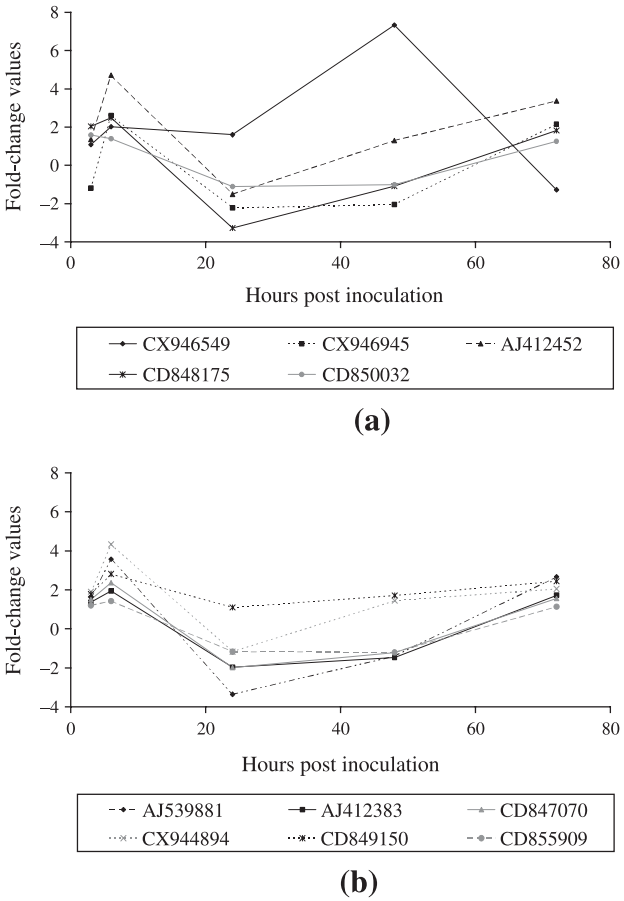


**Fig. 6** Kinetic accumulation for 11 differentially expressed genes in the partially resistant line using real-time RT-PCR. All gene transcripts (AJ539881, CX946549, CX946945, AJ412383, CD847070, CX944894, CD849150, CD855909, AJ412452, CD848175 and CD850032) had the same pattern of expression. The expression of the genes, compared with the control, increased between 3 and 6 h post inoculation, followed by a temporary decrease between 6 and 24 h and finally an increase between 24 and 72 h after contamination. The expression values represent changes of mRNA abundance in inoculated samples vs control. (a) Transcription factors; (b) defense-related genes and others.

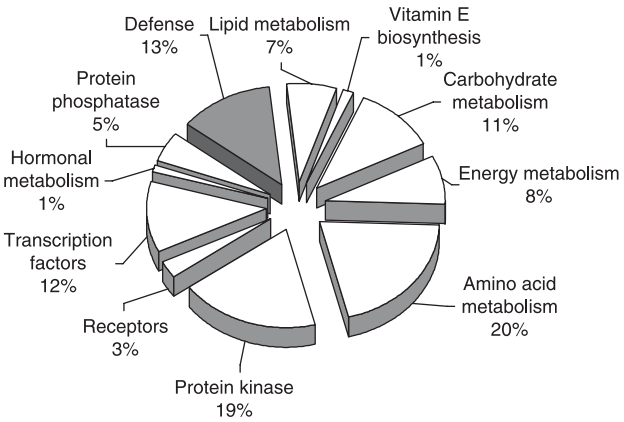


**Fig. 7** Functional classification of the selected expressed sequences tags (ESTs) spotted onto the array. These sunflower (*Helianthus annuus*) EST clones were selected from different cDNA libraries based on sequence homology to already known or predicted genes involved in major primary metabolic pathways, signal transduction and defense responses in *Arabidopsis thaliana*.

susceptibility 1 (*EDS1*) and phytoalexin-deficient 4 protein (*PAD4*), which encode lipase-like proteins, were found to be required for expression of multiple defense responses and basal plant disease resistance (Falk *et al.*, 1999; Jirage *et al.*, 1999; Feys *et al.*, 2001). In contrast to the expression profile of sunflower-like lipase, the transcripts of *EDS1* and *PAD4* were found to accumulate after pathogen infection or treatment with salicylic acid (SA) (Falk *et al.*, 1999; Jirage *et al.*, 1999). These observations, which clearly demonstrate the relationship between glycerolipid metabolism and defense response, suggest that sunflower-like lipase might play a role in plant responses to *P. macdonaldii* infection, possibly as an element of the susceptibility pathway, different from those described in *A. thaliana*.

By contrast, the clone encoding a putative MYB transcription factor was found to be up-regulated after inoculation. The MYB family is one of the largest families of transcriptional factors characterized in plants. Functional analysis of plant MYB transcription factors indicates that they regulate numerous processes, such as disease resistance (Yang & Klessig, 1996; Lee *et al.*, 2001; Vailleau *et al.*, 2002) and regulation of phenylpropanoid metabolism (Martin & Paz-Ares, 1997; Jin *et al.*, 2001). Phenylpropanoids are among the major secondary metabolites, involving modification of components derived from phenylalanine (Martin & Paz-Ares, 1997). A cDNA clone encoding PAL2 (EC: 4.1.3.5), a key enzyme involved in the phenylpropanoid pathway, was among the genes whose expression was induced after fungal treatment as revealed by our microarray analysis. These results provide an indirect potential link between activation of a MYB transcription factor and the defense response generated by *PAL2* as a defense-related gene. They are in accordance with the work of Sugimoto *et al.* (2000), who found that NtMYB2, a tobacco (Nicotiana tobaccum) MYB-related transcription factor, activated expression of the *PAL2* promoter in tobacco protoplasts. Also, it was found that overexpression of *NtMYB2* cDNA in transgenic tobacco plants induced expression of the *PAL* gene. This finding, together with our results, supports the hypothesis that MYB transcription factors positively regulate the plant defense response directly or indirectly via activation of secondary metabolism. Interestingly, the camalexin phytoalexin, an end product of the phenylpropanoid pathway, was found to be involved in resistance of *A. thaliana* to the fungus *Phoma lingam*.

A number of regulatory proteins and transcription factors are known to play important roles in disease signaling by controlling the transcriptional activity of defense-associated

 

**Table 3** Classification of the putative sunflower (*Helianthus annuus*) defense-related clones implicated in the salicylic acid (SA), jasmonic acid (JA) or ethylene pathway

| | Putative function |
|---|---|
| Salicylic acid | |
| At1g74710 | Isochorismate synthase 1 |
| Jasmonic acid | |
| At2g39940 | Coronatine-insensitive 1 |
| At1g17420 | Lipoxygenase, putative |
| At3g45140 | Lipoxygenase |
| At2g43710 | Acyl-[acyl-carrier-protein] desaturase |
| At1g19640 | Jasmonic acid carboxyl methyl transferase |
| At1g17990 | 12-Oxophytodienoate reductase, putative |
| At2g33150 | Acetyl-CoA C-acyltransferase CoA thiolase, putative |
| Ethylene | |
| At5g03280 | Ethylene-insensitive 2 |
| At3g61510 | 1-Aminocyclopropane-1-carboxylate synthase (ACC synthase) |
| At5g51690 | 1-Aminocyclopropane-1-carboxylate synthase (ACC synthase) |
| At3g61510 | 1-Aminocyclopropane-1-carboxylate synthase (ACC synthase) |
| At4g11280 | 1-Aminocyclopropane-1-carboxylate synthase 6 (ACC synthase 6) |
| At3g47190 | 1-Aminocyclopropane-1-carboxylic acid oxidase (ACC oxidase) family |
| At1g05010 | 1-Aminocyclopropane-1-carboxylate oxidase (ACC oxidase) |

Putative functions are based on sequence homology to already known or predicted genes in *Arabidopsis thaliana*.

genes. Three putative transcription factors that belong to the WRKY, AP2 and homeobox-leucine zipper families were induced 48 h after infection. These transcription factors have been suggested to be involved in defense responses (Maleck *et al.*, 2000; Schenk *et al.*, 2000; Chen *et al.*, 2002; Marathe *et al.*, 2004). Chen & Chen (2002) found that *A. thaliana* plants expressing AtWRKY18 showed higher resistance to a bacterial pathogen. They also demonstrated that the WRKY18 transcription factor enhanced induction of PR5 (Chen & Chen, 2002), a thaumatin-like pathogenesis-related protein with antifungal activities (Anand *et al.*, 2004).

Surprisingly, we did not observe changes in the expression levels of the 15 genes implicated in the SA, JA and ethylene pathways (Table 3) in the response of sunflower to *P. macdonaldii*. However, this does not imply that this response is independent of SA, JA and ethylene signaling. The complexity of plant defense against necrotrophic pathogens is further increased by the fact that resistance against most necrotrophic fungal pathogens does not follow a gene for gene type of interaction, but rather is multigenic. McDowell & Dangl (2000) proposed that the lifestyle of a pathogen might be a predictor of whether or not the pathogen will be affected by the jasmonate response. Their hypothesis is based on the fact that the salicylate response, which is frequently implicated in plant defense mechanisms, is associated with the hypersensitive response (HR), a form of programmed cell death. If the HR restricts the growth of biotrophs, it may fail to restrict necrotrophic pathogens.

It is well known that phosphorylation and dephosphorylation of proteins regulated by the opposing activities of protein kinases and phosphatases control many cellular processes (Smith & Walker, 1996). An increasing number of protein phosphatases have been reported to be involved in defense mechanisms. Forty-eight hours after inoculation, an increased level of transcripts encoding serine/threonine protein phosphatase 2A (PP2A) was observed. The same PP2A can perform different functions when associated with different regulatory subunits, being involved, for example, in metabolism, cell cycle control and telomerase activity. It has been demonstrated that PP2A activation inhibits localized cell death (He *et al.*, 2004). In our interaction, control of cell death could be an important factor in limiting the propagation of the necrotrophic fungus *P. macdonaldii*. At least one clone, putatively encoding tyrosine/serine/threonine protein phosphatase, also named dual-specificity phosphatase (dsPTP), exhibited different patterns of gene expression under control and treatment conditions depending on the genotype. After inoculation, its expression level was largely down-regulated in the partially resistant line and up-regulated in the susceptible line. Dual-specificity phosphatases are able to dephosphorylate both phosphotyrosine and phosphoserine/threonine residues. These dsPTPs have been shown to regulate MAPK activity (for a review, see Keyse, 2000). Among the MAPKs regulated by dsPTPs, AtMPK4 (Gupta *et al.*, 1998) and AtMPK6 (Ulm *et al.*, 2002) play a role in plant defense responses. AtMPK6 appears to be implicated in the activation of both local disease resistance, regulated by specific *R* genes, and basal resistance (Menke *et al.*, 2004). Moreover, a number of different pathogenic stimuli activate AtMPK6 (Nhüse *et al.*, 2000), and SA inducible protein kinase (SIPK) in tobacco (orthologous to MPK6 in *A. thaliana*). Therefore, defense mechanisms in sunflower against *P. macdonaldii* could be mediated, in part, via down-regulation of this dsPTP, resulting in the activation

of the MAP kinase cascade. This could trigger the defense response under the control of different transcription factors belonging to the MYB, WRKY, AP2 and homeobox-leucine zipper protein families. Further detailed analysis to identify the target protein of dsPTP could be of great interest in deciphering the mechanism by which this gene controls defense responses in sunflower against *P. macdonaldii* infection.

A time-course accumulation of selected transcripts allowed us to validate, with three biological replicates, the microarray results. The biphasic expression profile obtained, which is typically observed in plant responses to pathogen infection, could correspond to the recognition of a specific fungal elicitor by the plant (3 h) and a delayed response (48–72 h) to pathogen infection. The expression profiles of the 11 cDNA clones were similar, suggesting that coregulation events occurred.

Unravelling the mechanisms of partial resistance to *P. macdonaldii* in sunflower remains an ambitious endeavour. Nevertheless, by using a cDNA microarray approach, we report here, for the first time, the identification of several genes in sunflower that are regulated in response to this necrotrophic fungus. We propose a model in which negative regulation of a dual-specificity MAPK phosphatase is involved in the defense mechanisms of sunflower to *P. macdonaldii*. The resulting activation of the MAP kinase cascade could trigger defense responses (e.g. thaumatin biosynthesis and PAL activation), under the control of transcription factors belonging to the MYB and WRKY families. Concurrently, the activation of PP2A, which is implicated in cell death inhibition, could limit pathogen development. Such a model is a valuable first step towards understanding and analysing the *P. macdonaldii*–sunflower interaction. In future work, it would be interesting to link differentially expressed genes identified by microarray to the QTLs identified for *P. macdonaldii* partial resistance.

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## Supplementary Material

The following supplementary material is available for this article online.

**Table S1** List of the selected cDNA clones and their putative functions

This material is available as part of the online article from http://www.blackwell-synergy.com

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